My best regard, Microscopy—misc

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LABORATORY MANUAL FOR ACID-FAST MICROSCOPY

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CONTENTS

Frequently used abbreviations:

AFB	Acid-fast bacilli
BFM	Bright-field microscopy
FAM	Fluorescence acid-fast microscopy
Z-N	Ziehl-Neelsen

m	meter
cm	centimeter
mm	millimeter
μm	micrometer
kg	kilogram
g	gram
mg	milligram
l	liter
ml	milliliter
C	Celsius (Centigrade)
sec	second
min	minute
h	hour
g	gravity
lb	pound
in	inch

PREFACE

This manual may be used in training courses or for reference purposes in a laboratory. It is directed to laboratory personnel in various situations — from those working in field surveys and small hospitals in remote areas to those in large reference laboratories.

The entire manual will not be useful to everyone, but everyone should find in it the information needed to do acid-fast microscopy.

Since the July 1975 edition, some worthwhile changes have been suggested. These and other changes have been included in this 1976 revised edition.

I again thank Dr. Hugo L. David, former Chief, Mycobacteriology Branch, Bureau of Laboratories (BL), Center for Disease Control (CDC), Atlanta, Georgia, and now with the Institut Pasteur, Paris, for his encouragement and suggestions during the preparation of the original manual. I also thank Dr. Charles C. Shepard, Chief, Leprosy and Rickettsial Disease Branch, BL, CDC, for consultation on the preparation of the information on leprosy; and Dr. William B. Cherry, Bacterial Chemistry Branch, BL, CDC, for consultation on the preparation of the information on fluorescence microscopy.

I. INTRODUCTION

Tuberculosis and leprosy continue to be world health problems, and infection in humans and domestic animals by the so-called atypical mycobacteria is considered a serious health threat in many parts of the world.

Acid-fast staining with microscopic examination of specimen smears is usually the first, the simplest, and the most rapid bacteriologic method used to demonstrate infection by tubercle bacilli or other mycobacteria (*1,2*).

Now, greater emphasis is being placed on the use of acid-fast microscopy for finding new cases of tuberculosis and monitoring the effect of chemotherapy (*1,2,3,4*).

Many different acid-fast staining methods have been proposed since 1882 when Ehrlich and Ziehl employed the first such methods, and 1883 when Neelsen modified Ziehl's method (*5,6,7*). These techniques usually have basic fuchsin (magenta, aniline red) in an aqueous solution with phenol or other substances which aid the dye in penetrating the acid-fast organism's cell wall. Once the stain has penetrated the cell wall, it is difficult to remove it from within the cell with moderate solutions of mineral acids or with ethanol (ethyl alcohol).

The property of acid-fastness depends on the composition and integrity of the cell wall (*8,9,10*). Experiments have shown that mycolic acid, from mycobacterial cells, is weakly acid-fast and that acid-fast bacterial cells lose their strong acid-fast properties when the cell walls are broken (*11,12*).

A microscopist should not attempt acid-fast microscopy unless he or she examines at least 15 specimens per week for acid-fast bacilli. The future of patients depends on the laboratory's proficiency in processing specimens and in observing smears for acid-fast bacilli. Frequently, only a small percent of these specimens will be positive for acid-fast bacilli and although the temptation is great to do this microscopy, examing less than 15 smears a week does not provide enough experience for the microscopist to maintain proficiency in such work. Furthermore, in such circumstances staining solutions probably would not be used within a 6 month period, and unless unused stains were systematically discarded there would be a chance of using an old, poor quality stain. Therefore, these few specimens or heat-fixed smears of these specimens should be sent to laboratories that are examining a large number of specimens for acid-fast bacili.

II. SAFETY PRECAUTIONS

Tuberculosis infection in man is usually caused by droplet nuclei[1] containing tubercle bacilli being implanted in the lungs. Droplet nuclei are formed by the instantaneous drying of aerosol droplets produced by coughing, sneezing, or talking (*13*). These droplet nuclei may contain tubercle bacilli only if they are produced from a liquid that contains these microorganisms. Aerosols are also produced in the laboratory by disrupting liquid surfaces, for example, breaking bubbles, stirring or mixing liquids, vibrating or striking equipment while working with liquids, and the spattering of heated liquids and moist materials.

[1]Some of the less familiar terms used in this manual are defined in the glossary.

Laboratory infections have also been caused by puncturing the skin with contaminated equipment.

Laboratory personnel should be careful when handling specimens and transferring cultures. Most important, they must be aware of the potential hazards of their work and be aware of what is happening around them. Safety precautions must be taken to prevent infection. Five general rules to help prevent laboratory infection follow:

1. Try to avoid the production of aerosols.
2. Contain, as much as possible, those aerosols that are produced.
3. Establish an airflow pattern in the work area that will carry infectious particles away from laboratory personnel, and exhaust this air far away from other people.
4. Wear protective clothing and use protective equipment.
5. Use disinfectants and sterilizing equipment effectively.

To avoid the production of aerosols: 1) do not create bubbles or agitate liquid specimens or cultures in an open container, 2) do not strike or vibrate inoculating equipment, and 3) clean inoculating equipment in a flask containing 70% - 90% alcohol and sand before sterilizing in a flame.

Keep specimens and hazardous liquids or moist materials in sealed containers and add to or remove substances from these containers in a ventilated inoculation cabinet. This ventilated inoculation cabinet, or biological safety cabinet, is an enclosed area specially designed for working with infectious or potentially infectious materials. In front, it has an open slot or glove ports through which the arms are extended into the enclosed work area and has a glass shield to allow observation of the work. Air is drawn through the front opening and then on through the top or back by an exhaust fan located at an opening to the outside. The air may be passed through a bacteriological filter and an ultraviolet bactericidal chamber. Although some of the commercial ventilated inoculation cabinets are complex and very efficient, a simple boxed-in area on a table top with an exhaust fan to the outside can give some protection. Additional ducts may remove air from the laboratory area, thus giving the laboratory a lower air pressure than its surroundings and preventing infectious particles from escaping to other work areas.

Often, in remote areas or field hospitals, electricity and optimum facilities are not available, but specimens must still be processed. Breezes frequently supply the air currents needed to carry aerosols away from the work area. An isolated one-room building with free airflow through openings on all sides may be used. The technician must always work near and facing a large opening so that a good airflow comes from behind and then goes immediately to the outside and away from others. Hang cloth or paper strips in the openings or use smoke to demonstrate the flow of air.

Effective protective clothing must be worn when working with cultures. It should be worn even when preparing smears; however, in this case, it is not mandatory. The clothing, includes lab coats or gowns, latex surgeon's gloves, bacteriological masks (not cloth or gauze), and shoes that are worn only in hazardous areas.

Some materials used to clean and disinfect work areas contain substituted phenolic compounds, which are complex derivatives of phenol. These work well, but should be purchased from a reputable manufacturing firm that has tested the product by an established procedure for killing tubercle bacilli. All equipment exposed to possible contamination by infectious materials and aerosols should either be wiped with a disinfectant, autoclaved, boiled for 10-15 min, or burned before it is discarded or processed for reuse. See "Disinfection and Sterilization," p. 32.

Laboratory personnel must not smoke, eat, or drink in the laboratory area and should wash their hands after working with specimens and before leaving the laboratory.

They also should be tuberculin skin tested at least once a year. If they are skin-test positive, however, they should have a chest X-ray once a year. Those who must work under less than optimum conditions may be vaccinated with BCG if they are tuberculin-skin-test negative.

People who have active tuberculosis are the primary source of infection and contact with patients should be in the open air or in well-ventilated areas. It is difficult to keep patients from producing aerosols, but they can be instructed to cover their mouths and noses with paper or cloth handkerchiefs while coughing or sneezing. These handkerchiefs should be sterilized before they are discarded or cleaned. Patients' quarters should be ventilated to have 20 or more air exchanges per hour to reduce the infection hazard for those who must enter the room (*13*). This air should be ducted to the outside and away from areas frequented by other persons. Patients and personnel working with them should wash their hands frequently. Materials and equipment that are removed from the patients' quarters are not considered a serious health hazard but should be washed before reuse (*14*).

III. COLLECTION AND HANDLING OF SPECIMENS

Properly collecting and transporting a specimen to the laboratory is as important as properly processing it in the laboratory. A poor quality specimen will probably yield useless, misleading test results.

Specimens should be collected in clean containers that are free from paraffin and other waxes or oils. These materials may appear on the smear as acid-fast artifacts or may react with nonacid-fast bacteria and cause them to appear to be acid-fast (*15*).

Specimen containers should be sturdy and seal well to prevent leaks, especially if the specimen is to be transported to another laboratory. When the specimen is being prepared for transport, the container should be packed in material that will absorb any leakage caused by accidents. If the specimens are mailed, postal regulations should be followed.

Put the identification label on the side of the container and not on the lid or cap, which could be separated from the specimen. The identifying information should include the patient's name or number, the date, and where the patient can be found, *i.e.,* home address or hospital. It may also indicate the place of collection, the type of specimen, and the tests required.

The sputum collection container should have an opening that is 2 cm or more across and have at least a 50-ml capacity. This container should be one that can also be used for processing the specimen. If sputum specimens cannot be processed immediately, they should be refrigerated or substances such as sodium carbonate (0.05 g) or cetylpyridinium chloride (*16*) should be added to inhibit the growth of unwanted microorganisms.

It is best to obtain a sputum specimen early in the day, before the patient has eaten or taken medication. Food particles in smears make them difficult to examine, and medication may interfere with the growth of acid-fast bacilli (AFB) if attempts are made to culture them. Furthermore, in culturing AFB, a single, early morning specimen presents fewer contamination problems than a pooled one.

In collecting sputum instruct the patient as follows:

1. Rinse the mouth with water before giving the specimen.
2. Cover the mouth and nose with a tissue or handkerchief and cough the specimen from deep in the chest. (If the patient does not cough spontaneously, have the patient take several deep breaths and then hold the breath. Repeating this several times should induce coughing.)
3. Hold the specimen container to the lower lip and gently release the specimen from the mouth into the container, and avoid spills.
4. Place the specimen container in a holder. (The collector puts the cap on the container.)

The patient is asked to rinse the mouth with water to remove food and any saprophytic AFB. The specimen must be sputum from the lungs and not saliva or mucus from the nasal area. There is less chance of spilling if the patient holds the container and places it in the rack or carrier. The collector then applies the cap to the container.

Sputum is frequently thick and mucoid, but it may be fluid, with fine chunks of dead tissue from a lesion in the lung. The color may be a dull white or a dull light green. Bloody specimens will be red or brown. Thin, clear saliva is usually of little value, and an attempt should be made to collect material from the lungs.

Very old and young or uncooperative patients may swallow the sputum. This swallowed sputum may be removed from the stomach under a physician's supervision. For culture, this gastric washing should be processed immediately or the stomach acid should be neutralized with sodium carbonate to a pH of 7.0 to 7.6. These gastric washings are processed the same as sputum.

For urine, the collection container should have a 500-ml capacity and an opening more than 5 cm across. A single, first-of-the-morning specimen is preferred, and the genitals should be washed before the specimen is collected.

Blood and other body fluids are collected by physicians or other trained personnel. An anticoagulant should be added to blood specimens to prevent clotting. The concentration of AFB in blood is usually very low, and microscopy on it is rarely useful.

Biopsy and necropsy tissue may be submitted for microscopy and culture. These specimens are usually collected during surgery or at autopsy. Mycobacterial infection is suspected when tubercle-type lesions are found in the tissue.

Mycobacterial skin lesions are usually caused by one of three mycobacterial species: *Mycobacterium marinum, M. ulcerans,* and *M. leprae. M. marinum* can infect skin that has been punctured or scraped and then exposed to the organism, usually in open water or water in fish tanks. AFB are found in exudates from these ulcers, which frequently heal with no further complications (*17,18*).

In tropical areas ulcers caused by *M. ulcerans* begin as small swellings under the skin. These swellings enlarge until the overlying skin dies and spreading, open ulcers form. AFB are found in the subdermal and fatty tissue underlying the skin several centimeters back from the eroded edge. Specimens for detecting these AFB should be taken from exudates and this underlying tissue (*19,20*).

Leprosy is caused by *M. leprae,* and in persons with the lepromatous form of the disease, the organisms are found in the skin and are sometimes found in other parts of the body as well. Microscopy is the primary method of detecting these bacilli, and lepromatous leprosy may be confirmed by the "scraped incision" method. The skin is pinched toward the incision site from either side to prevent bleeding or oozing. While pinch pressure is maintained, a small incision is made with a sterile scalpel 4 to 5 mm long and 2 or 3 mm deep. Any blood or plasma is wiped away, and the sides of the incision are

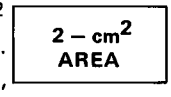
scraped with the edge of the blade held at a right angle to the incision. The tissue obtained is spread on a glass slide. The incision should then be dressed with a small adhesive bandage. In tuberculoid or indeterminate leprosy, bacilli may be very difficult to demonstrate in skin smears. In all forms of leprosy the laboratory diagnosis should be based on the histological finding in a skin biopsy specimen (*21*).

Tubercle bacilli can infect bone, and the lesion is composed of softened bone and dead tissue. The bone is exposed by surgery, and scrapings of the lesion are spread thinly over a glass slide.

Although laryngeal swabs are usually not desirable, swabbing may be the best method for obtaining specimens from small children and very ill patients when a gastric washing is not practical. A separate swab is used for each side of the throat. The tongue is held down with a flat depressor, and the throat area behind the tongue is swabbed as far down as can be reached easily. Smears are made directly from the swabs, or the swabs are sealed in sterile test tubes for future processing.

IV. PREPARATION OF THE SMEAR

Glass microscope slides for acid-fast staining should be new and clean. Used slides may retain material from previous smears, and this could cause a false positive report. The patient's name or identification number is written on one end of the slide so that it will not come off during processing. Some slides have a frosted end than can be labeled with a pencil; the identification is scratched on plain slides with a diamond or tungsten carbide tipped stilus. The specimen material is spread over approximately a 2-cm^2 area with a 3-mm wire loop, applicator stick, swab, or small pipette. Applicator sticks, swabs, and pipettes must be used for only one specimen, and then discarded. The wire loop may be reused after it is dipped in 70%-95% ethanol or methylated spirits, held in a flame until it glows and allowed to cool.



The ethanol may not kill acid-fast bacilli, but dipping the wire loop in ethanol dehydrates the remaining specimen so that it does not spatter in the flame. The flame should be colorless or blue, because an orange or red flame is usually not hot enough.

It is best to make smears for acid-fast microscopy from the sediment of specimens that have been concentrated by centrifugation. When a centrifuge is not available, a direct smear is made from the specimen. Chunks of tissue, pus, or the fine, pale-white, moist granular (caseous) material is the most likely source of tubercle bacilli and should be sought for preparing the direct smear. The caseous chunks are easier to find if the sputum is gently poured into a small dark-colored dish or a clear (Petri) dish that is placed on a dark background.

When sputum is cultured for tubercle bacilli, it is liquefied, decontaminated and concentrated with a centrifuge. Media is inoculated with the concentrate to grow the AFB, and smears are also made.

If the sputum is not to be cultured, a sodium hypochlorite solution (NaClO) can be used to liquefy the sputum and kill all of the microorganisms in the specimen, including tubercle bacilli (*22,23,24*). The advantages of this technique are that it concentrates the specimen and greatly reduces the hazard of infection by tubercle bacilli. Common washing bleach is suitable as long as it is 5% to 6% sodium hypochlorite. The sodium hypochlorite technique is as follows:

1. Place about equal volumes of sputum and sodium hypochlorite solution (5%-6%) in a screw-cap centrifuge tube. The sodium hypochlorite solution may be added to the collection container and used to help remove the sputum.
2. Tighten the cap and shake to liquefy and mix the sputum.
3. Let the mixture stand at least 10 min but not more than 30 min.
4. Add water to near the top of the tube and balance the tubes for the centrifuge.
5. Centrifuge at about 2,000 x gravity for 15 min.
6. Pour off the supernatant and retain the sediment.
7. Resuspend the sediment in several drops of water and prepare the smear.

Much of the solid organic debris in the specimen is broken down by the sodium hypochlorite. This material and the crystals that form as the smear dries will wash off during staining, but the AFB will remain on the heat-fixed smear.

A direct or concentrated sputum smear should appear cloudy before staining. But it is too thick if you cannot read print in a newspaper through the smear when it is held 5 to 10 cm from the print. Smears that are too thick often wash off during staining or the AFB are obscured by debris. A false negative or an incorrect low number will be reported if too little of the specimen is spread on the slide or if the area of the smear is so large that the smear is too thin.

Prepare smears from other specimens as for sputum, but do not expect all liquid specimens to produce a cloudy smear.

AFB will become nonacid-fast if they are exposed to ultraviolet light, direct sunlight, or overheating when fixing or autoclaving (*25,26*). Therefore, smears and specimens should not be exposed to these extreme conditions.

All liquid specimens, except blood, should be concentrated by centrifugation before smears are made.

Tissue specimens are frequently cut into thin sections on a microtome, are attached to a glass slide, and acid-fast stained. This procedure requires special equipment and training and will not be discussed in this manual.

Tissue specimens may also be ground to a fine pulp for culturing and microscopy. Grinding methods create aerosols, however, and grinders with sealing enclosures to contain the aerosols must be used. The glass tube and piston or the mortar and pestal grinders are not aerosol-free and must not be used.

If only smears are to be done and a proper grinder is not available, gently cut through the lesion in the tissue with a clean scalpel or razor blade and smear the lesion area on a slide. The tissue should be handled with forceps only. All equipment should be disinfected or autoclaved and cleaned after each use, and the remainder of the tissue autoclaved or burned.

The preparation of leprosy smears was discussed previously.

After the specimen has been spread on the glass microscope slide, the smear is dried without heat and then heat-fixed. Heat-fix the smear on an electric slide warmer at 65° to 75° C for at least 2 hours or over a flame as for bacteriological smears. Flame heat-fix the smear by holding the slide over a colorless or blue flame (produced by an alcohol or a gas burner) with the smear side up. Immediately after the slide is passed over the flame, it should be hot enough to cause slight pain when touched to the back of the hand. If there is no pain, the slide is too cold; if there is much pain, the slide is too hot. After some practice, the technician can easily judge how long to hold the slide over the flame to heat-fix the smear and will only have to occasionally test a smear for correct heating.

Heat-fixing does not always kill the AFB, and any of the smear rubbed from the slide may be a potential source of infection.

Saprophytic AFB are found in soil and water, and these organisms may contaminate the specimen during processing, resulting in a false positive test. Use only clean collection containers and equipment.

Remember, all used specimens and equipment should be disinfected or sterilized before discarding or cleaning for reuse.

V. ACID-FAST STAINING

A. GENERAL INFORMATION ON ACID-FAST STAINING

Many acid-fast staining techniques have been proposed (*6,27*). Some work well and may be substituted for those suggested in this manual. Others are unsatisfactory or require additional substances that add little to the staining quality. Any staining procedure that is not recommended by a leading laboratory or is not in an internationally recognized laboratory manual should be compared to the Ziehl-Neelsen procedure by examining several hundred specimens. If it compares favorably, it can be adopted for routine use.

Smears should be stained on staining racks or so that no material can be transferred from one smear to another. Staining jars or dishes should not be used. The transfer of a few acid-fast bacilli from one acid-fast positive smear to a negative one could cause the specimen to be incorrectly reported as positive; thus the patient would undergo unnecessary hardship and receive improper treatment.

During staining, water is used to rinse away used solutions so that the next solution can be applied. After each rinse the slide should be tilted slightly to drain off the excess rinse water which would dilute the next solution if it remained.

Do not blot acid-fast stained smears to dry them because AFB may be transferred from one smear to another during the process.

Staining solutions should be stored in dark bottles or in dark cabinets.

Occasionally, staining solutions will be contaminated with growths of microorganisms. If this occurs, the solution should be discarded, and the staining and stock bottles should be cleaned with acid alcohol and sterilized before reuse.

Precipitates or other solid material may be removed from staining solutions by filtering through glass wool or filter paper.

Food particles and precipitated stains may be confused with AFB, and waxes or oils may react with other bacteria and cause them to appear acid-fast. The microscopist should be alert for these artifacts and should become thoroughly familiar with the appearance of AFB.

Minerals and other substances are usually separated from water by either distillation or deionization. Distilled water is usually more pure than deionized water, but deionized water can be substituted for distilled water for the preparation of staining solutions. Saprophytic AFB can live in either type of water and can cause false positive results. Therefore, use only recently distilled or deionized water and keep the distiller or deionizer and storage containers clean.

Some manufacturers use a small quantity of methanol to denature ethanol. These methylated spirits can be substituted for ethanol in preparing staining solutions and acid alcohol.

Acids can react violently when mixed with other substances. Add acid to another liquid slowly while stirring. This quickly dilutes the acid and reduces the chance of a violent reaction. Stop adding acid if the diluting container becomes too hot to touch. Resume adding the acid when the container has cooled.

High concentrations of strong acids will destroy flesh, clothing, and metals. All spills and spatters should be washed with large quantities of water or neutralized with a sodium carbonate solution.

Stain powders are frequently not pure, and a corrected weight should be used to insure proper staining. Most manufacturers print the percent of available dye content on the label. The corrected weight is determined by dividing one by the decimal equivalent of the available dye. Multiply the resultant factor by the desired amount of dye to find the increased amount of impure stock dye powder to use.

Example:

a. 75% (.75) available dye
b. 5 g of dye needed

$$\frac{1}{.75} = 1.33 \text{ (factor)}$$

$$\begin{array}{r} 1.33 \\ \times\ 5\ g \\ \hline 6.65\ g \end{array}$$

of impure dye to use to obtain 5 g usable dye.

The factor for each container of stain powder should be noted on the label for future use.

Crystals of phenol (carbolic acid) are preferred for preparing acid-fast stains. The crystals may be weighed or melted (melting point is 41° C) and measured in a warm pipette. One ml of phenol will weigh about 1.07 g, and although there is a difference of 0.07 g, these units of measure can be interchanged for phenol when preparing acid-fast stains. When using melted phenol, do not overheat or allow it to recrystalize in the pipette while measuring. Pure phenol crystals are colorless; brown-tinted crystals should not be used because they may cause unsatisfactory staining.

Although the storing of fuchsin-phenol stained smears for future use is not recommended, some laboratories may have to do this. Immersion oil must be washed from the smears with xylene (xylol) because the oil removes the stain from the AFB. Fluorochrome-stained smears will not retain their fluorescence in storage.

Smears may be made of mycobacterial cultures and examined for contamination or cell morphology. A fuchsin-phenol stain should be used because bacterial morphology and staining characteristics appear more distinct than when fluorescence staining is used.

Most staining solutions deteriorate with age. A smear that is known to be positive for AFB should be stained as a control for each day's group of smears. These controls will assure the microscopist that the staining solutions and microscope are functioning properly and will serve as a morphology guide.

B. BRIGHT-FIELD MICROSCOPY

In bright-field microscopy (BFM) the light passes through the stained object, and the stains act as colored filters for the light. Structures or organisms can be differentiated if stains that contrast with others in the material on the slide are selective for these structures or organisms.

There are several acid-fast techniques for BFM. The two that are frequently used are the Ziehl-Neelsen and Kinyoun techniques.

Ziehl-Neelsen Acid-Fast Stain *(7,28,29)*

Reagents:

1. Fuchsin-Phenol: a) Dissolve 0.3 g basic fuchsin in 10 ml 90%-95% ethanol or methylated spirits; b) Add and mix 5 g phenol crystals in 95 ml distilled water; c) Mix solution a with 90 ml of solution b.
2. Acid Alcohol: Add 3 ml concentrated hydrochloric acid to 97 ml 90%-95% ethanol or methylated spirits.
3. Methylene Blue: Dissolve 0.3 g methylene blue chloride in 100 ml distilled water.

Procedure:

1. Prepare and heat-fix smears.
2. Cover the smear with absorbent paper and add approximately 5 drops of Ziehl-Neelsen fuchsin-phenol.
3. Heat the bottom of the slide until the stain begins to steam. Apply only enough additional heat to keep the stain steaming for 5 minutes. Do not boil or allow to dry. Add fuchsin-phenol if necessary.
4. Remove paper with forceps.
5. Rinse smears with tap water and drain.
6. Flood smear with acid alcohol and destain for 2 min. (Additional destaining may be necessary.)
7. Rinse smear with tap water and drain.
8. Flood smear with methylene blue and counterstain 1-2 min.
9. Rinse, drain, and air dry.

Kinyoun Acid-Fast Stain *(29,30)*

Reagents:

1. Fuchsin-Phenol: a) Dissolve 4 g basic fuchsin in 20 ml 90%-95% ethanol or methylated spirits; b) Dissolve 8 g phenol crystals in 100 ml distilled water; c) Mix a and b.
2. Acid Alcohol: Add 3 ml concentrated hydrochloric acid to 97 ml of 90%-95% ethanol or methylated spirits.
3. Methylene Blue: Dissolve 0.3 g methylene blue chloride in 100 ml distilled water.

Procedure:

1. Prepare and heat-fix smears.
2. Cover the smear with absorbent paper, add approximately 5 drops of Kinyoun fuchsin-phenol, and stain for 5 min. Do not heat.
3. Remove paper with forceps.
4. Rinse smear with tap water and drain.
5. Flood smear with acid alcohol and destain for 2 min. (Additional destaining may be necessary.)
6. Rinse smear with tap water and drain.
7. Flood smear with methylene blue and counterstain for 1-2 min.
8. Rinse, drain, and air dry.

Discussion

Leprosy bacilli are not strongly acid-fast. An accepted method for staining smears for detecting only leprosy bacilli is to stain the smear with the Ziehl-Neelsen fuchsin-phenol for 20 min without heat, and then destain with a 1% hydrochloric acid in 90%-95% ethanol for 2 min. Follow the Ziehl-Neelsen staining procedure except for the substitution of these two steps (*21*).

In the original Ziehl-Neelsen procedure, a 25% sulfuric acid in water solution was used to destain smears. The smears were flooded with acid and rinsed several times until only a faint pink color remained in the smear (*7*). Some bacteriologists still recommend the 20%-25% sulfruic acid destaining with and without an additional 95% ethanol destaining (*5*).

Several counterstains other than the one presented in this manual have been used with BFM acid-fast staining (*6*). Loffler's methylene blue is frequently used; others are malachite green, brilliant green, and picric acid. A counterstain is useful when it produces good differentiation between the AFB and the debris and if the counterstain is not so dark that it obscures the AFB.

Although 2-x 3-cm pieces of filter paper are preferred to cover the smears for staining, pieces of paper towel may be used for this purpose. Beware of chemicals in paper towels that interfere with staining and of AFB or debris that can transfer from the towel to the smear.

During Ziehl-Neelsen staining the smears can be heated with alcohol or gas flames as well as with an electric heater. Orange or red flames frequently leave black carbon deposits on the bottom of the slide; therefore, only colorless or blue flames should be used.

Old fuchsin-phenol stains may leave a dark red or brown deposit on the smear. If this happens, discard the stain solution and clean the stock and dropping bottles before refilling them with fresh stain. These bottles should be cleaned routinely with a few ml of acid alcohol and a water rinse before fresh stain is added.

C. FLUORESCENCE MICROSCOPY

Fluorescence acid-fast microscopy (FAM) is receiving greater recognition as a valuable diagnostic procedure, and improvements in microscopes and techniques have overcome many of the earlier objections to its use (*2,4*). This is acid-fast staining and is not fluorescent antibody microscopy. The specific staining solution contains a fluorochrome, usually auramine O, and phenol. When destaining, the acid concentration is usually less than that used for the fuchsin-phenol techniques. Some of the early techniques had no counterstain (*31*). In others potassium permanganate, which is not really a counterstain, was used to reduce the background fluorescence (*32*). Recently, acridine orange has been proposed as a true counterstain; it produces an orange-to-red fluorescence that contrasts with yellow fluorescing auramine O in the AFB (*27*). The orange or red fluorescing debris provides a visible field to search. This is better than having a dark field to search with little visible material as a guide.

Most investigators report that more acid-fast positive smears are found by fluorescence than by bright-field microscopy (*33*). Although the equipment for fluorescence acid-fast microscopy is more expensive and requires greater technical skill, many experienced microscopists believe that fluorescence microscopy is superior to bright-field microscopy and should be used when possible (*2,4*).

Several microscope light filter combinations have been recommended for FAM. Each microscope manufacturer has sets of these light filters and can supply the required set if given the fluorescent stain and technique to be used. The section, "The Microscope," p. 21, has more information on fluorescence microscopy.

Many fluorescent stains are altered by light and should be stored in dark bottles or dark areas. Auramine O is inactivated by temperatures above 40° C and should not be heated to prepare staining solutions or when staining (*34*).

Auramine (base compound) or auramine O (hydrochloride) is suspected to have caused cancer in people after prolonged exposure during manufacture (*35*). Large quantities also causes cancer in some susceptible laboratory animals (*36*). This indicates that slight exposure may not be harmful, but one should avoid direct contact with the powders or solutions and make sure that others are protected from them when they are discarded. This warning is not intended to discourage the use of auramine O, but to encourage care in using it.

Some factors which may interfere with staining or reduce fluorescence are: thick smears, using absorbent paper during staining, high chlorine content in rinse water, excess exposure of stained smear to potassium permanganate, and exposing fluorescent stains to solutions of heavy metal ions.

Some of the staining reagents drain off the smears before they are adequately stained. This causes unsatisfactory staining and should be avoided. Leveling the slide or staining rack usually helps.

Fluorochrome-stained smears usually lose their fluorescence with time. Refrigeration will slow this process, but these smears should be observed within 24 hours of staining. The smears should not be stained until they can be observed within this time.

A smear that has been examined by FAM may be restained by one of the fuchsin-phenol acid-fast techniques to confirm observations. It should be done routinely until the microscopist or laboratory supervisor has confidence in the fluorescence results; thereafter, it may be desirable for confirmation if no culture is attempted.

Once a smear has been stained by a fuchsin-phenol stain, will no longer give satisfactory results for fluorescence microscopy (*26,37*).

Clean old stain deposits from dropping bottles and stock bottles with acid alcohol and a water rinse each time new stain is made.

Blair Fluorescence Acid-Fast Stain (*29,38*)

Reagents:

1. Auramine O—Phenol: a) Dissolve 0.1 g auramine O in 10 ml 90%-95% ethanol or methylated spirits; b) Dissolve 3 g phenol crystals in 87 ml distilled water; c) Mix a and b.
2. Acid Alcohol: Add 0.5 ml concentrated hydrochloric acid to 100 ml of 70% ethanol or methylated spirits. (See "Measurement of Liquids," pp. 29-31, for preparation of 70% ethanol.)
3. Potassium Permanganate: Dissolve 0.5 g potassium permanganate ($KMnO_4$) in 100 ml distilled water.

Procedure:

1. Prepare and heat-fix smears.
2. Flood smears with auramine O–phenol and stain for 15 min. (Do not heat or use absorbent paper.)
3. Rinse with chlorine-free water and drain.
4. Flood with acid alcohol and destain for 2 min.
5. Rinse and drain.
6. Flood with potassium permanganate and allow to react for 2 min.
7. Rinse, drain, and air dry. (Do not heat.)

Richards (*31*) used a similar technique, but did not use potassium permanganate to quench the background fluorescence.

Smithwick Fluorescence Acid-Fast Stain (*27*)

Reagents:

1. Auramine O–Phenol: a) Dissolve 0.1 g auramine O in 10 ml 90%-95% ethanol or methylated spirits; b) Dissolve 3 g phenol crystals in 87 ml distilled water; c) Mix a and b.
2. Acid Alcohol: Add 0.5 ml concentrated hydrochloric acid to 100 ml of 70% ethanol or methylated spirits. (See "Measurement of Liquids," pp. 29-31, for preparation of 70% ethanol.)
3. Acridine Orange: Dissolve 0.01 g anhydrous dibasic sodium phosphate (Na_2HPO_4) in 100 ml distilled water, then add and dissolve 0.01 g acridine orange.

Prodecure:

1. Prepare and heat-fix smears.
2. Flood smear with auramine O–Phenol and stain for 15 minutes. (Do not heat or use absorbent paper.)
3. Rinse with chlorine-free water and drain.
4. Flood with acid alcohol and destain for 2 min.
5. Rinse and drain.
6. Flood with acridine orange and counterstain for 2 min.
7. Rinse, drain, and air dry. (Do not heat.)

Truant Fluorescence Acid-Fast Stain *(29,39)*

Reagents:

1. Auramine O–Rhodamine B–Phenol: Dissolve 1.5 g auramine O and 0.75 g rhodamine B in a solution of 75 ml glycerol (glycerine), 10 ml heated phenol crystals, and 50 ml distilled water. This staining solution is usually cleared by filtering through glass wool.
2. Acid Alcohol: Add 0.5 ml concentrated hydrochloric acid to 100 ml of 70% ethanol or methylated spirits. (See "Measurement of Liquids," p. 29-31, for preparation of 70% ethanol.)
3. Potassium permanganate: Dissolve 0.5 g potassium permanganate ($KMnO_4$) in 100 ml distilled water.

Procedure:

1. Prepare and heat-fix smears.
2. Flood smear with auramine O–rhodamine B–phenol and stain for 15 min. (Do not heat or use absorbent paper.)
3. Rinse in chlorine-free water and drain.
4. Flood with acid alcohol and destain for 2 min.
5. Rinse and drain.
6. Flood with potassium permanganate and allow to react for 2 min.
7. Rinse, drain, and air dry. (Do not heat.)

VI. SCANNING SMEARS AND REPORTING OBSERVATIONS

AFB in specimens are usually in the form of bacilli (rod-shaped), but may appear as cocci (spherical) or filaments (thread-like). They are frequently bent and usually contain heavily stained areas called beads. They may have alternating stained and clear sections, which make them appear banded. These and other characteristics are shown in the photographs in Figures 1 and 2.

As can be seen in smears of cultures, not all of the mycobacteria in any given smear are acid-fast. Some species of the genera *Norcardia* and *Corynebacterium* and some bacterial and fungal spores are acid-fast. In FAM some debris fluoresces yellow-green, and small particles may be mistaken for fluorescing AFB. Therefore, the microscopist should learn to recognize AFB morphology.

AFB stained with a fuchsin-phenol stain appear red or pink; those stained with auramine O-phenol will fluoresce yellow to yellow-green; and those stained with auramine O–rhodamine B–phenol will fluoresce yellow-orange, which contrasts with any yellow-green fluorescing debris.

The nonacid-fast background material in a sputum smear will contain human cells, usually lymphocytes; microorganisms, such as bacteria, yeast, and fungal filaments; food particles; and other debris. This nonacid-fast background material is stained by the counterstain and is blue if stained with methylene blue, fluoresces orange or red if stained with acridine orange, appears black or brown if exposed to potassium permanganate, or fluoresces a pale light-green if it receives no further treatment after destaining with acid alcohol. Figure 2 shows sputum smears stained by the various techniques.

AFB in specimens can usually be stained by either a fuchsin-phenol or a fluorescence acid-fast stain. On rare occasions they can be stained by only one type of stain rather than both. If at all possible, a laboratory should be prepared to use either method to confirm a negative result that is thought to be in error.

Occasionally, AFB dislodged from a smear will be suspended in the immersion oil during observation. The oil should be wiped from the oil immersion lens after each AFB positive smear is found to avoid reporting false positive results on any AFB negative smears that follow.

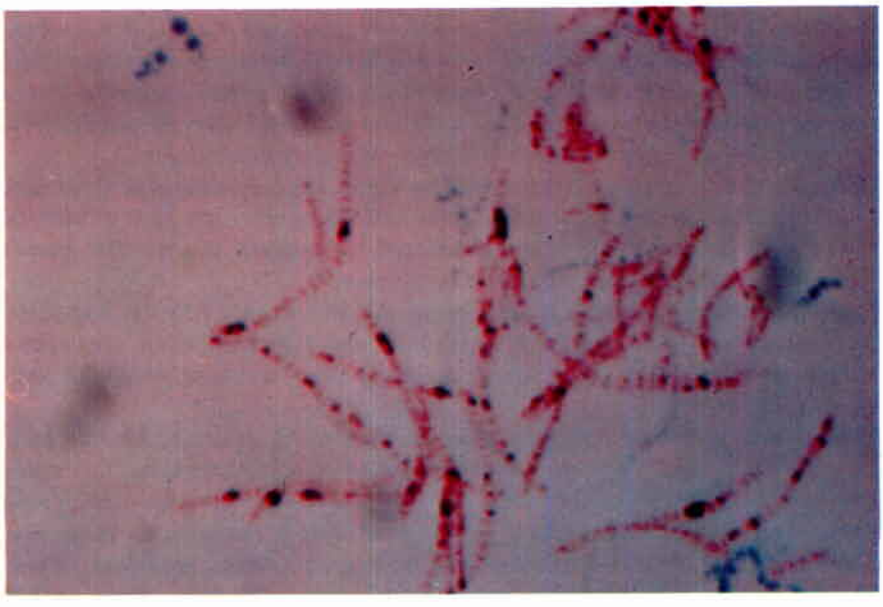
Certain vision problems may keep a person from being an acceptable microscopist. One is color blindness. There are various degrees of color blindness, and someone with this problem is not necessarily excluded from doing acid-fast microscopy. Such a person may find a staining procedure that produces colors which can be easily differentiated. This person should not attempt to change from a compatible procedure to another without the aid of a competent microscopist with normal color vision. Some other vision problems can be overcome with corrective lenses. If the microscopist wears eyeglasses, he or she must use a microscope with eyepieces designed for use with eyeglasses.

Some binocular microscopes have one adjustable eyepiece to compensate for minor differences of focus in an individual's eyes. This lens may have to be readjusted when others use it.

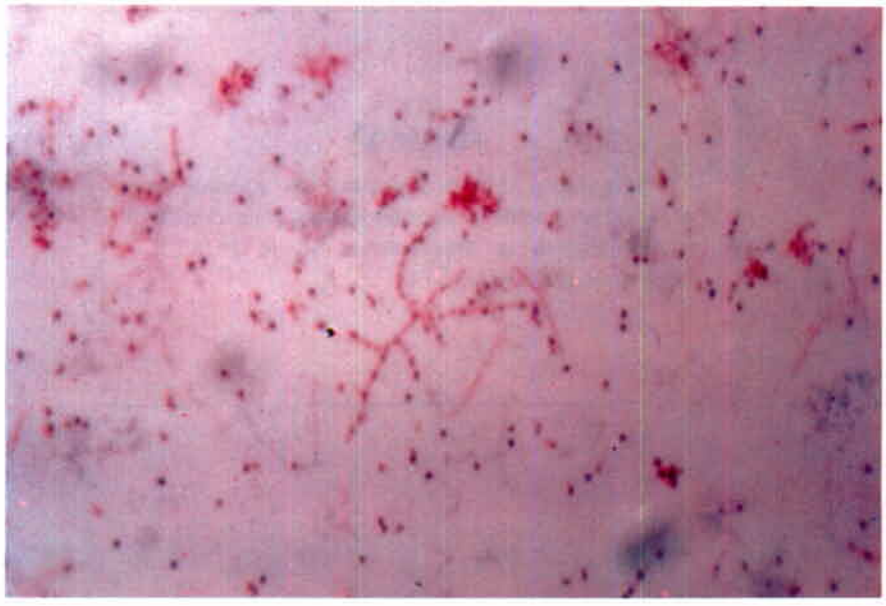
To insure that an area is observed only once, search the smear in an orderly fashion by making a series of vertical or horizontal parallel sweeps; attempt to sample all parts of the smear. Search each field thoroughly with a rapid change to the next field; scanning with a continuous movement of the slide may cause motion sickness. Mechanical devices to control the movement of the slide across the microscope stage are available for most microscopes.

FIGURE 1.

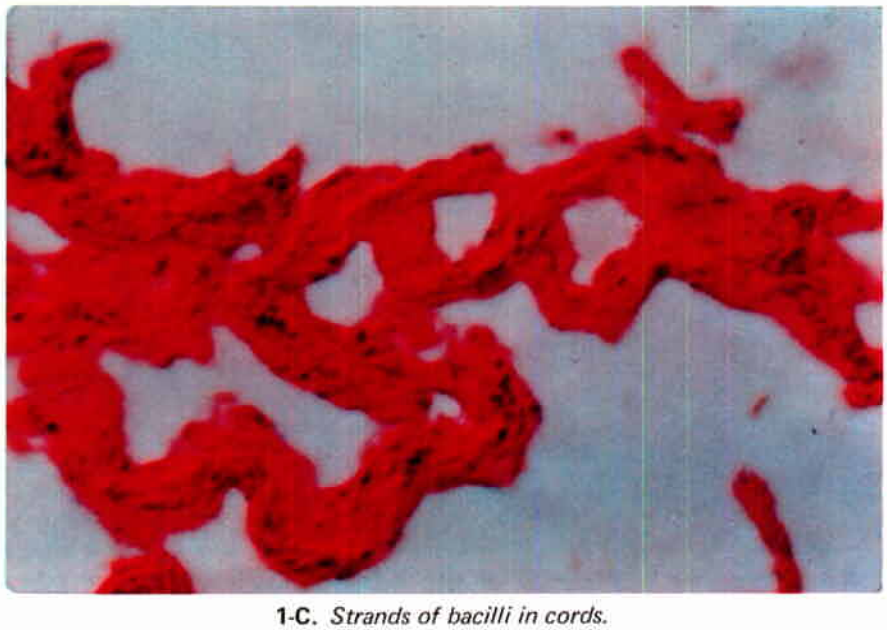
Photographs of mycobacterial culture smears that demonstrate some characteristics of acid-fast bacilli stained with a fuchsin-phenol stain (1,000X original magnification).



1-A. *Alternating clear and pink-stained parts of the bacilli that show the characteristic of banding. Note the dark-stained parts called beads.*



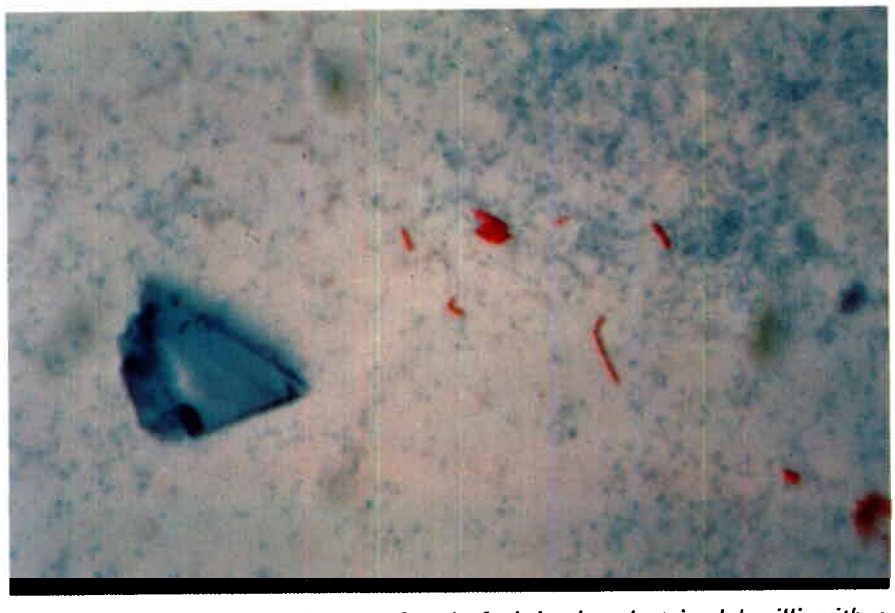
1-B. *Branching filaments with beading and small coccoid forms.*



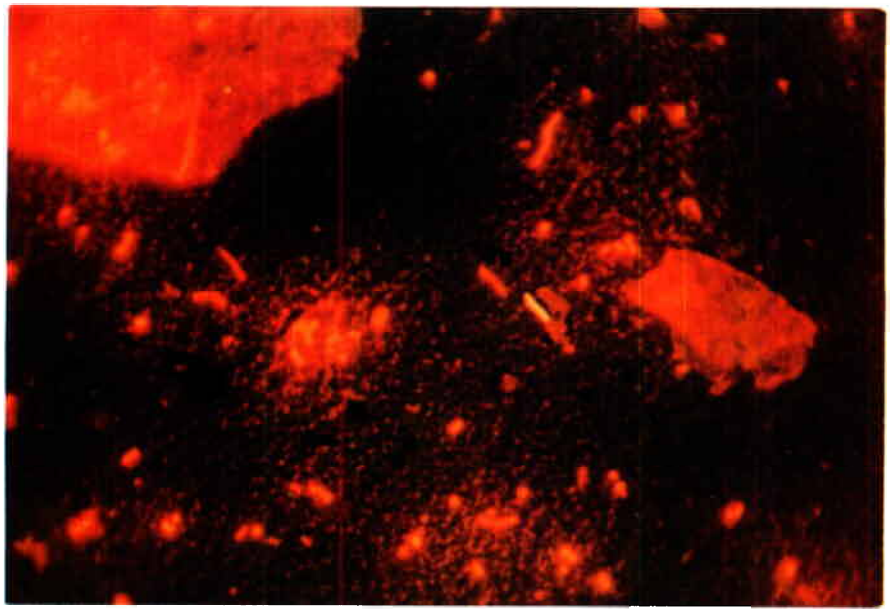
1-C. *Strands of bacilli in cords.*

FIGURE 2.

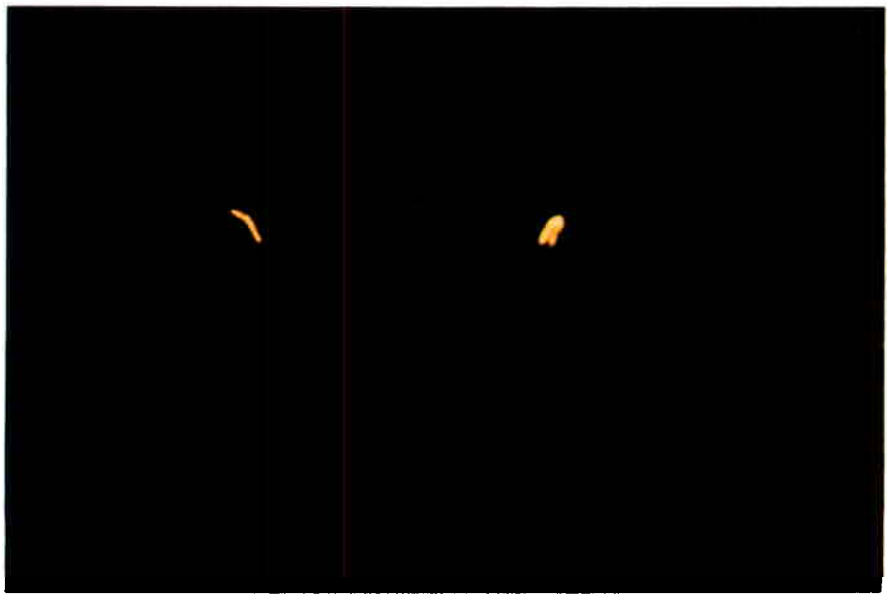
Photographs that demonstrate the appearance of acid-fast bacilli in sputum smears stained with fuchsin-phenol or fluorescence stains (1,000X original magnification).



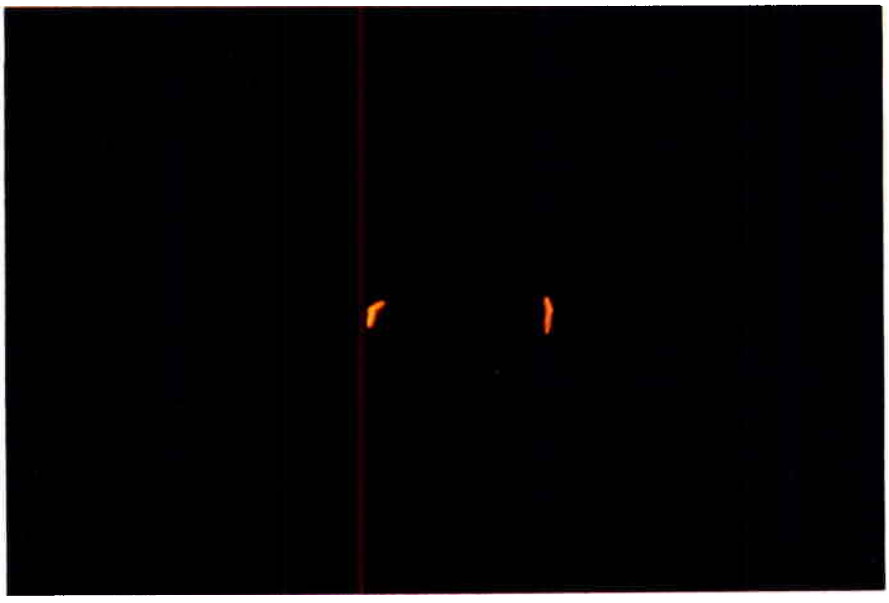
2-A. *Short, long, and clumps of red, fuchsin-phenol stained bacilli with a methylene blue stained background.*



2-B. *A yellow fluorescing, auramine O-phenol stained bacillus with an orange fluorescing, acridine orange stained background.*



2-C. *Yellow fluorescing, auramine O-phenol stained bacilli. The background fluorescence is quenched with potassium permanganate.*



2-D. *Yellow-orange fluorescing, auramine O-rhodamine B-phenol stained bacilli. The background fluorescence is quenched with potassium permanganate.*

A field of view that is seen in the microscope covers only a very small area of a smear, and the smear represents a very small portion of the total specimen. The microscopist should understand the relationship between the number of AFB in the specimen and those that are seen in the smear.

Since acid-fast artifacts or saprophytic AFB may be present in the smear, a smear should not be reported as positive for AFB unless at least three AFB have been seen (*28*). The required minimum count of three AFB reduces the chance of giving a false positive report.

To go further, although a 3-mm opening of a wire inoculating loop delivers about 0.004 ml of water, it delivers about 0.01 ml of the more viscous sputum or concentrated sputum. If a hypothetical smear is prepared from this 0.01 ml of sputum and is 1 cm x 2 cm, we can determine the minimum number of AFB per ml of sputum needed to have a 50% chance of finding at least 3 AFB in 300 oil immersion fields.

The diameter of the field of a 100X oil immersion objective lens in combination with a 10X eyepiece is about 0.2 mm, with an area of about 0.03 mm^2. Three horizontal sweeps (*28*), of a 2-cm wide smear will give about 300 consecutive fields with a total area of about 9 mm^2. This 9 mm^2 area represents 9/200 of the smear and 9/20,000 of a 1-ml sample. Therefore, there must be about 6,000 AFB per ml of sputum to have a 50% chance of finding at least three AFB in the 9-mm^2 area that is observed. This result is based on the assumption that the observed number of AFB in a sample follows the Poisson distribution.

Theoretically, a sputum specimen could initially have a lower number of AFB per ml and there would still be a 50% chance of finding at least three AFB after it has been concentrated to about 6,000 AFB per ml.

Smears that are stained for FAM are usually scanned at a lower magnification (250X-450X) than fuchsin-phenol stained smears (800X-1000X). When a 25X objective lens in combination with a 10X eyepiece was chosen to compare with the 100X oil immersion lens just described, the field was 0.67 mm in diameter and had a 0.35-mm^2 area. A search of 26 fields covered about the same area as 300 fields with the oil immersion lens. In 1950, Jennison and Morgan (*40*) reported a similar ratio of field areas.

Table 1 gives a recommended minimum number of fields to observe for several of the magnifications used for acid-fast microscopy.

TABLE 1. Recommended minimum number of fields to search at selected magnifications before reporting an acid-fast stained smear as negative for AFB.

Magnification[a] :	Number of Fields:
250X	30
400X	55
450X	70
630X	130
1,000X	300

[a]This final magnification represents the objective lens magnification multiplied by the eyepiece magnification.

The scanning of smears for fluorescence acid-fast microscopy at a final magnification of less than 200X is not recommended, because the field may not be filled with exciting light and the relatively very small fluorescing bacilli are difficult to see. The morphology of the AFB should be confirmed at a final magnification of 450X or greater to avoid a false positive report for fluorescing debris that is mistaken for AFB.

There is a striking difference in the time required to observe smears by FAM and BFM. Table 2 illustrates this difference.

TABLE 2. An illustration of the theoretical difference in the time required to observe 50 AFB negative smears by fluorescence and bright-field microscopy. The arbitrary time chosen for observing each field is 3 sec. Actual time will vary with laboratory conditions.

Conditions:	Fluorescence:	Bright-Field:
Final magnification	250X	1,000X
Maximum number of fields	30	300
Time to observe one smear	1.50 min	15.00 min
Time to change slide and record observations	0.25 min	0.25 min
Total time for one smear	1.75 min	15.25 min
Total time for 50 smears	1 h, 28 min	12 h, 42 min

Observe only enough fields to obtain a representative average number of AFB. Count a clump of bacilli as one; give separate counts only for AFB that are not touching others. A clump is considered a single colony-forming unit, and this method of counting clumps will be useful for diluting inocula for media. Large numbers of clumps should be noted in the report to indicate that the true number of AFB is actually greater than the number reported.

Several systems are useful for reporting the results of acid-fast microscopy. One such system is shown in Table 3.

TABLE 3. A suggested method for reporting the average number of acid-fast bacilli found in smears when scanning at 800X to 1,000X[a]

Number of AFB found:	Report:	Alternate:
0	Negative for AFB	–
1 - 2 / 300 fields	Number seen[b]	±
1 - 9 / 100 fields	Number / 100 fields	1+
1 - 9 / 10 fields	Number / 10 fields	2+
1 - 9 / field	Number / field	3+
> 9 / field[c]	> 9 / field	4+

[a]Use of magnifications of less than 800X should be clearly stated on the report or should be adjusted as follows: near 650X, divide count by 2; near 450X, divide count by 4; near 250X, divide count by 10. Example: observed average of 8 AFB per 10 fields at 450X, adjusted to 2 AFB per 10 fields.

[b]Any number of AFB that is less than three per 300 fields is not considered positive, but it does indicate that another specimen should be processed if available.

[c]> = greater than.

This method of reporting communicates an approximation of the relative number of AFB from one smear to another and is useful in determining dilutions of inocula or for monitoring the effect of medication.

If the specimens remain at room temperature after they are collected, microorganisms contaminating the sample will continue to multiply. Specimens processed within an hour of collection will have a relatively small amount of this overgrowth. Although a specimen which is processed immediately after collection is negative for AFB, the microscopist should report any abundant microorganisms seen in the smear. This report may lead to an early recognition of other pathogenic organisms.

Report only the number of AFB seen; you cannot identify *M. tuberculosis* with only acid-fast microscopy.

Keeping accurate records is an important part of laboratory operation. These records must be well organized and must contain enough information to be useful. The organization of records may differ from laboratory to laboratory, but information should be easy to find.

VII. THE MICROSCOPE

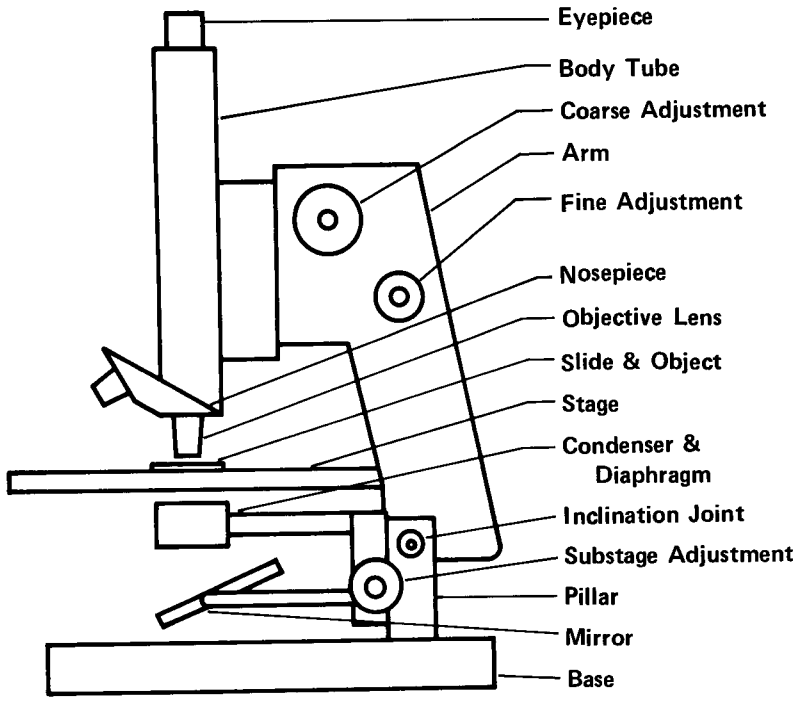
Because the human eye cannot perceive two points less than 0.1 mm apart (*41*) (limit of resolution of the human eye), objects smaller than this must be observed with a microscope. The microscope is a complex optical instrument which makes these small objects appear larger (magnification). The principle of its function is based on the fact that light is bent (refracted) when it passes at an angle from one transparent light conductor into another with a different light bending property. Air is a transparent light conductor and in the microscope, the glass lenses are the others. Magnifying lenses have uniform, circular, curved surfaces that are thicker in the center (convex). Two or more of these lenses are used in combination for magnification.

It is important to learn how to use the microscope correctly. Improper operation could result in costly damage.

A. MICROSCOPE COMPONENTS

1. **Base:** The structure which rests on the table top and supports the rest of the components.
2. **Pillar:** Upright portion extending from the base.
3. **Inclination joint:** The hinge between the pillar and the arm.
4. **Arm:** The structure above the inclination joint which supports other components.
5. **Body tube:** The cylinder which conducts light from the objective lens to the eyepiece.
6. **Nosepiece:** The offset, circular structure at the lower end of the body tube which supports the various objective lenses.
7. **Light source:** Origin of useable light. Usually this is an electric lamp, but skylight may be used; *direct sunlight should not be used* because it may damage the eye.
8. **Mirror:** A reflecting surface for directing the light into the condenser. The surface may be flat or concave (curving inward).
9. **Diaphragm:** A structure which regulates the amount of light passing through the condenser. The diaphragm may be a piece of material with a series of different sized holes, which can be selected, or a single hole of variable size (iris).
10. **Bright-field condenser:** A lens or group of lenses located beneath the stage which concentrates the light on the object to be magnified.
11. **Stage:** The flat surface on which the glass slide supporting the object is placed. The stage may have metal clips to hold the slide in place, or it may have a mechanical device which moves the slide to different positions.
12. **Object:** The material or structure which is magnified.
13. **Objective lens:** The lens which magnifies the object and is attached to the nosepiece.
14. **Eyepiece (ocular):** A lens or group of lenses located at the upper end of the barrel which magnifies the image from the objective lens. The observer looks into this lens to see the magnified object.
15. **Substage adjustment knob:** Turns to raise or lower the condenser and thus change the position of the focal point and, therefore, the intensity or character of the light at the object.
16. **Coarse adjustment knob:** Turns to give a large change of the distance (focusing) between the object and the objective lens.
17. **Fine adjustment knob:** Enables very small changes of the distance (focusing) between the object and the objective lens.

FIGURE 3. Schematic of a Microscope



B. GENERAL INSTRUCTIONS

Always read the specific instruction manual for the microscope before attempting to operate it.

When not in use, the microscope should be protected from dust by a covering or case.

A microscope should be picked up or carried with two hands, one grasping the arm firmly and the other under the base for added support. Never carry a microscope with only one hand.

Microscope lenses may be scratched by dirt or grit. The lenses should be cleaned only with clean, dry *lens* tissue. Cloth or facial tissues may contain grit and should not be used. Moistening the lens tissue with some clean water before using it may be helpful, but this should *not* be done frequently. Never use soap, alcohols, xylene, or other solvents. If lens tissue will not clean the lenses, ask the microscope manufacturer what may be used to safely clean the lenses.

Immersion oil should be wiped from the objective lenses after each day's work. Use non-drying, low viscosity, synthetic, not natural (balsam, cedar oil, etc.) immersion oils, because they are easier to remove.

Unexpected objects may be seen when using the microscope. If these artifacts move only when the slide is moved, they may be: 1) materials occasionally found in the specimen or object; 2) precipitated fixatives or stains; 3) contaminants from the stain or other reagents; or 4) objects suspended in the immersion oil. If the artifacts move while the slide is stationary, they may be: 1) materials suspended in the liquid in the eye; 2) eyelashes or dust on the eyepiece; or 3) materials suspended in the immersion oil. Artifacts that move only when the eyepiece is rotated are in the eyepiece or on its lenses. Artifacts may also be caused by material on the condenser lenses, mirror, or light source.

The object will not be seen clearly if dirt is on the lenses, if the oil immersion lens does not have a layer of immersion oil between it and the object, or if there is a film of oil on the wrong objective lens.

Some microscopes have a single eyepiece (monocular); others have two eyepieces (binocular). Most microscopists recommend keeping both eyes open while looking into a monocular microscope with one eye. This prevents fatigue from holding the other eye closed. Although it is difficult to learn to do this the effort is usually worthwhile.

Binocular microscopes are designed for using both eyes. The eyepieces can be adjusted for the distance between the eyes and, in many, the focus of one eyepiece can be adjusted to compensate for focusing differences in the microscopist's eyes.

Pointers or measuring grids may be placed in some eyepieces.

C. METHODS OF MICROSCOPY

Microscopes may be used in many ways. A few of these methods will be mentioned simply to familiarize the users of this manual with them. A text must be consulted for detailed descriptions.

In reflected light microscopy the object is lighted from above (epi-illumination), and the surface of the object is the most prominent feature.

In transmitted light microscopy the light passes through the object, and variations in color due to staining plus differences in refraction and transparency show the form, structure, and other properties of the object.

Bright-field microscopy is the most common form of transmitted light microscopy. The light passes directly through the object and is altered only by the object and any stains that are present.

Phase contrast microscopy is similar to bright-field microscopy, but it is used to observe unstained structures that are almost transparent. A green light transmitted from below the condenser is altered by partial blocking at the center; differences in the refractive qualities of various parts of the specimen appear to be differences in light conduction. Thus, the parts of the almost transparent specimen are seen in greater contrast than could be seen in regular bright-field microscopy.

In dark-field microscopy the light is directed on the object from below at an angle from the sides rather than from directly below. The refraction and reflection of the light by the object shows its structure in shades of light and dark against a black background.

Fluorescence microscopy is discussed on pages 24-25.

D. PROCEDURE FOR USING A MICROSCOPE

1. Be sure the light source is working properly.
2. Be sure the lenses, mirrors, and other light-conducting surfaces are clean.
3. Check for broken or damaged parts.
4. Adjust the light, mirror, condenser, and diaphragm so that a strong beam of light is directed towards the objective lens.
5. Turn the coarse adjustment knob to move the objective lens away from the stage.
6. Rotate the nosepiece so that a low power objective lens (5X or 10X) is directly over the condenser.
7. Place the slide on the stage so that the object is under an objective lens.
8. Look from the side of the stage so that you can see the space between the slide and the objective lens. Slowly turn the coarse adjustment knob to bring the objective lens very near but not near enough to touch the object, or until the knob stops turning.
9. Adjust the light so that it is bright but not uncomfortable when you look into the eyepiece. This may be done by changing the intensity of the lamp, changing mirror surfaces, using dark filters, adjusting the diaphragm, or adjusting the condenser. Usually, adjusting the diaphragm is sufficient.
10. While looking into the eyepiece, slowly turn the coarse adjustment to separate the objective lens and the stage. The object should come into focus within several turns. If not, go back to step 8.
11. Turn the fine adjustment knob until the object is seen most clearly.
12. While looking from the side, turn the nosepiece to select a higher power lens. Be sure the lens does not touch the slide. The object should be almost in focus, and the best focus can be achieved by turning the fine adjustment knob. A light adjustment may also be needed.
13. To use the oil immersion lens, turn the nosepiece so that no lens is over the object. Let a drop of oil fall on the object, and do not touch the slide with the dropper. Turn the nosepiece so that only the oil immersion lens comes in contact with the oil. Turn the fine adjustment knob to focus.
14. Move the slide slowly and systematically across the stage to observe the object.
15. Scan the slide as directed for your work or as needed.
16. Report only what you see and not what you think you are supposed to see.
17. When you have finished with the slide, raise the objective lens and remove the slide.
18. When you have finished with the microscope, clean the oil off the lens, place the low power objective lens as if it were to be used, turn off the light source, and cover the microscope or place it in its cabinet.

E. FLUORESCENCE MICROSCOPES

This is simply an introduction to fluorescence microscopes; it is not intended to be a thorough discussion. Textbooks, manufacturers' manuals, and scientific publications should be consulted for specific techniques and operation instructions.

Fluorescence stains are usually organic substances which are excited by specific wavelengths of light and then emit light of a longer wavelength. The stains commonly used are excited by violet or ultraviolet light and fluoresce red, yellow, or green. Some stains used for fluorescence techniques are fluorescein, rhodamine, auramine O, and acridine orange. Poor fluorescence of the stains may be caused by improper pH of the staining system, reaction of heavy metal ions with the stain, exposing the stain to strong oxidizing or reducing substances, precipitation of the stain, and exposing sensitive stains to excess heat or light.

The lens system of a fluorescence microscope is the same or similar to that of a standard microscope and, after some adjustments, usually may be used for transmitted light microscopy. There are several lighting systems to choose from. The high intensity tungsten filament lamp can be used for most fluorescence acid-fast techniques, but not for fluorescent antibody. This is the least expensive and the easiest system to maintain. The halogen lamp system can be used for most fluorescence microscopy methods, including fluorescent antibody. The high pressure mercury vapor lamp has been the most widely used system, but it is costly and requires advanced technical skill to maintain. Some xenon lamps and carbon arcs have been used, but they have not been popular. All of these lighting systems require one of the standard electrical power supplies which has little or no voltage fluctuation.

The lamp must have its maximum output in that part of the light spectrum that includes the desired waveband and at an intensity that will excite the stain to its maximum fluorescence. The position of the lamp can be adjusted within its housing to focus the light source on the stained object. There is usually a reflector and/or a collecting lens in the lamp housing to aid in concentrating the light on the object.

Many lamps produce enough heat to damage the glass filters transmitting the light. A heat absorption filter is placed between the lamp and the other glass filters to protect these filters.

An exciter filter is chosen to absorb the unwanted red, yellow, and green light and to transmit the waveband of light that excites the stain to fluoresce. These exciter filters are colored glass, usually dark blue, or are the interference type of light filters; they are placed between the heat absorption filter and the stained object. In some microscopes the exciting light is transmitted down through the body tube and the objective lens to excite the fluorescent material in the object (epi-illumination). This allows the microscopist to scan objects that would not transmit light well, such as rocks, minerals, or thick specimens. This type of illumination may also be used for fluorescence microscopy of smears and tissue sections.

A mirror is usually used to direct the light into the condenser; the mirror must have a front surface of aluminum or silver if it is to reflect ultraviolet light.

The condenser must be designed to give maximum illumination in the area of the object directly under the objective lens. It is usually an oil immersion, dark-field condenser. Some techniques require only a bright-field, dry condenser that focuses the light source on the object (critical illumination).

There must be a light filter between the objective lens and the eye which absorbs the light passed by the exciter filter, but allows the light emitted by the fluorescing stain to pass. This is the barrier filter. It is usually made of colored glass or colored gelatin that has been laminated between two pieces of plain glass, and these filters usually appear yellow or orange. The exciter and barrier filters must be properly matched to block out all light from the lamp and still allow the light from the fluorescing stains to reach the eye.

VIII. THE CHEMICAL BALANCE

The chemical balance is an instrument for accurately determining the weight (mass) of substances. This instrument has fragile components and must be used with care. This outline is an introduction to the use of the balance.

The basic unit of weight measure used with the chemical balance is the gram, which is abbreviated g. One thousand g is the kilogram (kg), and one-thousandth of a g is the milligram (mg).

A. COMPONENTS

1. **Pan:** A platelike receptacle for the substance to be weighed that is suspended from or supported by the beam. A second pan may hold the standard weights that are used to match the weight in the other pan.
2. **Beam:** A long, solid structure that rests and is balanced on one edge of a wedge (knife edge) and can rock for a short distance on this edge. This supporting edge is easily broken and must be protected from shocks.
3. **Support:** The structure that holds the knife edge on which the beam rests.
4. **Pointer:** A free-swinging needle which indicates the balance of the beam.
5. **Pointer scale:** A scale of numbers which shows the range of swing of the pointer but does *not* indicate weight.
6. **Rider:** A known weight that is moved across a scale on the beam to balance the beam.
7. **Rider scale:** A series of numbers that indicates weight according to the position of the rider when the beam is balanced.
8. **Weight:** Known standard weights which are usually placed in the right pan to balance the beam. The weights should be picked up with forceps only and *not* with the fingers.
9. **Beam arrest:** A mechanism to lift the beam from the supporting edge when changing weights or materials or when the balance is not in use.
10. **Pan arrest:** A mechanism to stop unwanted swinging of the pans.
11. **Vernier:** A sliding double scale found on some balances that increases the accuracy of the reading.

FIGURE 4. Schematic of a Chemical Balance

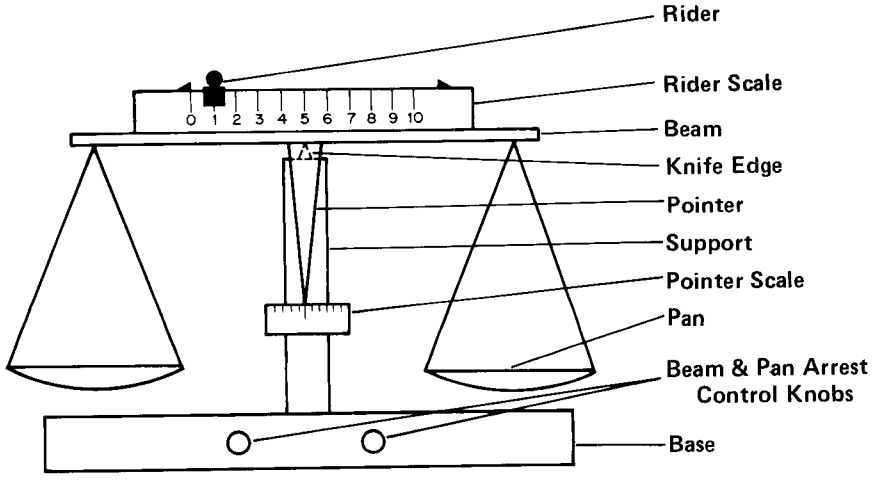
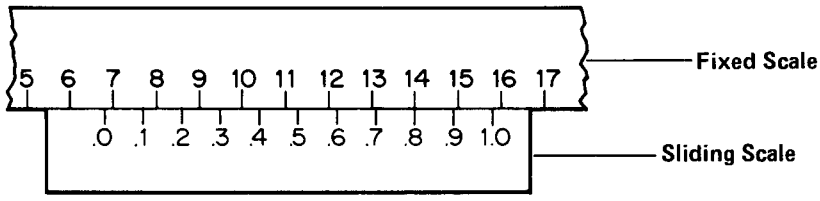


FIGURE 5. A Vernier Scale



To read a Vernier scale, read to the highest number on the fixed scale before the 0 on the sliding scale (example is 6), then look up the sliding scale to the point where a line on the sliding scale most closely matches a line on the fixed scale; this line on the sliding scale is the last part of the reading (example is .8). Final reading on the example is 6.8.

B. GENERAL INSTRUCTIONS

Some balances may not have all of the components listed, and others may have additional components. The single pan and double pan balances function on the same principles, but for the single pan balance, the weights are added to the end of the beam opposite the pan by a mechanism that is controlled by a series of knobs or sliding weights. Always consult the manual for detailed operating instructions.

Always keep the balance and weights clean and dry to protect them from corrosion. Any change in the surface of the parts may affect the accuracy of the balance.

Engage the beam arrest and pan arrest when adding or removing weights or material, when you have finished using the balance, and when moving it.

Do not put material to be weighed directly on the pan; always use a container or weighing paper for this material.

Subtract the weight of the container or weighing paper (tare weight) from the combined weight of the container and material weighed.

As a general rule, unused substances should not be returned to the stock bottle to prevent contamination of stock materials. Each laboratory will have to determine what is to be done with unused substances from stock bottles.

Protect the balance from drafts of air. The more accurate balances have individual cases which are to be closed at the final reading. Air moving across the pans will cause an inaccurate reading.

C. PROCEDURE

1. Have all materials ready: balance, pen or pencil, note paper, substance to be weighed, weighing container or paper, and spatula or spoon.
2. Be sure the balance is clean and the pointer indicates that the initial reading is zero when the beam and pans are released.
3. Be sure the spatula or spoon is clean.
4. Engage the beam and pan arrests.
5. Place the weighing container or paper on the left or single pan.
6. Place a similar standard weight on the right pan or move the rider to an approximate position to equal the weight in the left or single pan.
7. Slowly release the beam arrest and then the pan arrest.
8. Gently move the rider or change small weights until the pointer swings equally to either side of zero, or rests on zero, if the balance has a damper. Engage the beam and pan arrests before adding or removing large weights.
9. Record the tare weight of the weighing container or paper.
10. Engage the beam and pan arrests.
11. a. To find the weight of a given volume of a substance, do the following. With a spatula or spoon, put the material to be weighed in the weighing container or on the paper and proceed to weigh the material as in steps 5-10 and then subtract the weight of the weighing container or paper from the final weight.

    b. To obtain a desired weight of a substance, do the following. Add the tare weight and the desired weight to determine the combined weight needed. This will be the final weight onthe balance. Place the appropriate standard weights in the right pan and/or use the rider so that the weights on the balance are the same as the calculated total. Place an appropriate amount of material in the weighing container, then gently release the beam and pan arrests. If the instrument is not balanced, engage the beam and pan arrests and add or remove some of the material as needed. Release the beam and pan arrests. Continue in this manner until the instrument is in balance. Add only minute amounts of material without engaging the beam and pan arrests.
12. Engage the beam and pan arrests and remove the weighing container and material.
13. Remove the standard weights and put them away. Place the riders on zero. Clean up any spilled material with a powder brush.

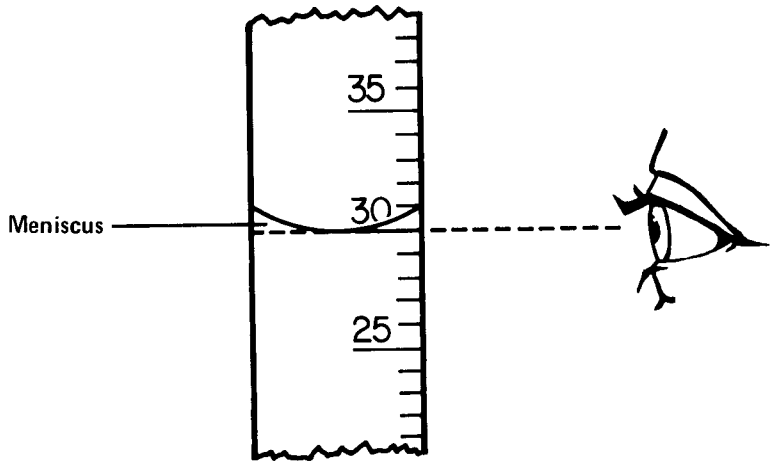
IX. MEASUREMENT OF LIQUIDS

Liquids are usually measured in volume units called liters or one-thousandths of a liter, milliliters. They are abbreviated "l" for liter and "ml" for milliliter. Many large vessels, such as beakers and flasks, are marked in *approximate* volumes but graduated cylinders and graduated pipettes must be used for more accurate measurements. Very accurate volumes are measured with volumetric flasks and volumetric pipettes. Graduated cylinders and graduated pipettes are satisfactory for most applications, when used properly.

A. GRADUATED CYLINDERS AND PIPETTES

1. Be sure the cylinder or pipette is clean and dry before use.
2. Do not use graduated cylinders for mixing liquids.
3. Cylinders and pipettes may be reused for measuring the same liquid if there are no deposits from drying between measurements.
4. For general laboratory use, the cylinder may be stored for reuse after it is rinsed with three or four small volumes of distilled water and air dried. It should be stored with the open end down to exclude dust.
5. Pipettes should not be reused for different kinds of liquids unless they have been cleaned, rinsed in distilled water, and dried.
6. Be careful not to contaminate the liquid being measured with cleaning materials or deposits from other liquids.
7. Liquids tend to cling to the sides of containers and the surface appears to curve downward in the middle when viewed from the side. This downward curve is called the meniscus.
8. When measuring liquids, align the bottom of the meniscus with the line of the desired volume. The measuring vessel should be vertical and your eye should be at the same level as the bottom of the meniscus.
9. Usually, the meniscus is easier to see if a light colored card with a wide, dark, horizontal band is held behind the cylinder near the area of the meniscus.
10. Allow the cylinder to drain well when pouring out the measured contents.
11. Graduation on pipettes vary according to how they are to be used. The numbers may indicate the number of milliliters remaining in the pipette or how many have been dispensed. Some pipettes are designed to have some liquid remain in the tip after dispensing; others, including most serological pipettes, are designed to have all of the liquid expelled.
12. Mechanical suction and dispensing devices, such as a rubber bulb, should be used with pipettes. Do not mouth-pipette caustic and flamable liquids, any specimens, or cultures of microorganisms.
13. Grasp the pipette firmly near the suction end when attaching it to a rubber bulb or other suction device. This reduces the chance of breaking the pipette and cutting the hand. Slightly moisten the opening of the bulb or pipette with water, which acts as a lubricant for inserting the pipette. A simple suction device for pipettes is a short piece of thin latex tubing that will fit snugly over the pipette and has a stopper in one end.

FIGURE 6. Sighting the Meniscus in a Graduated Cylinder



B. DILUTING ALCOHOLS

At times concentration of alcohols and other solutions must be reduced; that is, they must be diluted. A liquid that is 100% means that 100 parts of 100 is that liquid only; a liquid that is 70% means that only 70 parts are that liquid and 30 parts are something else. Determining the portions for diluting a 100% liquid is not difficult. Diluting a liquid that is not 100% however, requires some calculations. In the examples, the stock concentration is 95% alcohol and the desired concentration is 70%.

Example 1:

Assume 100% = 100 ml

Assume that 100 ml less 70 ml (70%) = 30 ml distilled water to be used to dilute the 95% alcohol.

3. Then the amount of 95% alcohol used must be increased to account for the 5% that is water. Divide one by the decimal equivalent of the percent concentration of the stock alcohol and multiply this factor times the 70 ml of pure alcohol needed to obtain the increased amount of alcohol to use.

$$\frac{1}{0.95} = 1.05$$

$$\begin{array}{r} 70 \text{ ml} \\ \times\ 1.05 \\ \hline 350 \\ 70 \\ \hline 73.50 \text{ ml of 95\% alcohol needed} \end{array}$$

$$\begin{array}{r} 73.5 \text{ ml of 95\% alcohol} \\ +30.0 \text{ ml distilled water (diluent)} \\ \hline 103.5 \text{ ml 70\% alcohol} \end{array}$$

Although the final concentration will not be exactly the one desired, the method in this example can be used for most dilution applications. Just substitute the appropriate numbers for those in the example and complete the computations.

Example 2:

1. Stock is X% (95%) and the desired concentration is Y% (70%).
2. Use the numbers representing the percent concentrations involved to represent milliliters of stock solution and desired solution in the following formula.

Y ml of stock + (X – Y) ml of diluent = Y + (X – Y) ml of Y%

70 ml of stock + (95 – 70) 25 ml of diluent = 95 ml of 70%

To obtain larger volumes, just multiply the volumes in the formulas by the same appropriate number.

X. THE CENTRIFUGE

A centrifuge is a machine that creates an artificial gravity by spinning an object or, more often, a liquid, at the outer edge of a circular head. The amount of artificial gravity or relative centrifugal force is determined by both the rate of spin or revolutions per min (r/min), and the radius of spin or distance from the center of the spinning head to an outer point where the force is to be measured. The relative centrifugal force is increased by increasing either the rate of spin or the length of the radius. Either change requires more energy. The maximum force occurs at the part of the centrifuge tube farthest from the center of the shaft while it is spinning.

The relative centrifugal force can be determined by using the formula:

$RCF = 1.118 \times 10^{-5} \times R \times N^2$

RCF is the relative centrifugal force, which is an expression of multiples of standard gravity (g). An RCF of 2,000 = 2,000 x g.

R is the radius of the circular path of the bottom of the centrifuge tube in a line perpendicular to the rotating axis as it would be during operation, and it is measured in centimeters.

N is the number of revolutions per minute.

There are other formulae for determining RCF, but they are based on other systems of measurement.

The numbers on most speed controls on electric centrifuges do not always indicate the r/min of the centrifuge. A tachometer must be used to determine the r/min of a centrifuge. Consult the manual or ask the manufacturer about the rated r/min or RCF of your centrifuge.

Substances can be described by their ratio of weight to volume; this is called density. Liquid and solid substances separate if their densities differ, and the greater the difference, the easier the separation. If the solid is more dense than the liquid, it will sink; if less dense, it will float.

Liquids become more dense if substances of greater density are dissolved in them. Therefore, the more material that is dissolved in the water during the centrifugation of specimens, the more difficult it is to have the particles settle to the bottom of the tube. Further, adding sterile distilled water to water solutions that are more dense than pure water improves the sedimentation by lowering the density of the solution.

As specimens are centrifuged, they are usually separated into the sediment of solid material at the bottom of the tube and the liquid supernatant.

Glass tubes may break under the stress of centrifugation, safety, screwcap centrifuge cups, as well as screwcap centrifuge tubes should therefore be used for potentially infectious material. If a centrifuge tube breaks in an open cup, the liquid will splash or be blown out and aerosolized.

Be sure that shock absorbing pads are in the bottom of the centrifuge cups.

The centrifuge head must be in balance while spinning. An out-of-balance head vibrates and may break. The heads are balanced at the factory. If any component or tube is added to one side of the head, an equivalent weight must be added to the opposite side. Tubes used in processing specimens will usually be balanced satisfactorily if matched pairs have matched levels of liquid in them.

Tubes should be wiped dry of any spills before they are placed in the centrifuge.

Do not touch any centrifuge head while it is spinning. Touching it may not only cause injury, it may also cause rapid or erratic stops which stir the sediment and resuspend it. Some centrifuges are equipped with a brake to gradually slow the spinning head.

Again, read the manual before operating a piece of equipment for the first time, and keep the manual with he equipment for ready reference.

XI. DISINFECTION AND STERILIZATION

Disinfection is the process of killing most of the organisms whch can cause disease; sterilization is the process of killing all organisms, whether they can cause disease or not.

Methods of disinfection and sterilization, especially in mycobacteriology, will be briefly discussed. Consult microbiology texts for more complete information.

Phenol (carbolic acid) was one of the first chemical disinfectants used; and the effectiveness of other disinfectants are compared to about a 1% solution of phenol under standardized conditions. Pure phenol is a colorless, clear crystal, but is frequently sold as a 90% liquid solution for use as a disinfectant or medical preservative. A 5% solution kills tubercle bacilli very well, but will also cause severe burn-like lesions on the skin after a few minutes exposure.

Substituted phenols, such as cresol, are usually very good disinfectants. They can be used effectively at lower concentrations than phenol and are not as destructive to human tissue.

Formaldehyde is a good chemical disinfectant for some purposes. Liquid solutions can be harmful to the skin, and the vapors can damage the eyes and breathing passages. It is not desirable for general use in a mycobacteriology laboratory.

Sodium hypochlorite (NaClO) has good disinfectant properties but is not used as a general disinfectant because of its strong odor at effective concentrations. It is also inactivated by organic material. It is used in some specific applications.

Ethanol and isopropanol are used as disinfectants in some situations but are not recommended for general use, because they evaporate too rapidly when used on open surfaces.

The quaternary compounds are good general disinfectants, but are not effective against mycobacteria. In fact, several have been used as decontaminants to isolate live tubercle bacilli by culture (*16,42,43*). The quaternary compounds are not as irritating to human tissue as most other disinfectants and are used as surgical skin antiseptics and for disinfecting kitchen and dining utensils.

Many chemical disinfectants are effective only under specific acid or alkaline conditions and when used in adequate concentrations and for sufficient exposure time. Further, many are inactivated by organic material or metals, and some do not affect bacterial and fungal spores.

Ultraviolet (UV) light is used to disinfect or sterilize the surface of objects or the air in a room. The most effective rays are in the waveband near 254 nanometers. These rays cannot be seen, though other light from a lamp is visible; and the UV light production of a lamp must be checked with a UV light meter to determine when a lamp is no longer effective. The rays can cause severe irritation (sunburn) to the eyes and skin, so direct exposure should be avoided. Most of the effective UV rays do not pass through ordinary glass. Therefore, glass will inhibit disinfection, and eyeglasses can give some protection from eye irritation.

The sun radiates UV light and sunlight contributes to the disinfection of air and surfaces exposed to it.

The effect of UV irradiation is governed by both the time of exposure and the distance from the lamp. The longer the exposure or the closer to the lamp, the greater the effect.

Heat is used for disinfection and sterilization. Many microorganisms, including tubercle bacilli, are killed in liquids, such as milk or wine, by a moderate heating process called pasteurization. Those liquids would be damaged by the more effective boiling. Used sputum containers and other contaminated articles to be boiled should be placed in a pot of unheated water and then heated until the water has boiled for 10-15 minutes.

Incineration, or burning, refers not only to the disposal of waste; it is also a method of removing organic debris from fire-resistant equipment, such as bacteriologic transfer needles and loops. Liquids and moist substances will spatter when exposed to these high temperatures. This spattering can create hazardous aerosols if the spattered material contains infectious organisms. Liquid or moist, potentially infectious refuse should be boiled or autoclaved before it is incinerated. Bacteriologic needles and loops should be rinsed in 70%-95% ethanol before being incinerated.

Two additional methods of heat sterilizing are dry heating and autoclaving (*44*).

Objects to be sterilized by dry heat are placed in an oven and heated at 160°-180° C for 1 or 2 hours. A disadvantage of dry heat sterilization is that heat penetrates large or low density items slowly; this means that for these items the exposure time must be increased.

Autoclaving is the most efficient means of sterilizing equipment and heat-stable liquids. The autoclave is a sealing chamber which can contain steam at more than 1.06 kg/cm^2 (15 lb/in^2). The significance of the 1.06 kg/cm^2 pressure is that steam is most saturated at this pressure and the temperature will be 121° C at sea level.

The time required to kill the most resistant spores under these conditions is 10 minutes. The usual time for autoclaving is 15 minutes.

The reason autoclaving is so efficient is the latent heat of steam. The steam releases not only heat energy as hot air would, but also the additional heat energy required to change water to steam as the steam condenses to water on objects in the autoclave.

Two important factors for the optimum function of an autoclave are that: (1) all of the air in the chamber should be replaced by steam and (2) the temperature must be 121° C. The air is released from the chamber, usually from the bottom, as the chamber fills with steam from the top. Any air remaining in the chamber will reduce the efficiency of the steam. Pans of material to be sterilized must have some water added to turn to steam as the pans are heated. This prevents air pockets from forming in the pans.

The 1.06 kg/cm^2 (15 lb/in^2) pressure in the autoclave is shown as a comparison to atmospheric pressure by most pressure gauges and control devices. Atmospheric pressure decreases as altitude increases. At an altitude of 1,500 m (5,000 ft) above sea level the pressure would be reduced by 0.21 kg/cm^2 (3 lb/in^2) and the internal temperature would be 117.6° C although the gauge showed a 1.06 kg/cm^2 pressure. In sterilizing at high altitudes, it is best to maintain a temperature of 121° C without exceeding the pressure limits of the autoclave.

The time for autoclaving must be increased if the temperature does not reach 121° C or if the material to be sterilized is so large that heat cannot rapidly penetrate it.

An autoclave can be checked for proper heating or sterilization in several ways. A tape with a dye that changes color at 121° C can be applied to objects to be sterilized, or a vial with a material that melts at 121° C can be placed in the autoclave. The best method is to put a vial of selected bacterial spores in the autoclave with the material to be sterilized and to test the spores for growth after autoclaving.

Pressure gauges and thermometers may not function properly, so autoclaves should be tested periodically.

Improper operation of the autoclave can be very dangerous. It can explode if the pressure is too great, if a part is defective, or if the door or cover is opened while under pressure. The caps of containers of liquid or moist material should be left loose during autoclaving because the container may explode when the autoclave is opened if the caps are tight.

Large pressure cookers may be used as substitute autoclaves.

Be sure to follow the instructions for operating these sterilizers.

XII. GRAM STAIN

The Gram stain is a general differential bacterial stain, not an acid-fast stain. It has been included for those familiar with its use and will not be discussed.

Reagents:

1. Crystal Violet: Dissolve 2.0 g crystal violet in 20 ml 90%-95% ethanol or methylated spirits; then add 80 ml aqueous 0.1% ammonium oxalate.
2. Gram's Iodine: Dissolve 2 g potassium iodide in 300 ml distilled water; then add and dissolve 1 g iodine crystals.
3. Safranin: Dissolve 0.25 g safranin O in 10 ml 90%-95% ethanol; then dilute with 90 ml distilled water.

Procedure:

1. Prepare and heat-fix smear.
2. Flood smear with crystal violet solution and stain for 1 min.
3. Rinse with tap water and drain.
4. Flood with Gram's iodine and let stand for 1 min.
5. Rinse and drain.
6. Flood with 90%-95% ethanol and let stand for 30 sec.
7. Rinse and drain.
8. Flood with safranin solution and counterstain for 30-60 sec.
9. Rinse, drain, and dry.

Gram-positive bacteria are dark blue; Gram-negative bacteria are red or pink.

XIII. LIST OF MATERIALS AND EQUIPMENT FOR ACID-FAST MICROSCOPY

Items may be deleted or added, depending on the situation.

microscope, with mechanical stage
immersion oil & dropper bottle
lens tissue
microscope slides
slide storage boxes
basic fuchsin
methylene blue chloride
auramine O
rhodamine B
acridine orange
crystal violet
safranin O
potassium permanganate
dibasic sodium phosphate (anhyd.)
potassium iodide
iodine crystals
ammonium oxalate
phenol crystals
ethanol
methanol
hydrocholoric acid, concentrated
sodium hypochlorite solution (5%-6%)
sodium hydroxide
sulfuric acid
alcohol lamps
Bunsen burners
slide warmer
staining racks
centrifuge, 1,800 - 2,400 X g
test tubes, screwcap
test tube racks
balance, chemical, accurate to 0.01 g
weighing paper
chemical spatula
tongue depressors, wood
powder brush for balance
pipettes, tip delivery, 1, 5, & 10 ml
capillary pipettes
pipettors (bulb, teat, tubing)
pipette washer
graduated cylinders
beakers
flasks, screwcap & Erlenmeyer
brown bottles, glass or plastic
dropping bottles, dark
specimen containers
specimen transport boxes
interval timer
large plastic bottles (20l)
latex or plastic tubing
pinch clamps, tubing
glass tubing
towels, cotton
cleaning cloths
paper towels
applicators, wood
cotton sponges
disinfectant
disinfectant soap
hand soap
glassware detergent
filter funnels & filter
biological safety cabinet
smocks or surgical gowns
bacteriological filter masks
surgical gloves, latex
work gloves, rubber or cotton
distiller, water
deionizer, water
tool kit
wire inoculating loops
reference manuals
laboratory manuals
record books
pens & pencils
glass marking stilus
glass marking pencil, wax
tape, paper, adhesive (masking tape)
autoclave, sterilizer
discard pans w/lids, for autoclave
tape, paper, adhesive, heat indicator

FIELD LABORATORY

electric generator, fuel, and tools
transformers
autoclave/pressure cooker, sterilizer
discard pans w/lids, for autoclave
stove, fuel cans, and funnel

XIV. GLOSSARY

AEROSOL: The fine mist or small droplets produced when a liquid is mechanically disrupted.

ANTICOAGULANT: A substance added to blood specimens to prevent clotting.

ARTIFACT: In microscopy, an unnatural condition or object seen in the field that is not desirable or interferes with the work.

AQUEOUS: Containing or related to water.

ATYPICAL MYCOBACTERIA: See mycobacteria.

BACTERICIDAL: Something that kills bacteria.

BCG: An abbreviation for Bacillus Calmette-Guerin. BCG is the name given to those strains of modified, attenuated *Mycobacterium bovis* that are used to immunize against tuberculosis.

BIOPSY: A surgical procedure to take living tissue from a patient for examination.

CASEOUS: A term used to describe a substance that has the appearance of milk curd in the initial step of making cheese.

CONTAMINANT: An unwanted living organism or substance in a specimen or other material.

CULTURE: To grow, or the growth of, microorganisms on or in a specified nutritious substance (medium, media).

DEBRIS: The name given to nonspecific or useless material.

DILUENT: A substance, usually liquid, which reduces the concentration of another specified substance when added.

DISINFECTANT: Any substance that is used to kill unwanted microorganisms.

DROPLET NUCLEI: The minute solid particles that remain after droplets of liquid have evaporated while suspended in the air.

DYE: A colored substance that penetrates various materials, is difficult to remove, and is used to give color to designated objects.

EXUDATE: A substance, usually liquid, that is given off gradually by a specific area or structure in or on the body. This term is frequently used to refer to discharge from lesions.

FOCAL POINT: The point at a specified distance from the central plane of a lens where rays from a light source converge after passing through the lens.

FUNGI: A group of organisms whose structures are usually composed of long filaments, are not motile, may reproduce by sexual or asexual spores and exist on other dead or living organisms. Examples are mushrooms, toadstools, molds, mildew, and also the single celled yeasts.

GASTRIC: A term used to describe something related to or coming from the stomach.

GENITALS: The external sexual organs or structures.

INFECTIOUS: A term used to describe organisms that can enter another type of organism and cause disease and are frequently transmitted from one individual to another.

INOCULUM: (PL. - INOCULA) Material that usually contains microorganisms that is deposited intentionally or accidentally in or on culture media or introduced into the body.

LESION: An area in or on the body where tissue destruction has taken place.

MEDIUM: (PL. - MEDIA) See culture.

MICROORGANISM: See organism.

MICROTOME: An instrument that is used to slice tissue into very thin sections for microscopic examination.

MORPHOLOGY: The form or appearance of an organism.

MYCOBACTERIA: A group of microorganisms that are characteristically acid-fast, usually appear as bacilli, do not form spores, and are resistant to strong acids and alkalies. *Mycobacterium tuberculosis* (the tubercle bacillus) is the species of mycobacteria most frequently seen in sputum specimens. The term "atypical mycobacteria" has been used to refer to those species of mycobacteria that are not the typical tubercle bacilli.

NECROPSY: The examination of a dead body.

ORGANISM: Any living being. Microorganisms are very small organisms.

PATHOGEN: Anything that causes disease.

PRECIPITATE: A substance that was in solution and then was caused to form solid particles and settle to the bottom of the container.

pH: A symbol which concerns the acidity or alkalinity of a material, usually water solutions. pH 7 is neutral, pH 1-6 is acid, and pH 8-14 is basic or alkaline.

QUENCH: To reduce intensity such as heat or light.

SAPROPHYTE: An organism which usually feeds on nonliving organic material.

SPECIMEN: In medicine, a sample from a body either given off naturally or taken from it for examination.

STAIN: See dye.

STERILE: To be free of living organisms.

SUBDERMAL: A position just under the skin.

TISSUE: Living or once living material composed of similar cells and cell products that has a consistent nature and is usually combined with other types of tissue to form organs.

TUBERCULIN: A material that has been isolated from a *Mycobacterium tuberculosis* culture. This, or a purified protein derivative (PPD) of tuberculin, is injected into the skin to test for previous mycobacterial infections.

TUBERCLE BACILLI: See mycobacteria.

YEAST: See fungi.

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