

TUBERCULOSIS

Laboratory Methods
in
Diagnosis

By
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With the assistance of
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Tuberculosis and Related Pulmonary Disease

General Aspects

Tuberculosis is an infectious disease that spares neither age, sex, race nor nationality. Although capable of infecting almost any organ of the body, tuberculosis is most generally associated with the lungs. Because of this pulmonary localization, the disease generally spreads from man to man via the respiratory route – coughing, sneezing, and expectoration all serve as vehicles of transportation for the organism carrying it from man to man. Once the tubercle bacilli have gained access to the lung tissue, the process of disease is generally slow; the development is so slow that often the disease goes undetected until the individual has developed an advanced case of tuberculosis. Treatment, then, may consist of surgery, prolonged hospitalization and/or treatment with a battery of antibiotics. It may be necessary for treatment to continue for months and sometimes years before a return to good health is achieved. Present evidence seems to indicate that once a person is infected there is almost never complete elimination of the tubercle bacillus from the body.

Following infection with tubercle bacilli, man becomes hypersensitive to the protein of the tubercle bacillus, i.e., if tubercle bacillus protein were injected into his skin he would react with a delayed (1-4 days) allergic response as evidenced by inflammation – the reaction would be localized at the site of injection and would be characterized by a red, inflamed area and firmness at the center. This is what is referred to as the tuberculin skin test. The skin test serves several useful purposes. Since a positive tuberculin reaction indicates presence of tubercle bacilli, the test can be used to screen large groups of people; the positive reactors are further followed up with X-rays. A positive reaction does not tell how old or how bad the infection is, nor does it tell whether the disease is active or healed – it merely indicates that the tuberculosis organisms are present in the body. Because of this fact a positive test in an adult may not mean much unless accompanied by clinical symptoms of disease; however, a positive test in young children may be useful in diagnosis of tuberculosis as it may indicate active, unhealed tuberculosis, or a recently acquired infection.

What about immunity to tuberculosis? Can we immunize or protect against the infection just as we do with smallpox, tetanus, and diphtheria? There is a vaccine for tuberculosis called BCG (the **B**acillus of **C**almette and **G**uerin) which contains a bovis variety of tubercle bacillus (the type that primarily infects cows); the organisms in the vaccine are still viable but they have been grown in an unfavorable environment so that they no longer will produce disease.

BCG has the disadvantage of causing reaction to tuberculin, thus removing an important screening and diagnostic tool. The protection it confers is not considered to be sufficient to justify wide use in the United States, where other control methods have reduced risk of exposure to a very low level. It is recommended here only for persons unavoidably exposed to tuberculosis where they live or in their work (laboratory workers, for instance). In other countries, where tuberculosis mortality is high and the risk of infection considerable, BCG is routinely given to young children. It is the fond hope of many research investigators that a vaccine will be developed to protect all people against possible infection with tubercle bacilli.

Despite the fact that the organism responsible for the infection was not found until 1882, tuberculosis is an old disease, having been described by Hippocrates about 400 B.C. The tubercle bacillus, discovered by Robert Koch, has a rather impressive biological classification: Class – Schizomycetes; Order II – Actinomycetales; Family I – Mycobacteriaceae; Genus I – Mycobacterium; Species – tuberculosis. In bacteriology, however, this extensive classification is simplified by merely referring to the organism by its genus and species name, i.e., *Mycobacterium tuberculosis*. Clinically and bacteriologically several species of mycobacteria are important. Thus, we have *M. tuberculosis* as the principal cause of tuberculosis in man; *M. bovis* infects cattle primarily, although it can infect man and indeed in some European countries is a major cause of human tuberculous infection, while *M. avium* infects fowl.

Unlike other microorganisms, the mycobacteria stain only with difficulty, requiring either several minutes in contact with the dye or application of heat to force the dye into the cells. Once stained, however, the mycobacteria resist decolorization with strong mineral acids; this property of tubercle bacilli has led to their designation as acid-fast bacilli – i.e., once stained they resist decolorization with strong acids.

The tubercle bacilli are very resistant to many chemicals and disinfectants, consequently, they pose quite a problem in hospitals and sanatoria where one is faced with the problem of sterilizing or disinfecting equipment. Fortunately, the organisms are readily killed by heat, so that equipment which can be treated by boiling or sterilization by steam under pressure can readily be disinfected. Certain equipment, such as thermometers, cannot be sterilized by heat, hence, a disinfectant must be used. There probably is not complete agreement as to the disinfectant of choice, but those that have been used include 70 percent alcohol, such phenol-containing agents as cresol, lysol or amphy, and certain iodine containing compounds, to mention a few.

Since methods for the laboratory diagnosis of tuberculosis will be discussed in detail in subsequent sections of this manual, at this point it is sufficient to say that the major tools available for bacteriologic identification of tubercle bacilli are microscopy, cultural characteristics and animal pathogenicity.

What is the importance of tuberculosis from the medical viewpoint? The tubercle bacillus probably causes more deaths in the United States than does any other single microbial agent, in spite of the fact that the death rate has steadily declined since the middle 1800's. World Health Organization statistics reveal that tuberculosis also is the most potent international disease known, alone accounting for three-fourths of all deaths from infectious diseases occurring after age fifteen. In the United States tuberculosis once claimed the lives of 200 people per 100,000 population, but today, thanks to medical research, refined surgical procedures, and the advent of such chemotherapeutic agents as isonicotinic acid hydrazide (INH), streptomycin and para-aminosalicylic acid (PAS), the tuberculosis mortality figures have dropped to their present level of 7/100,000. This figure is still extremely high when compared to such communicable diseases as diphtheria, scarlet fever, smallpox, polio and others where the death rate is less than one per 100,000. Thus, it can be seen that tuberculosis is an important public health problem which still requires extensive cooperative research on the part of clinicians, bacteriologists and administrators before it can be listed as one of the less important causes of human mortality.

The successful treatment and partial eradication of one disease producing agent frequently brings another to the fore — tuberculosis is no exception to this statement. We cannot be absolutely certain, however, if effective control of true tubercle bacilli has increased our awareness of other types of acid-fast bacilli, but regardless of the cause, there is no denying that both clinician and bacteriologist have become more aware of a somewhat nebulous group of acid-fast bacilli that have been dubbed the anonymous or atypical acid-fast bacilli. These organisms produce a disease which is clinically very much like tuberculosis, however, culturally they exhibit several unique characteristics. It was on the basis of these unique properties that the term "atypical" was introduced, encompassing all those cultures in which one or more of the following features existed:

1. Frequently, but not always, the colonies were pigmented and smooth.
2. A large percentage of the strains were resistant to para-aminosalicylic acid and often moderately resistant to streptomycin and isoniazid.
3. These strains generally have a lowered degree of virulence for guinea pigs and mice than do human and bovine strains.

Because of the fact that these organisms were not able to produce progressive disease in the guinea pig, a great deal of skepticism hung over early reports of their pathogenicity for man. However, repeated isolation of these organisms from sputum and resected lung tissue of the same patient, in the complete absence of other pathogenic organisms, is strong evidence that in these patients the

“atypicals” are the causative agents of human disease. With the almost universal acceptance that some of these organisms may be agents of disease, certain problems arise, including the following:

1. Are there different kinds or types of anonymous acid-fast bacilli, and if so, how are they differentiated?
2. Are they closely related in any way to known mycobacteria? If not, how can they be distinguished from existing named species?
3. From whence in nature do these organisms originate?

Let's attempt to answer these questions in order. First, there apparently are different kinds or types of anonymous acid-fast bacilli. At the present time their classification on the basis of speed of growth and pigment production has offered the most feasible means of differentiation, however, as we learn more about these organisms, a more clear-cut and exacting taxonomic scheme will unquestionably be developed. The existing method of classification was devised by Dr. Ernest H. Runyon and provides for the following four groups:

Group I – Photochromogens. The strains of this group are sufficiently distinct and homogeneous that they have been named *Mycobacterium kansasii* Hauduroy. Unless a search of the literature discloses a prior name, the foregoing will be accepted as the valid name for those organisms heretofore classed as Group I Photochromes. These organisms have a rather unique property of photochromogenicity; they are never pigmented if grown in the dark, however, if, while the cultures are still young and actively growing, they are exposed to light, yellow pigmentation occurs within the next 6-24 hours. If grown in continuous light, this pigment often deepens to orange or even brick red. These organisms grow both at room temperature and 37° C. but their rate of growth is slow, on the order of that seen with typical human tubercle bacilli. These same growth characteristics apply also to the next two groups in this schema.

Group II – Scotochromogens. As the name implies, these microorganisms are pigmented both in the light and in the dark. The pigment, a yellow-orange, frequently deepens on exposure to continuous light. There seems to be general agreement among mycobacteriologists that the scotochromes still represent a rather heterogeneous group, requiring more definitive tests before they can be accurately typed and justifiably given a name. Distinguishing Group II strains from pigmented Group III cultures is one major problem still to be solved. Aside from the fact that these organisms grow very slowly, their pigment production often leads to their confusion with pigmented saprophytes.

Group III – Non-photochromogens or Battey strains. By and large these strains seem to be non-chromogenic on primary isolation, however, not infrequently tan, pink or yellowish colonies appear and still others acquire pigment after subculture or prolonged incubation.

One characteristic sometimes seen in this group is that of actinomycete-like mycelial-patterns. Many of the Battey strains bear striking resemblance to avian tubercle bacilli in certain respects.

Group IV – Rapid Growers. Most of these strains grow within 2-4 days like the known rapidly growing saprophytes, and, needless to say, many group IV strains are undoubtedly saprophytes. Designation by some group number is needed only for anonymous or un-named organisms, and should not include strains which have been identified as some named species. Already one species of the heterogeneous rapid growers has been named: *Mycobacterium fortuitum*.

In a subsequent section of the manual more data will be presented for the differentiation of these groups from one another, and methods for differentiating the atypicals from known mycobacteria also will be discussed. While it is true that representatives of all four groups of anonymous acid-fast bacilli have been isolated from sources indicating pathogenicity for man, it is generally conceded that *M. kansasii* (Group I, Photochromogen) and Battey strains are the most virulent, while scotochromes and rapid growers are less frequently isolated under circumstances indicating complicity in human disease. It should be noted that there is little evidence that infection with the anonymous acid-fast bacilli is spread from man to man. This latter would suggest some source in nature, other than man, as harboring these agents of disease. Other than some recent skin hypersensitivity surveys which indicate that infection with Group III organisms may be rather widespread in the southeastern United States, epidemiologic investigations probing the source of atypicals in nature are rare and undocumented at this time.

From this brief discussion it can be seen that there are a variety of acid-fast bacilli capable of causing tuberculosis-like disease. As we learn more about these bacteria, some clinical signs and symptoms doubtless will be found to be different. Already there are some: e.g., poor results of drug therapy in infections with the atypicals. For many reasons – clinical, bacteriologic and epidemiologic – it is important that all such organisms isolated from man be carefully and properly identified. It behooves clinician and bacteriologist alike to be aware of these different types of acid-fast bacilli so that more effective methods of identification and control may be realized. Further, it is hoped that research in fields of treatment, prevention, immunity and proper identification may lead to continual declines and ultimate eradication, not only of tuberculosis but of similar pulmonary infections caused by other mycobacteria.

Safety Measures

In the tuberculosis laboratory today, with the increased handling of viable organisms – for culture, animal studies and chemical tests – the possibilities of laboratory infection are greatly increased. Because of this fact, it becomes necessary for laboratorians to examine and analyze all factors that may jeopardize their safety and then to take measures to counteract these hazards.

The primary purpose then for observing the safety measures listed below is to protect ourselves and those around us from infection with the tubercle bacillus. Always remember that your own safety, as well as the safety of others, depends upon **your** techniques.

Precautionary measures in the tuberculosis laboratory may be divided into several major categories.

A. Medical and Health Program

1. Physical examination, including chest X-ray, is required before employment.
2. Tuberculin tests are done on all new personnel, and non-reactors are urged to be vaccinated with BCG.
3. It is emphasized that fatigue, emotional stress, and malnutrition act as contributory factors to the development of tuberculosis after exposure.
4. Chest X-rays are recommended every three to six months, depending upon the nature of an employee's work.
5. Illnesses are reported immediately.
6. Accidents are reported immediately.
7. Fifteen-minute rest periods are allotted in morning and afternoon.

B. Contaminated Areas (Definition)

Rooms where specimens are received, cultures transferred, infected animals are housed, and where animals are inoculated and autopsied, are considered contaminated, as are the outsides of containers of specimens, needles, syringes, metal trays and all objects used in the transfer of cultures.

C. Procedures in Contaminated Areas

1. Smoking and eating are prohibited in contaminated rooms.
2. Safety hoods equipped with ultraviolet light and exhaust fan are provided for processing of specimens, handling pure cultures of organisms, and for the inoculation and autopsy of animals. The ultraviolet light is turned on for 10 minutes before and after the hood is used. Since ultraviolet light can injure the eyes and can cause burns, it must be turned off when the hood is in use. As an added precaution the work area is swabbed before and after use with 5 percent phenol or 2 percent saponated cresol.

3. Before entering any contaminated area, make certain you have all the equipment you will need. ONCE IN THE CONTAMINATED ROOM, DO NOT LEAVE UNTIL YOUR TASK IS COMPLETED. Should you need additional equipment, ask someone outside the room to get it for you.
4. ALWAYS don gown, mask, rubber gloves, sneakers and hat before entering a contaminated area.
5. REMEMBER — Everything within the limits of a contaminated room is potentially infectious.
 - a. Do not touch or handle anything in a contaminated area with your bare hands. Should you inadvertently handle contaminated materials with your bare hands, wash very carefully, using plenty of soap; finally, rinse hands in some suitable disinfectant (see Section D which follows).
 - b. NOTHING is to be brought from a contaminated area out into a noncontaminated area unless it is first autoclaved or otherwise decontaminated.
6. Make sure the hood is on during all culture or specimen manipulations and animal examinations. ALWAYS bear in mind, however, that NO HOOD IS 100 PERCENT EFFECTIVE. THERE IS NO SUBSTITUTE FOR CAREFUL ASEPTIC TECHNIQUE.
7. When handling infected materials in the hood, work over a $\frac{3}{4}$ inch deep pan containing cheesecloth liberally soaked in 2 percent cresol.
8. For personal protection in hoods:
 - a. Use safety pipettors for all liquid cultures; use of oral pipettes is prohibited.
 - b. Before flaming transfer loops or spatulas, clean them carefully in flask containing sand and alcohol.
 - c. Heat-fix all smears immediately.
9. The outside of specimen bottles may have been contaminated at the source or by leakage in transit. Therefore, they should be disinfected with 2 percent saponated cresol or 5 percent phenol before handling. The inner metal mailing tube should be autoclaved before re-use.
10. All contaminated materials or dead animals are to be placed in completely covered containers which should be swabbed with 2 percent cresol before leaving the room.
 - a. All contaminated fluids are discarded into a splash-proof can containing 2 percent saponated cresol or 5 percent phenol.
 - b. All contaminated equipment (glassware, etc.) is placed in covered metal cans which are autoclaved.
 - c. Discarded autopsied animals are wrapped in wax paper

and incinerated.

- d. After use, needles, syringes and autopsy instruments are placed in covered containers and autoclaved.
- e. Laboratory request slips accompanying specimens are placed in a paper bag and autoclaved before they are handled by the record clerk.

11. Before leaving your work area:

- a. Place the cresol-soaked cheesecloth back into a receptacle containing cresol.
- b. Swab around the work area with 2 percent cresol.
- c. Turn on ultraviolet lights.

12. Remove gown, mask, rubber gloves, sneakers, and hat and place them in a covered GI can which is inside the contaminated area. AUTOCLAVE THE CAN BEFORE REMOVING ANY CLOTHING THEREFROM.

13. Upon leaving contaminated rooms, hands are washed for two minutes with soap and water, and rinsed with an efficient disinfectant (see below). If deemed advisable, take a shower before dressing in street clothes.

D. Disinfectants

Although a number of different chemical agents have been suggested as disinfectants for the tubercle bacillus, an examination of the literature reveals that most results are equivocal. An adequate disinfectant should:

1. Kill the tubercle bacillus rapidly.
2. Be able to act in the presence of organic materials, such as sputum, tissues, etc.
3. Be relatively nontoxic to tissues (in the event that it is to be used for skin disinfection).
4. Maintain its disinfectant properties (i.e., remain stable) for prolonged periods of time and under diverse conditions.

A word of caution at this point. Each laboratory or clinic has its own unique problems insofar as disinfection is concerned, and it is the responsibility of each Unit to evaluate the effectiveness of proposed germicides under conditions simulating actual everyday usage.

Since the primary purpose of the study reported below was to find a disinfectant which would work in **this** laboratory, an attempt was made to simulate, as nearly as possible, the work surface over which most routine diagnostic work is performed. At the present time, two types of work surfaces are used: one is a hood with metal-surfaced work area, the other a hood area covered with several thicknesses of disinfectant-soaked gauze. Hence, the surfaces on which disinfectants were tested included: 1) gauze, and 2) metal tabs of the same composition as our hood-work surfaces.

Twelve-ply gauze sponges and metal scraps were cut to 1/4 x 3/8 inch size and seeded with one drop each of an 8-day old broth culture of human, bovine or avian tubercle bacilli and allowed to dry overnight. Plate counts showed these inocula to contain $10^6 - 10^7$ organisms. The gauze and tabs then were dipped into various disinfectants (see accompanying tables) for varying time intervals: 15 seconds, 30 seconds, 60 seconds, 5 minutes, 15 minutes, 30 minutes. Upon removal from the disinfectants, the gauze or metal tabs were dipped four times into sterile saline to stop the action of the disinfectant and subsequently added to a tube of tween-albumin broth.

Broth tubes were read weekly and all positive tubes were subcultured onto Lowenstein-Jensen medium to be sure that the growth was not due to contaminants. Any subsequent growth on Lowenstein medium was confirmed as acid-fast by making smears and staining by Ziehl-Neelsen technique. (See table I).

Conclusions

From the foregoing data the following points seem to be indicated:

1. It appears best to have a disinfectant-soaked gauze over the immediate work area, preferably, using one of the phenolic compounds as disinfectant.
2. Cresol and phenol appear to be the best disinfectants, however, they are noxious to the skin and should not be used where direct skin contact occurs.
3. The alcohols (70-95 percent ethanol) are generally ineffective, primarily because they would have evaporated long before their germicidal action was manifested.
4. Both Wescodyne and Amphyl appear to offer good tuberculo-cidal action, however, the effectiveness of Amphyl is curtailed on metallic surfaces while the activity of Wescodyne is not altered markedly by the metal.
5. There seems to be a variation in resistance of different varieties of tubercle bacilli to certain disinfectants; generally speaking, the bovine and avian varieties are more resistant than the human.
6. Warexin, which has been highly praised by others, was not one of the better disinfectants in our hands. It has the added disadvantage that (because of rapid deterioration) it must be prepared shortly before use, hence, it would not be effective in our laboratory where disinfectants are prepared in 5-gallon batches.

TABLE I
EFFECTIVENESS OF DISINFECTANTS ON GAUZE WORK SURFACES

Disinfectant	CULTURES					
	HUMAN		BOVINE		AVIAN	
	Living After Exposure for	Dead After	Living After Exposure for	Dead After	Living After Exposure for	Dead After
70% Ethanol	30 sec.	60 sec.	60 sec.	5 min.	60 sec.	5 min.
95% Ethanol	30 min.	—	30 min.	—	30 min.	—
2% Cresol	—	15 sec.	—	15 sec.	—	15 sec.
5% Phenol	—	15 sec.	—	15 sec.	—	15 sec.
1-1/2% Warexin*	30 sec.	60 sec.	60 sec.	5 min.	60 sec.	5 min.
Wescodyne**	15 sec.	30 sec.	—	30 sec.	30 sec.	60 sec.
2% Amphyl***	—	15 sec.	30 sec.	60 sec.	30 sec.	60 sec.
Saline control	All Control Tubes Positive					

* Brand of Clorpactin; Guardian Chemical Corp., Long Island City 1, New York.

** Brand of organic iodophor; West Disinfecting Co., Atlanta, Georgia. Prepared as follows:
Wescodyne — 10%, 95% Ethanol — 50%, Water — 40%.

*** Phenolic compound; Lehn & Fink Products Corp., Bloomfield, New Jersey.

TABLE II
EFFECTIVENESS OF DISINFECTANTS ON METAL SURFACE

Disinfectant	CULTURES					
	HUMAN		BOVINE		AVIAN	
	Living After Exposure of	Dead After	Living After Exposure of	Dead After	Living After Exposure of	Dead After
70% Ethanol	60 sec.	5 min.	60 sec.	5 min.	60 sec.	5 min.
95% Ethanol	30 min.	—	30 min.	—	30 min.	—
2 % Cresol	15 sec.	30 sec.	30 sec.	60 sec.	60 sec.	5 min.
5 % Phenol	15 sec.	30 sec.	30 sec.	60 sec.	15 sec.	30 sec.
1-1/2 Warexin	15 sec.	30 sec.	30 min.	—	15 min.	30 min.
Wescodyne	15 sec.	30 sec.	60 sec.	5 min.	15 sec.	30 sec.
2 % Amphyl	15 sec.	30 sec.	5 min.	15 min.	15 min.	30 min.
Saline control	All Control Tubes Positive.					

Collection and Shipment of Specimens

A. Sputum

1. A clean, sterile container should be used for collection of the specimen.
2. If the specimen is to be sent through the mail, it must be packaged and shipped according to postal regulations (see sheet at end of this section, page 14).
3. A 5-10 ml. sample of sputum (not saliva) is adequate for a single examination; there is no advantage in collecting a larger single sample.
4. An early morning sample of sputum is preferable.
5. If the early morning sample is not adequate in volume, sputum may be collected over a 12-24 hour period. Collection of a single sample over a longer period of time or the pooling of several days' production has no advantage and in fact may be disadvantageous for several reasons: a) the sputum itself may be toxic to tubercle bacilli on prolonged exposure; b) associated organisms also may be harmful to the tubercle bacillus; c) the degree of contamination of cultures may be increased; d) all of a large sample or "pool" of sputum cannot easily and efficiently be concentrated. If some of the successive increments to the "pool" are negative, as is likely to be the case, these negative increments dilute the positive portions, making it actually more difficult to find the organisms.

B. Gastric Washings

1. The sample should be collected early in the morning on a fasting stomach.
2. Tubercle bacilli may die rapidly in gastric washings. Therefore, these samples should be processed as soon as possible (at most within 4 hours) after collection.
3. If gastric washing specimens have to be submitted with delay, as by mailing, they should be neutralized or preserved when collected.

C. Urine

1. An early morning voided or catheterized sample is preferable.
2. The 24-hour pooled sample of urine is more likely to be contaminated and to contain a lower concentration of living tubercle bacilli.
3. Urine also is toxic to tubercle bacilli.

D. Other Materials

Since a tuberculous infection may localize in almost any anatomical site in the body, the variety of clinical materials which may be submitted for examination is considerable. Samples of cerebrospinal fluid, pleural fluid, joint fluid, pus, bronchial washings, pharyngeal swabs, and even feces are received with varying frequency in the tuberculosis diagnostic laboratory. With the refined surgical techniques of today, portions of resected lung may be submitted for bacteriologic study. The collection of these kinds of specimens is not commonly a problem for the bacteriologist, nor does their collection involve active cooperation by the patient; with the exception of feces, these are surgical specimens, to be collected or taken by the physician or surgeon, with at least surgical asepsis. They should of course be transported to the laboratory in bacteriologically sterile containers.

Certain of these specimens, e.g. tissues, will not well tolerate a prolonged transportation time (decomposition) and, since chemical preservatives and tissue fixatives would make cultural bacteriologic examination impossible, these examinations are most productive in the hospital or sanatorium laboratory where prompt transportation and delivery by messenger or orderly is the common practice.

In the bacteriologic examination of resected lung or other surgically removed tissue, it is well to enlist the aid or guidance of the surgeon who can personally indicate the portion of tissue or lesion most likely to be rewarding of bacteriologic examination.

The examination of fecal specimens for tubercle bacilli is not frequently requested and is not often of much value. The presence of tubercle bacilli in the feces does not necessarily indicate intestinal tuberculosis, but is more likely to mean the presence of pulmonary tuberculosis, where sputum has been swallowed.

Postal Regulations on Shipment of Diseased or Contaminated Materials

The latest edition (1954) of the U. S. Official Postal Guide on the subject of packaging and mailing states:

PART 124: Nonmailable Matter

124.2 Harmful Matter

124.21 General Provisions of Law. Any articles, compositions, or materials which may kill or injure another, or injure the mails or other property, are nonmailable. This includes but is not limited to:

- a. All kinds of poison or matter containing poison
- b. All poisonous animals, insects, and reptiles
- c. All disease germs and scabs
- d. All explosives, inflammable material, infernal machines, and mechanical, chemical, or other devices or compositions which may ignite or explode.

124.23 Acceptability if Properly Packed. When authorized by the Postmaster General, various of the articles specified in this part as being nonmailable may be sent through the mails if they conform to special regulations as to preparation and packaging and if they are not outwardly dangerous, or of their own force dangerous or injurious to life, health, or property.

Despite the above reference to disease germs there is no description in the new Postal Guide of the proper method of packaging diseased tissues or cultures of microorganisms. In spite of the fact that the 1953 edition of the Postal Guide is now obsolete, it was very specific in its description of how diseased tissues and/or specimens were to be packaged and mailed. From the standpoint of presenting a proper method of packaging of such materials, Article 39 of the July 1953 edition of the U. S. Official Postal Guide, Chapter IV, Mailability and Packaging, is reproduced below.

Diseased Tissues and Other Specimens

- A. Specimens of diseased tissues, blood, serum, and cultures of pathogenic micro-organisms may be admitted to the mail for transmission to United States, State, municipal, or other laboratories in possession of permits issued by the Solicitor, certifying that said laboratory has been found to be entitled to receive such specimens only when enclosed in mailing cases as prescribed in this article.

However, bacteriologic or unfixed pathologic specimens of plague and cholera shall not be admitted to the mails except when prepared specifically as follows:

1. Pathologic specimens of plague and cholera which have been immersed for at least 72 hours in four times their volume of 4 percent formaldehyde gas in water, or other fluid of equal or superior disinfecting power for a period sufficient to fix or harden the central portions of the specimen, may be admitted to the mails if packed in the same manner as herein prescribed for other unfixed pathologic tissues in paragraphs 3, 4, and 5 of this article.
2. Cultures and infectious material of plague, cholera, anthrax, undulant fever, and tularemia may be admitted to the mails if enclosed in stout glass tubes sealed by fusion of the glass and packed in a large stout glass container with a layer of absorbent cotton soaked in 4 percent formaldehyde surrounding the inner container. The outer glass container shall be closed with a rubber stopper or cork of good quality or by fusion of the glass. This double glass container shall then be packed in accordance with the provisions of paragraphs 4 and 5 of this article.
3. Specimens of sputum, feces, pus, unfixed diseased tissue, or other infectious material fluid in nature or shipped with non-disinfecting fluid shall be placed in stout glass containers of suitable size (but not more than three inches in diameter) closed with a metal cover having a rubber, cork, or paraffined paper leakproof washer or with a cork or rubber stopper of good quality, or by fusing the glass. Large fixed specimens of diseased tissue may be prepared for shipment outside mail bags when packed in accordance with the provisions of the following paragraph.
4. The aforesaid glass container shall then be placed in any of the following described containers: (1) a cylindrical sheet-metal box with soldered joints, closed by a metal screw cover; (2) a paraffin-impregnated heavy cardboard container, with ends made of metal or a suitable substitute. A sleeve type of closure may be employed provided that the overlap is at least one-third the length of the cylinder and in any case at least 2 inches. The closure shall be sealed with tape; or (3) a one-piece bored wooden cylinder at least three-sixteenths of an inch thick in its thinnest part with a threaded screw top.

The screw-top covers shall be provided with rubber or felt washers and shall be threaded with sufficient screw threads to require at least one and one-half full turns before they will come off.

The glass tubes in the above containers shall be completely and evenly surrounded by absorbent cotton or other suitable absorbent in quantity sufficient to absorb the contents of the glass container if broken.

5. The sheet-metal box with its contents shall then be enclosed in a closely fitting wooden or papier-mache box or tube, at least three-sixteenths of an inch thick at its thinnest part, or in a sheet-metal box or tube of sufficient strength to resist rough handling and support the weight of the mails piled in bags. The tube shall be tightly closed with a screw-top cover with sufficient screw threads to require at least one and one-half full turns before it will come off.
6. a. Cultures in solid media, blood, serum, spinal fluid, fixed and completely disinfected diseased tissue, and infectious materials on swabs shall be transmitted in a stout glass container of suitable size (but not more than 3 inches in diameter), closed with a plastic or metal cover having a rubber, cork, or paraffined paper washer, or with a stopper of rubber, paraffined cork, or cotton, the last sealed with paraffin or covered with a tightly fitting rubber cap. The tube shall then be packed in a single wooden or papier-mache cylindrical box or tube, at least three-sixteenths of an inch thick in its thinnest part, or in a sheet-metal box or tube of sufficient strength to resist rough handling and support the weight of the mails piled in bags. The glass container in this box or tube shall be completely and evenly surrounded by absorbent cotton or other suitable absorbent packing material. Cultures in media that are fluid at the ordinary temperature (below 45 C. or 113 F.) may be mailed if packed in stout glass vials closed by fusing the glass and enclosed as in paragraphs 4 and 5 of this article.
b. Large specimens of fixed diseased tissue shall be placed in securely sealed glass containers, or in securely closed (hermetically sealed or screw-top or approved patent-top) metal containers with the necessary preservative fluid. The container shall be surrounded by sawdust or other suitable absorbent material to protect against breakage or leakage.
7. Specimens of blood dried on glass microscopic slides for the diagnosis of malaria or typhoid fever by the Widal test or of other conditions shall be sent in any strong mailing case which is not liable to breakage or loss of the specimen in transit.
8. Large pathological specimens of fixed disease tissue and shipments of large numbers of small specimens may be prepared for shipment outside mail bags. Small specimens of sputum, blood, serum, spinal fluid, pus, feces, fixed or unfixed diseased

tissue, or other material fluid in nature or shipped with fluid, forming part of such a shipment shall be placed in stout glass containers as in paragraph 3 of this article and individually evenly wrapped in absorbent cotton or other suitable absorbent material in sufficient quantity to absorb all the fluid in case of breakage. Small and large specimens so prepared shall be shipped in a strong, securely closed box endorsed in accordance with article 59 (f), and marked "Specimen for Bacteriological Examination," and be transported outside mail bags.

9. Upon the outside of every package of diseased tissue, blood, serum, or cultures of pathogenic micro-organisms admitted to the mails shall be written or printed the words, "Specimen for bacteriological examination. This package shall be pouched with letter mail," except that when dispatch is made to non-stop trains in catcherpouches or large specimens or shipments are prepared as prescribed in paragraph 8 of this article they shall be marked "Specimen for Bacteriological Examination."

Laboratory Methods for Processing Clinical Materials

A. Routine Laboratory Procedures for the Treatment of Sputum Specimens

1. Number each specimen as it is received in the laboratory.
2. Mark a sterile Petri dish, a sterile 15 ml. screw-cap centrifuge tube (16 mm. x 125 mm. culture tube), and a clean microscope slide with the same number.* The screw-cap tube should be of resistant glass, should be inspected for chips and weak points, and the cap must contain a good liner. Remove the cover of the Petri dish and lay it upside down on the work surface. Place the slide in a horizontal position across the edge of the inverted cover and pour the specimen into the bottom half of the dish. Discard the sputum bottle in a metal can to be autoclaved.
3. Pick up a sterile wooden applicator stick by grasping it in the middle. Then break it and use the two pieces to divide and cut the sputum. Violent agitations and spattering are to be avoided. Over a black background, select small bits of necrotic, cheesy, rusty or bloody material from various portions of the specimen and deposit them in the centrifuge tube until it contains from 2 to 4 ml. If the specimen is thin, a capillary pipette with rubber bulb may be used in place of the applicator sticks. In like manner, place selected particles on the slide and spread to make a smear of convenient thickness.
4. The smears are stained by the Ziehl-Neelsen technique as follows:
 - a. Allow the smear to dry, and fix to the slide by means of gentle heat over a flame or electric slide warmer.
 - b. Place a strip of filter paper, the size of the smear, on the slide.
 - c. Flood the slide with carbolfuchsin and heat to steaming. Allow it to stand for 5 minutes without further heating and remove filter paper. Slides should be stained individually. Mass staining in a common container may allow cross transfer of acid-fast organisms.
 - d. Wash twice with water.
 - e. Decolorize in two or three successive solutions of acid-alcohol until no more color appears in the washings. (Approximately 2 minutes.) A longer period may be required for thicker smears.

*One must always use NEW slides in tuberculosis work.

- f. Wash with water.
- g. Counterstain with aqueous methylene blue or other appropriate stain for 20-30 seconds.
- h. Wash with water.
- i. Dry in the air over gentle heat.
- j. Examine smear with oil immersion lens, taking care to wipe the lens well after each examination of a positive smear. Three long lines the length of the smear, or 9 short lines the width of the slides, are examined.
 - 1) Some laboratories prefer to examine slides for a given length of time, in which case 15-30 minutes is the usual period. However, examination by area covered is more thorough.
- k. Results may be reported as follows:

Number of Bacilli	Report
0	No acid-fast bacilli found
1-2 (in entire smear)	Report number found and request repeat specimen
3-9 (in entire smear)	Rare acid-fast bacillus found; report number seen
10 or more (in entire smear)	Many acid-fast bacilli found
10 or more in most oil immersion fields	Innumerable acid-fast bacilli found

Far more important than number of bacilli in preparation, however, is the fact that they **are** present. If clumps of bacilli appear, the number of individual bacilli is estimated. It is desirable, if **time allows**, to make culture inoculations according to the microscopy findings. If a slide is positive, the sediment is diluted in order to get well isolated colonies which may be more easily identified. (See #10 below.)

5. Remove cap from centrifuge tube, flame lip of the tube, and add to the specimen a volume of digesting agent equal to the amount of sputum. The digesting agent most commonly employed is heat sterilized 4 percent NaOH, containing 0.004 percent phenol red indicator.
6. Tighten all caps and invert to test for leakage. If a tube leaks, transfer contents to another sterile tube.
7. Place tubes in a shaking machine and homogenize for 10 minutes.
8. Centrifuge tubes for 15 minutes at 2,500 to 3,000 rpm.
9. One by one, remove caps from tubes, pour off supernatant fluid

into a splash-proof can containing 2 percent cresol, flame lips of the tubes and replace caps.

10. Neutralization should be done immediately so specimens will not be in contact with NaOH for more than 40 minutes at most. Treat tubes individually. With a sterile capillary pipette, add sterile 2N HCl* slowly, drop by drop, to each sediment until a definite yellow color is obtained. Back titrate with a sterile 4 percent NaOH* solution in the same manner until the first persistent pink tinge appears. Mix each sediment well and make a concentrate smear if desired.

For the culture procedure it is recommended that at least 2 tubes each of 2 different media be used; these 4 tubes are arranged in pairs so that each pair consists of one tube of each medium. If the microscopy report is **negative**, pour off the water of condensation from all 4 tubes and inoculate each tube with approximately 0.1 ml. of the undiluted, neutralized sediment.

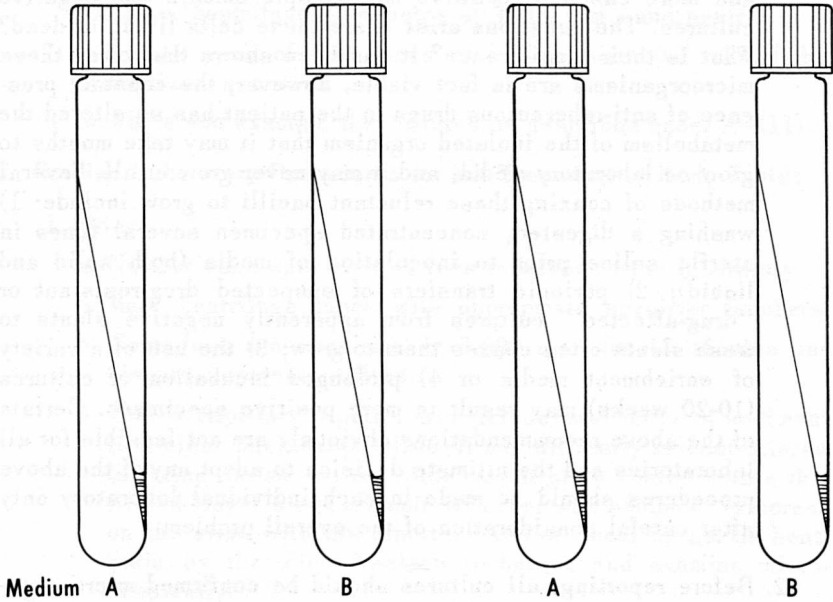
If the microscopy report is **rare** or **many**, pour off the water of condensation from the first pair of tubes and inoculate this pair of tubes without dilution. Add one drop of sediment to the water of condensation in the third medium tube; then discard the remainder of the sediment from the pipette. Mix well, draw up the diluted sediment in the third medium tube and divide this diluted sediment evenly between the third and fourth medium tubes, spreading the inoculum over the surface, having first discarded the water of condensation from the fourth medium tube.

If the microscopy report is **innumerable**, pour off the water of condensation from the first pair of tubes, and inoculate this pair of tubes with the sediment without dilution. Expel all the sediment from the pipette, and rinse the inside of the pipette with the water of condensation in the third medium tube by drawing it up into the pipette several times. Use this rinsing to streak the third and fourth medium tubes, after having discarded the water of condensation from the fourth medium tube. Refer to the diagrams in figure 1 for clarification.

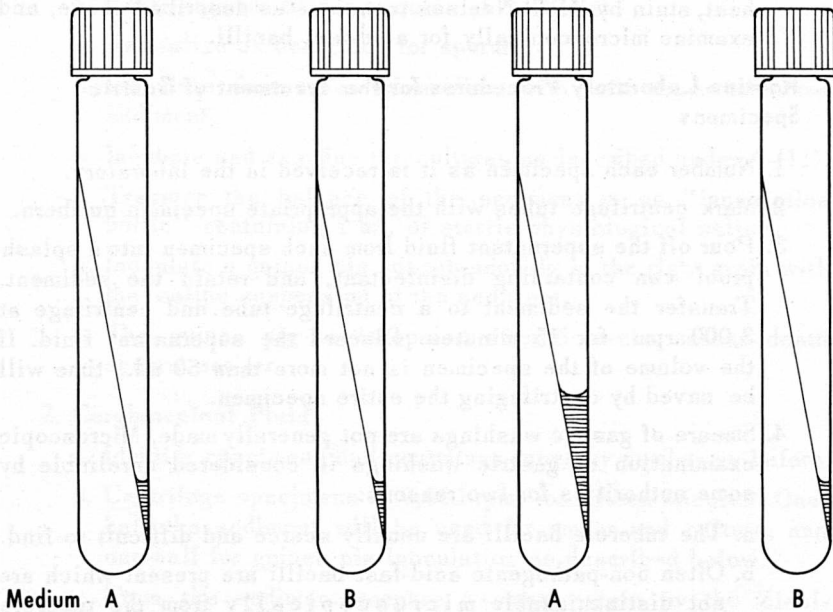
11. The inoculated culture tubes should be incubated overnight in a nearly horizontal position to insure uniform distribution of inoculum over the surface of the medium. The next day, bind the four (4) tubes with a rubber band, stand them upright in a wire basket and reincubate. Examine the cultures after 2 weeks and every week thereafter until the cultures are 6 weeks old. Negative cultures are commonly reported at 6 weeks and discarded.

*A good practice is to partially fill some clean screw-cap tubes with HCl and NaOH and autoclave them. After use, they can be discarded with little loss.

FIGURE I
Water of Condensation and the Dilution of Inoculum



Here the water of condensation has been poured off from all 4 tubes.



Here the water of condensation has been retained in the third tube to serve as diluent for an otherwise excessively heavy inoculum.

One problem facing many laboratories today is a direct result of the modern drug era. Many laboratories are finding more and more cases of positive microscopic smears and negative cultures. The questions arise – are these cells living or dead? What is their significance? It has been shown that often these microorganisms are in fact viable, however, the constant presence of anti-tuberculous drugs in the patient has so altered the metabolism of the isolated organism that it may take months to grow on laboratory media, and it may never grow at all. Several methods of coaxing these reluctant bacilli to grow include: 1) washing a digested, concentrated specimen several times in sterile saline prior to inoculation of media (both solid and liquid); 2) periodic transfers of suspected drug-resistant or “drug-affected” cultures from apparently negative slants to fresh slants often coaxes them to grow; 3) the use of a variety of enrichment media or 4) prolonged incubation of cultures (10-20 weeks) may result in more positive specimens. Certain of the above recommendations obviously are not feasible for all laboratories and the ultimate decision to adopt any of the above procedures should be made in each individual laboratory only after careful consideration of the overall problem.

12. Before reporting, all cultures should be confirmed microscopically. Place a drop of distilled water on a clean slide. Pick a typical colony from the culture with a spade (appendix 17) and suspend it in the drop of water. Make a smear from surface of “negative” cultures also. Allow the smear to dry, fix by gentle heat, stain by Ziehl-Neelsen technique as described above, and examine microscopically for acid-fast bacilli.

B. Routine Laboratory Procedures for the Treatment of Gastric Specimens

1. Number each specimen as it is received in the laboratory.
2. Mark centrifuge tubes with the appropriate specimen numbers.
3. Pour off the supernatant fluid from each specimen into a splash-proof can containing disinfectant, and retain the sediment. Transfer the sediment to a centrifuge tube and centrifuge at 3,000 rpm. for 15 minutes. Discard the supernatant fluid. If the volume of the specimen is not more than 50 ml., time will be saved by centrifuging the entire specimen.
4. Smears of gastric washings are not generally made. Microscopic examination of gastric washings is considered unreliable by some authorities for two reasons:
 - a. The tubercle bacilli are usually scarce and difficult to find.
 - b. Often non-pathogenic acid-fast bacilli are present which are not distinguishable microscopically from the tubercle bacillus.

5. Treat the sediment with an equal volume of 4 percent NaOH.
6. Process gastric washings exactly as was described above for sputum specimens. Homogenize 10 minutes in the shaking machine, centrifuge 15 minutes at 3,000 rpm., and neutralize.
7. Inoculate each of four tubes of medium with 0.1 ml. of the neutralized sediment.
8. Incubate and examine the cultures as described under A-(11).

C. Routine Laboratory Procedures for the Treatment of Body Fluids

1. Urine

- a. Number each specimen as it is received in the laboratory.
- b. Mark centrifuge tubes with appropriate specimen numbers.
- c. Centrifuge the specimen at 3,000 rpm. for 15 minutes and discard supernatant fluid.
- d. With a capillary pipette, transfer several drops of sediment to a clean microscope slide. If any difficulty is encountered in fixing the smear, the slide may first be coated with a thin film of Mayer's egg albumin fixative. The sediment is spread on the slide with the pipette. Fix the smear by gentle heat. Stain by the Ziehl-Neelsen technique and examine microscopically.
- e. Add to the sediment of each specimen an equal amount of 4 percent NaOH and homogenize ten minutes in the shaking machine.
- f. Centrifuge for fifteen minutes at 3,000 rpm.
- g. Neutralize as described for sputum.
- h. Inoculate four tubes of medium with half the neutralized sediment.
- i. Incubate and examine the cultures as described under A-(11).
- j. Transfer the balance of the sediment to an "inoculation bottle" containing 1 ml. of sterile physiological saline.
- k. Inoculate a guinea pig subcutaneously in the right groin with the saline suspension of the sediment.
- l. The guinea pig is autopsied after 6 weeks unless death occurs earlier.

2. Cerebrospinal Fluid

- a. Identify specimens and centrifuge tubes by number as before.
- b. Centrifuge specimens at 3,000 rpm. for fifteen minutes. One-half the sediment will be used for smear and culture, and one-half for guinea pig inoculation as described below.
- c. From the sediment prepare a smear, stain by the Ziehl-Neelsen stain, and examine microscopically for acid-fast

bacilli. At the same time note the presence of any other organisms revealed by the counter-stain.

- d. If the sediment appears to be microscopically free from pyogenic or contaminating organisms, inoculate half of the sediment, without chemical treatment, on the usual media, and inject one-half into a guinea pig.
- e. If the sediment contains organisms other than acid-fast bacilli,
 - 1) culture for other pathogens, and
 - 2) treat with 4 percent NaOH as for sputum. Inoculate the usual media and a guinea pig.

3. Pleural Fluid

If fluid contains a clot, cut it into small pieces using sterile forceps and scissors. Treat specimens as a sputum.

D. Miscellaneous Clinical Materials

1. **Pus and bronchial washings** – treat as described for sputum specimens.
2. **Feces** (modified Petroff's method) – Add three volumes of distilled water to the specimen and thoroughly mix. Add NaCl to saturation, let stand 30 minutes, then all bacteria should be floating on the surface of the top layer. Skim the top layer with a wide spoon. Add an equal volume of 4 percent NaOH to the skimmings and homogenize in the shaker for 30 minutes. Neutralize with 2N HCl and centrifuge at 3,000 rpm. for fifteen minutes. Inoculate five tubes of media with the top layer of the supernatant fluid and add 1 ml. of the supernatant fluid to an inoculation bottle. Decant supernatant fluid, use one-half of the sediment to inoculate 5 tubes of media. Add the other half of the sediment to the supernatant sample in the inoculation bottle and use this to inoculate a guinea pig. A larger number of media tubes are used because of the increased likelihood of contaminants. Presence of acid-fast bacilli in feces must be carefully interpreted, since the organisms may result NOT from tuberculosis of the gastrointestinal tract, but merely from swallowed sputum!
3. **Tissue** (tonsils, lymph nodes, etc.) – place specimen in a sterile mortar and cut it into small pieces with sterile scissors. Add sterile distilled water a drop at a time and grind the material thoroughly. Transfer to centrifuge tube with a sterile capillary pipette and treat with 4 percent NaOH in the usual manner, homogenizing ten minutes in the shaker. Centrifuge and neutralize the sediment. Prepare smear from the sediment. Inoculate culture media and guinea pig. (If sterile sand is used

in the grinding process, allow time for sand and large tissue particles to settle. Transfer the supernatant suspension to a centrifuge tube and process as above.)

- 4. Swab** – transfer swab to centrifuge tube and half-fill with 4 percent NaOH solution. Homogenize 10 minutes in the shaker, remove swab and centrifuge. Prepare smear from neutralized sediment. Inoculate culture media and guinea pig.

Technique for Subculturing

Subculture should be prepared if, on primary isolation, the colonies are too numerous, too few, or so atypical that culture identification by colonial characteristics is difficult. For adequate study of colonial characteristics, at least five well-isolated colonies are desirable.

1. Number centrifuge tubes and microscope slides for all cultures which are to be subcultured.
2. With a sterile capillary pipette, place four drops of sterile distilled water in each centrifuge tube.
3. Pick a colony from the culture with a sterile wire loop or spade and suspend it in the water. This is done by slowly grinding the organisms against the side of the tube with the loop or spade and gradually suspending them in the water.
4. Transfer a drop of the suspension to a slide, within a circle which has been marked with a glass marking pencil. Allow smear to dry, fix with gentle heat, stain by the Ziehl-Neelsen technique, and examine microscopically for acid-fast bacilli.
5. Add about three drops of 4 percent NaOH to each centrifuge tube. (Invisible contaminants may be present in the colonies.)
6. Recap tubes and agitate gently to mix.
7. Incubate 10 minutes at room temperature and shake occasionally.
8. Neutralize with 2N HCl and add 2 to 3 ml. of sterile distilled water.
9. With a sterile capillary pipette, inoculate four tubes of medium in the following manner:
 - Tube 1:** Discard condensed water from medium tube and streak slant with one drop of undiluted sediment.
 - Tube 2:** Thoroughly mix one drop of undiluted sediment with the condensed water in tube 2 and streak the slant with a portion of it.
 - Tube 3:** Thoroughly mix one drop of the diluted sediment from tube 2 with the condensed water in tube 3. Use a few drops of this material to inoculate the tube.
 - Tube 4:** Expel all of the diluted sediment from the pipette and rinse it with the condensed water in tube 4 by drawing it up into the pipette several times. Use part of this to streak the slant.
10. Incubate all tubes at 37° C. Examine after 3 days and after 1 week and every week thereafter for a total of 6 weeks.

Cultural Characteristics of the Acid-Fast Bacilli (Modified Lowenstein Medium)

The acid-fast nature of all colonies must be confirmed by the Ziehl-Neelsen or other acid-fast technique of staining before they can be identified as mycobacteria or related microorganisms.

A. Colonies of **human** type tubercle bacilli generally appear in 12 to 25 days and, because of their luxuriant growth, are termed "eugonic." They are dry, friable, somewhat rough, buff in color and after a few weeks generally measure from 3 to 5 mm. in diameter. Typical colonies have a flat irregular margin with a rough, heaped-up, "cabbage-head" center. They are easily detached from the surface of the medium but are difficult to bring into even suspension.

Human type tubercle bacilli on rare occasions exhibit dysgonic growth characteristics. To differentiate these dysgonic strains from bovine tubercle bacilli, it is necessary to make further tests, including animal inoculations and the niacin test both of which will be discussed later.

With a moderate amount of training using modified Lowenstein medium one can identify, by colony characteristics and acid-fast staining, typical human type mycobacteria, provided it is known by previous observations that the cultures are not rapid growers or photochromogens. If there is any doubt, however, final typing should always be made by the use of laboratory animals.

B. Colonies of **bovine** bacilli do not grow as rapidly as the human type. They usually appear in 25 to 40 days. They are tiny, dysgonic, translucent, pyramidal and are usually less than 1 mm. in diameter. They adhere to the surface of the medium but are readily brought into suspension.

C. Colonies of **avian** bacilli generally appear in 14 to 21 days. They are eugonic, smooth, butyrous or viscid, and hemispherical and may have a faint yellow or pink pigment. They are somewhat larger (1 to 2 mm. in diameter) than bovine type colonies, with a mucoid, tenacious consistency but are easily suspended.

D. Saprophytes include several species of various colonial characteristics. Many are smooth, chromogenic, and rapid growing. Others lack these characteristics, and it is likewise true that some rapid-growers and some chromogens are disease producers. The only test of saprophytism is demonstration of its inability to grow on living tissue (man or animal). A strain which will not parasitize a laboratory animal and is not closely associated with lesions in man may tentatively be assumed to be a saprophyte.

The most commonly occurring saprophytes in clinical laboratories appear to be smooth chromogens which grow at about the same rate as avian bacilli. Hence, speed of growth is no indication of culture virulence, and it becomes necessary to determine other identifying characteristics before reporting results. Most saprophytes grow equally well at 37° C. and room temperature. Colonies of saprophytic mycobacteria can be smooth and either chromogenic or nonchromogenic. The majority of these suspend readily, others only with difficulty. Smooth, hemispherical, nonchromogenic colonies should be inoculated into guinea pigs and chickens since these characteristics would lead one to suspect avian tubercle bacilli.

It is never safe casually to categorize a mycobacterium as a saprophyte. At most, one may report "resembles a saprophyte." Consideration should be given to the number of colonies obtained from the original specimen and other evidence concerning possible association with human lesions. What a clinician needs to know is not whether a given organism is a saprophyte but whether it is a pathogen in his special patient.

Saprophytes include well-defined species such as *M. phlei* and *M. smegmatis* and certain strains of anonymous Groups II, IV, and perhaps some Group III strains (see below).

E. Colonies of *M. kansasii* Hauduroy (Group I Photochromogens) grow at about the same rate as tubercle bacilli, or perhaps slightly more rapidly at 37° C. from small inocula; growth at room temperature (20° to 25° C.) appears in three to four weeks. Colonies usually are smooth and disperse more easily than typical human tubercle bacilli; on occasion completely rough strains are encountered. When grown in the dark, the photochromes have little or no pigment; however, if grown in continuous light they are bright yellow to orange or brick red. If young, actively growing, nonpigmented cultures are exposed to light for one hour, yellow pigmentation will appear after further incubation of from 6 to 12 hours. It is imperative that actively growing cultures (about 1 week old) be used to observe this phenomenon, for older, mature colonies show little or no response to light. There are other species of mycobacteria that may show intensification of pigment upon exposure to light. However, the property of photochromogenicity herein described is a rapid, unique phenomenon among the acid-fast bacilli. Microscopically the organisms are long, strongly acid-fast and often appear banded or beaded.

F. Colonies of Group II Scotochromogens have a speed of growth comparable to *M. kansasii* at both 37° C. and room temperature. As the name implies, their yellow to orange pigment is present in the light and in the dark, although it usually becomes more reddish-orange if grown in continuous light. These scotochromogens retain their orange pigment when grown on the 7H-9 or 7H-10 medium. As opposed to some of the more rapidly growing saprophytes, scotochromes usually fail to grow at 45° C. Colonies are regularly smooth

and may be either eugonic or dysgonic. Microscopically these strongly acid-fast organisms vary in size and rarely form cords.

G. The growth rate of Group III Nonphotochromogens (Battey strains) at 37° C. and room temperature is similar to that of the previously described groups of anonymous acid-fast bacilli. One characteristic which may help differentiate these strains from the culturally similar avian tubercle bacilli is that most Battey strains do not grow well at 45° C., whereas this temperature is generally optimum for most avian strains.

Colonies of nonphotochromes are smooth, hemispherical and generally have little or no pigment, but occasional strains may have a tan or light yellow which is markedly different from the pigment observed in the preceding two groups. On rare occasions, most notably on prolonged culture, Battey strains may acquire an orange pigment indistinguishable from that seen in Group II. Scotochromogens of Group III, however, are less deeply pigmented and the color is more slowly developed than in the case of Group II Scotochromogens, especially on such media as 7H-9. Microscopically these highly pleomorphic cells are not as strongly acid-fast as the other groups; not infrequently these cells are seen to possess branching filaments similar to species of *Nocardia*.

H. The most distinguishing characteristics of the Group IV ("Rapid Growers") is the fact that they grow out in 4 or 5 days from digested clinical material and following subculture onto fresh medium, growth appears in 48 hours at both room temperature and 37° C. On further testing some or most of these strains may prove to be *M. fortuitum*.

Biochemical methods of differentiating these strains will be discussed in the section on cytochemical tests. Colonies are non-pigmented and either rough like tubercle bacilli, or smooth and moist. The organisms are strongly acid-fast. The rough colony strains generally form serpentine cords.

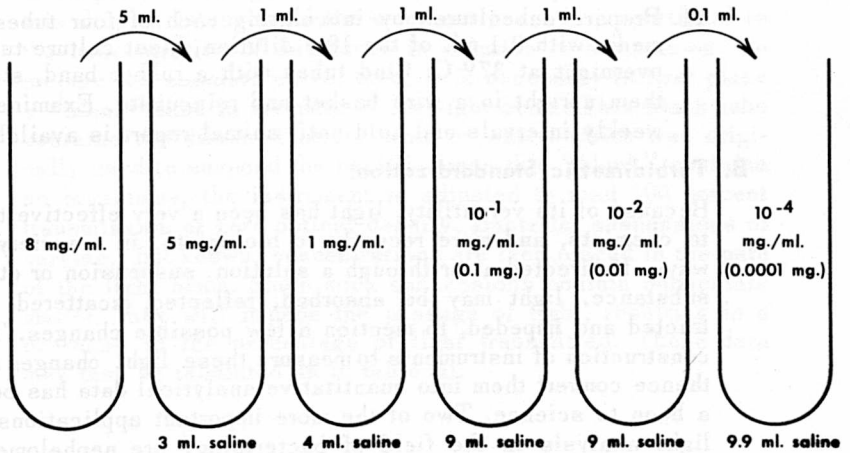
With a moderate amount of experience in observing these colony characteristics on modified Lowenstein medium, one can become quite adept at limiting the possible mycobacterial types of any given colony. Following this preliminary limitation, further confirmatory tests can be set up to provide a final diagnosis as to species or group of the isolated acid-fast organism.

Preparation of Cultures for Typing by Animal Inoculation

The type identification of cultures of mycobacteria by animal inoculation is predicated upon the development of characteristic lesions and course of disease and is, therefore, to some extent, related to the numbers or dosage of organisms inoculated. To insure proper interpretation of animal results, the inoculum should be standardized. A variety of methods is available for standardizing suspensions of acid-fast bacilli for animal inoculation.

- A. One which combines simplicity with a fair degree of accuracy is that involving the use of a **Hopkins vaccine centrifuge tube**.
1. Place 0.1 ml. of a sterile physiological saline in a sterile centrifuge tube.
 2. With a stiff wire loop or spade (appendix B-Item No. 16) transfer several large typical colonies (6 to 8 spadefuls of organisms) from the culture to the tube and grind them against the side of the tube, slowly suspending the bacilli in the saline.
 3. Add saline to the tube until there is a volume of about 7.0 ml.
 4. Allow the tube to stand at room temperature for 1 to 2 minutes; by this time, the larger clumps of bacilli will have settled leaving a relatively fine homogeneous supernatant suspension.
 5. With a sterile pipette, equipped with rubber bulb, transfer the supernatant suspension to a second sterile tube.
 6. Transfer 1 ml. of the suspension to a clean Hopkins vaccine tube. The tube need not be sterile since the aliquot is to be used for standardization purposes only and will be discarded after measurements are made.
 7. Centrifuge at 3,000 rpm. for 15 minutes (horizontal type centrifuge).
 8. Measure the packed organisms in the tip of the tube. Each .01 ml. division of the Hopkins tube is assigned a wet weight of 10 mg. (as determined by experimentation). Once the concentration of organisms is known, the necessary dilutions can be prepared, using the remainder of the original suspension.
 9. Assume that the wet weight of organisms has been calculated to be 8 mg./ml. saline; prepare serial dilutions as shown in figure 2.

FIGURE 2
Preparation of Serial Culture Dilutions



Thoroughly mix the contents of each tube before withdrawing the desired amount. The mixing and measurements involved in the serial dilutions are made with a 1.0 ml. graduated pipette, equipped with a tight-fitting rubber bulb. A single pipette can be used for each set of dilutions without introducing an appreciable error.

In calculating the degree of dilution of the original suspension necessary to yield the desired concentration in mg. per ml. in the first dilution tube, the following principle is observed on the basis of the foregoing example: Since the concentration of organisms in the original suspension is 8 mg./ml., the desired concentration of 5 mg./ml. represents $\frac{5}{8}$ of the original concentration. Therefore, to arrive at the desired concentration take five parts of the original suspension and dilute to eight parts by adding three parts of saline. It is only necessary to choose the volume of the "parts" in such a way that the final volume of diluted suspension will be adequate for the needs of the test.

10. It is important that subcultures be made each time a suspension is prepared for typing by animal inoculation, in order to:

- a. Have a fresh culture of the organisms available for repeating virulence tests in the event the animal dies prematurely.

- b. Check the viability of the culture.
- c. Correlate colony characteristics with animal results.

11. Prepare subcultures by inoculating each of four tubes of media with 0.1 ml. of the 10^{-4} dilution. Slant culture tubes overnight at 37° C. Bind tubes with a rubber band, stand them upright in a wire basket and reincubate. Examine at weekly intervals and hold until animal report is available.

B. Turbidimetric Standardization:

Because of its versatility, light has been a very effective tool to chemists, and more recently to biologists, in a variety of ways. If directed at or through a solution, suspension or other substance, light may be absorbed, reflected, scattered, refracted and impeded, to mention a few possible changes. The construction of instruments to measure these light changes and thence convert them into quantitative analytical data has been a boon to science. Two of the more important applications of light analysis in the field of bacteriology are nephelometry and turbidity. Nephelometry is the science of measuring the amount of light **scattered** or **reflected** by the particles of a suspension. In bacteriology this becomes an important tool for measuring growth rates. Nephelometry is a very delicate technique and finds important application in the study of low population bacterial suspensions wherein the turbidity of the broth culture is often below that visible to the human eye. Generally speaking, the bacteriologist in a tuberculosis laboratory is more apt to be dealing with a very dense bacterial suspension of known concentration from which he can make appropriate dilutions for animal or culture inoculations. Where the bacterial suspension is sufficiently concentrated to interfere substantially with the transmittance of light, the concentration of the suspension can be measured by determining its percent transmission. This latter technique is called **Turbidity**, and is defined as the proportion of incident light stopped by unit depth of sample. If turbidity measurements were to be used to measure bacterial concentration, one would merely have to set up a standard curve of percent transmission (or optical density) vs. either number of viable organisms (as determined by bacterial plate counts) or wet weight of cells (determined by Hopkins tube); all subsequent standardization of suspensions could be made using the standard curve. An example of the preparation and use of a standard curve is given below. The data were made using a carefully suspended culture of the human strain H37Rv.

The culture is first ground up very carefully in saline or other diluent as described under the Hopkins tube technique. The turbidity or percent transmission of the suspension can be determined on a variety of instruments — colorimeter, nepho-

colorimeter, spectrophotometer, etc. — all of which operate on the principle that the intensity of light passing into a solution or suspension emerges decreased in intensity, and further that this drop in intensity of the emergent beam is proportional to the concentration of substance in solution or suspension. To prepare the standard curve, then, it is necessary to first place a “blank” tube in the path of the light beam. This blank tube contains the diluent (saline, broth or water) which was originally used to suspend the bacilli. Since this “blank” contains no organisms, the instrument is adjusted to read 100 percent transmission or zero optical density. Bacterial suspensions of varying, but known, concentrations are then placed in the path of the light beam. Since such suspensions contain particulate matter, they will impede the passage of light, resulting in a decrease in the percentage of light transmitted. These data may then be tabulated as in table III.

TABLE III*

COMPARISON OF THE WET-WEIGHT OF BACTERIAL CELLS WITH OPTICAL DENSITY (OR PERCENT TRANSMISSION)**

Weight of Cells	Optical Density	Percent Transmission
22 mg.	1.1	8
11	0.56	27.5
5.5	0.28	52.5
2.75	0.125	75.0
1.375	0.06	87.2
1.0	0.05	89.3
0.69	0.032	93.0

* Data obtained from a Coleman Model 9 Nepho-colorimeter, using a #8-209 green filter, transmitting at 525 mμ.

** Percent transmission may be converted to optical density using the formula:
 $\log_{10} \frac{(100)}{\% T} = \text{Optical Density.}$

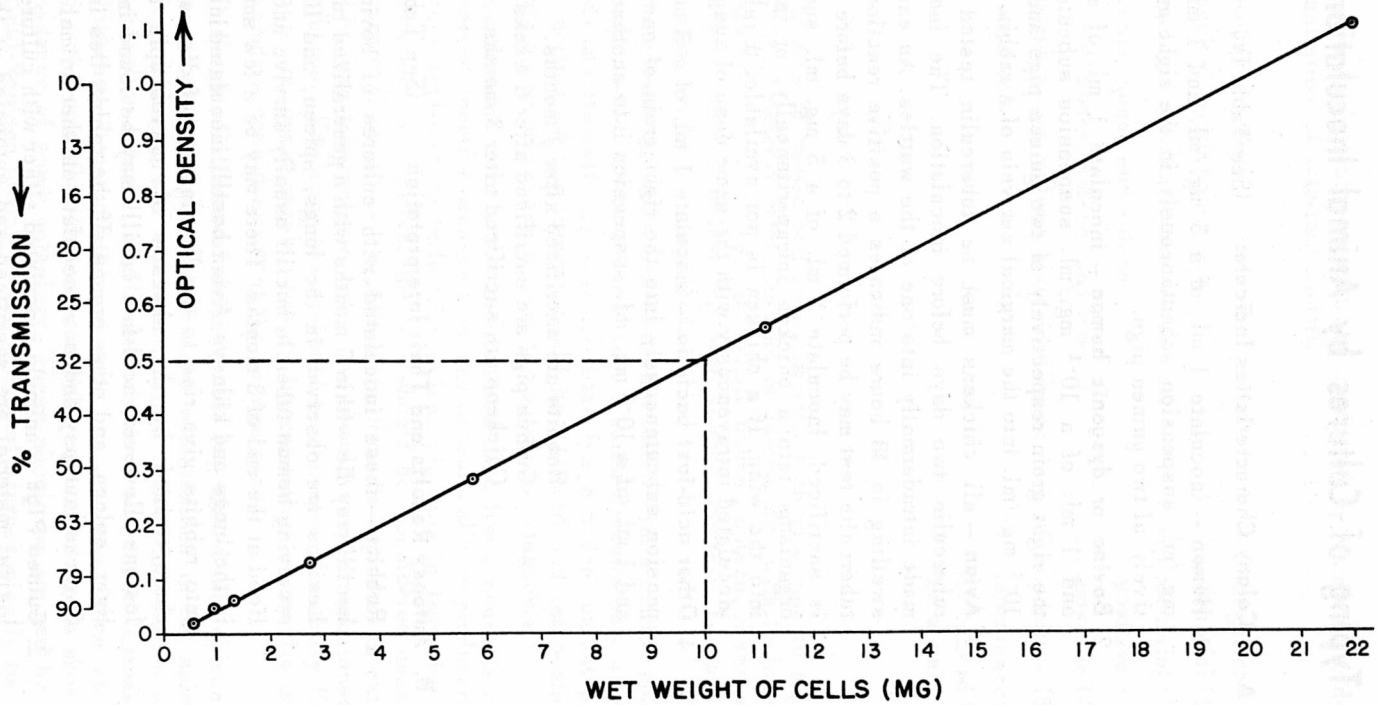
By plotting this data on a graph we obtain the curve shown in figure 3. Now then, how is this curve used? Suppose a bacterial suspension is prepared which has an optical density of 0.5; by checking the standard curve, it can be seen that such a suspension would contain about 10 mg./ml. Suitable dilutions could then be made to contain 5 mg./ml. and 10^{-1} mg./ml. as described under the Hopkins tube procedure. If the standard curve is to be plotted on ordinary graph paper, it is best to use optical density measurements, since they will yield a straight line curve whereas percent transmission vs. weight of cells produces a curved graph which is sometimes more difficult to interpret.

Care must be taken in using turbidity measurements, since the data varies depending upon particle size in suspension; for this reason a standard curve must be prepared in **each** individual laboratory. Since some acid-fast bacilli can be suspended more readily and in finer dispersion than others, it is suggested that standard curves be prepared for major types of cultures with which one might deal: human, bovine, avian, and saprophyte. Once the standard curves are prepared, the turbidity technique offers many attractive advantages:

1. Ease, speed and time saved.
2. None of the sample suspension need be lost (if sterile turbidity tubes are used) whereas the Hopkins tube entails loss of a 1 ml. aliquot for cell weight determination.
3. Accuracy and reproducibility.

FIGURE 3

Comparison of wet weight of bacterial cells with optical density or percent transmission



Typing of Cultures by Animal Inoculation

A. If Colony Characteristics Indicate: (See Table IV)

1. **Human** — inoculate 1 ml. of a 5 mg./ml. and 1 ml. of a 10^{-1} mg./ml. suspension subcutaneously in the right groin respectively of two guinea pigs.
2. **Bovine or dysgonic human** — inoculate 1 ml. of a 5 mg./ml. and 1 ml. of a 10^{-1} mg./ml. suspension subcutaneously in the right groin respectively of two guinea pigs and 1 ml. of a 10^{-1} mg./ml. into the marginal ear vein of a rabbit.
3. **Avian** — all chickens must be tuberculin tested with avian tuberculin two days before inoculation. The inoculation is made intradermally into one of the wattles. An easily visible swelling in 48 hours indicates a positive reaction. A repeat tuberculin test may be performed 2 to 3 days before the chicken is sacrificed. Inoculate 1 ml. of a 5 mg./ml. suspension of organisms into a chicken intraperitoneally, or intravenously into the wing. If a chicken is not available, a rabbit may be inoculated intravenously with the same dose of suspension.
4. **Other acid-fast bacteria** — inoculate 1 ml. of a 5 mg./ml. suspension subcutaneously into the right groin of one guinea pig and 1 ml. of a 10^{-1} mg./ml. suspension into another.

Rabbits are sacrificed after 3 months.

Guinea pigs are sacrificed after 6 weeks.

Chickens are sacrificed after 3 months.

B. Autopsy Results and Their Interpretation (See Table V)

1. **Rabbits** — those inoculated with cultures of bovine tubercle bacilli may die within 3 months with a generalized tuberculosis. Lesions are observed in the lungs, spleen, and liver. Those receiving **human** tubercle bacilli usually survive and are sacrificed at the end of 3 months. There may be a few small lesions in the lungs and kidneys. Avian **bacilli** inoculated intravenously into rabbits give rise to the Yersin type of disease which is characterized by the absence of macroscopic tuberculous lesions. However, acid-fast bacilli may be found in smears of liver, spleen, and other organs. If the rabbit dies in less than 6 weeks, autopsy and/or reinoculate another animal.
2. **Guinea Pigs** — animals inoculated either with cultures or pathological material are sacrificed and autopsied at the end of 6 weeks unless they die prior to that time. Record the extent of infection in the following manner, based on the distribution of

macroscopic lesions noted and confirmed by microscopic demonstration of acid-fast bacilli.

No involvement	. . .negative
Inguinal lymph nodes only	. . .doubtful (1+)
Iliac and inguinal lymph nodes only	. . .doubtful (2+)
Lymph nodes and spleen	. . .positive (3+)
Lymph nodes, spleen, and liver	. . .positive (4+)
Lymph nodes, spleen, liver, and lungs	. . .positive (5+)
Lungs only	. . .spontaneous (not caused by inoculum)

Cultures of human and bovine bacilli both give rise to a progressive, usually fatal, disease in guinea pigs with lesions in inguinal lymph nodes, abdominal nodes, spleen, liver, and lungs. Other acid-fast bacteria seldom progress beyond the inguinal and iliac lymph nodes.

If a guinea pig which has been inoculated with a routine specimen of pathological material (not a culture) shows only a minimal involvement (inguinal and iliac lymph nodes) on autopsy, a portion of the tissue should be cultured and reinoculated into a second animal in order to confirm the diagnosis.

Involvement of only the inguinal and iliac lymph nodes is considered doubtful because this may occasionally be produced by other acid-fast bacteria. If a guinea pig dies in less than 28 days and is negative, the test should be considered unsatisfactory. If on autopsy a guinea pig presents a picture of extensive lung tuberculosis only, one should suspect spontaneous infection. Virulence tests should be repeated if autopsy findings are questionable. Proper interpretation of results must include a consideration of the dosage used.

- 3. Chickens** - Cultures of avian bacilli inoculated into chickens give rise to progressive disease with lesions in the spleen, liver, kidneys, and mesenteric lymph nodes. Inoculation with mammalian tubercle bacilli usually does not cause lesions in chickens. Occasionally, however, an abscess forms at the site of inoculation. Smears should be made from organs showing evidence of gross pathology. These should be stained by the Ziehl-Neelsen technique and examined microscopically for the presence of acid-fast bacilli. If the chicken dies in less than 6 weeks it should be autopsied and if results indicate, another chicken should be inoculated.

TABLE IV
ANIMAL INOCULATION CHART

Culture Type	Dosage	Route	Animal	Days Until Autopsy
Human or Atypical Human	10 ⁻¹ mg.	subcutaneous	guinea pig	42
	5 mg.	subcutaneous	guinea pig	42
Bovine or	5 mg.	subcutaneous	guinea pig	42
	10 ⁻¹ mg.	subcutaneous	guinea pig	42
Dysgonic Human	10 ⁻¹ mg.	intravenous	rabbit	90
Avian	5 mg.	intraperitoneal or intravenous	chicken	90
Other Acid-fast Bacteria	5 mg.	subcutaneous	guinea pig	42
	10 ⁻¹ mg.	subcutaneous	guinea pig	42

TABLE V
AUTOPSY RESULTS

Culture Type	INOCULATED INTO		
	Guinea Pig	Rabbit	Chicken
Human	+++	+(1)	-
Bovine	++++	+++	-
Avian	-	± (Yersin) ⁽²⁾	+++

(1) Normally the only disease seen, if any, following intravenous inoculation of rabbits with human variety tubercle bacilli, is isolated lesions in the lung and kidney.

(2) The Yersin reaction is characterized by absence of macroscopic disease but presence of large numbers of acid-fast organisms in microscopic sections of the spleen, liver, lung and/or kidney.

C. Pathogenicity of the Anonymous Acid-Fast Bacilli for Laboratory Animals.

In the experience of this laboratory there is no laboratory animal which consistently and reproducibly demonstrates the virulence of these organisms. For the main, clinicians and bacteriologists have been satisfied that consistent isolation of the same organism from repeated samples of sputa and later perhaps from resected lung tissue – in the absence of other disease agents – constitutes significant evidence of the pathogenicity of these strains for man, Koch's Postulates notwithstanding. The results of pathogenicity studies in laboratory animals are indicated below.

1. *M. kansasii* (Group I Photochromogen) is undoubtedly the most pathogenic of the anonymous strains for laboratory animals. The usual subcutaneous and intraperitoneal routes of inoculation generally are innocuous for the guinea pig, even with as much as 5 mg. of inoculum. Some investigators report that intracardial inoculation of 1 mg. of organisms into guinea pigs often results in death within 4 to 5 weeks. Only rarely has the latter been observed in this laboratory and indeed, in our experience; it is more usual to find little progressive infection following intracardial inoculation. On the other hand, mice seem to be a little more susceptible to infection than guinea pigs. The intravenous inoculation of 0.01 to 0.1 mg. of organisms may result in the formation of macroscopic lesions in lungs and spleen and more rarely in the liver and kidneys. As in the case of guinea pigs, mice are not sufficiently susceptible to these microorganisms to warrant their use as the sole laboratory animal.
2. Group II Scotochromogens exhibit essentially no pathogenicity for the usual laboratory animals.
3. Group III Nonphotochromogens are less consistent than strains of *M. kansasii* in producing infection in laboratory animals. Some strains appear to be highly virulent while others behave like saprophytes, indicating either that this group is still a heterogeneous mixture or that there are several variants within the group. As in the case of *M. kansasii*, Battey strains are more pathogenic for mice than for guinea pigs, but in neither case is the animal data reliable enough to warrant their use for pathogenicity tests.
4. Group IV Rapid Growers appear to be comprised both of true pathogenic species and of saprophytic strains recoverable from sources unassociated with disease. Of the apparent pathogenic strains, the rough colony variants and *M. fortuitum* show virulence for mice following intravenous inoculation. It is not uncommon for the mice to die following inoculation. At autopsy the most notable lesions are seen in the kidneys.

From the foregoing it can be seen that there is no laboratory animal whose pathologic picture, following infection, consistently and reliably demonstrates the pathogenicity of the atypicals for man. One technique, **the intracutaneous inoculation of guinea pigs**, appears to offer better correlation of cultural pathogenicity for man than do any of the other animal virulence tests herein described. Until some more reliable pathogenicity test is obtained, this intracutaneous technique offers considerable promise as an economical relatively rapid test of culture virulence. The intracutaneous inoculation of guinea pigs has been used in the past to determine the virulence of saprophytic and mammalian acid-fast bacilli. It has been pointed out previously in this section of the manual that the groups of anonymous mycobacteria are relatively innocuous for guinea pigs when administered via the usual subcutaneous, intraperitoneal, and often intracardial routes. Studies in this and other laboratories have shown that the results of intracutaneous challenge of guinea pigs with these strains possibly may provide closer correlation of culture virulence for man. Results of this test may be read in 7 to 21 days and as many as four cultures may be inoculated into one animal, so the obvious advantages of speed and economy are readily recognized. In performance of this procedure, culture suspensions containing 1.0 mg./ml. and 0.1 mg./ml. are prepared and 0.1 ml. volumes of each are inoculated into the quadrants of the shaved abdominal surface of the guinea pig. To avoid possible errors arising if the inoculum is inadvertently administered subcutaneously, this laboratory prefers to duplicate all inocula. From the standpoint of economy, however, it is possible to use only one inoculum per culture (1.0 mg./ml. is recommended) and if one is careful to raise a bleb at the site of each inoculum, it is not necessary to duplicate inocula.

The site of inoculation is observed at 3- or 4-day intervals and any changes noted are recorded. A positive test is indicated by the formation of a persistent open ulcer at the site of inoculation. Using this technique all virulent human, bovine, and avian strains produce open ulcers, whereas saprophytes produce no lesion, or, at most, an area of induration which soon fades. In the experience of this laboratory about 85 percent and 75 percent respectively of strains of *M. kansasii* and Group III Nonphotochromogens yield open ulcers. These two groups generally are considered the most virulent of the anonymous acid-fast bacilli for man. The scotochromes and rapid growers, considered to be much less virulent than the other two groups, cause fewer open lesions by following this procedure. Until such time when a laboratory animal can be found which will consistently and reliably indicate the virulence of the atypical acid-fast bacilli for man, the intracutaneous route of challenge appears to offer much promise.

Cytochemical Tests

At the outset it should be pointed out that final typing and virulence determination of the mycobacteria (human, bovine, avian, and saprophyte) should be reported only after adequate biochemical and/or animal pathogenicity tests have been performed and recorded. It is recognized that the foregoing thesis falls down in two major areas. First, there is no laboratory animal which can provide a reliable measure of the pathogenicity of atypical acid-fast bacilli. Secondly, it is realized that many laboratories are ill-equipped to perform animal pathogenicity tests. It is for this latter reason primarily that cytochemical tests are being discussed in this manual. It is hoped that those laboratories which are unable to perform animal pathogenicity tests may be able to achieve some degree of typing and virulence determination of acid-fast bacilli by performing a number of relatively simple cytochemical tests.

For many years research workers have sought simple and rapid *in vitro* tests for the differentiation and virulence determination of acid-fast bacilli. Because of inconsistencies in results many of these "test-tube procedures" have been disregarded. However, certain of these tests have had widespread acceptance and others are looked upon with much favor. Those cytochemical tests which appear most popular and most reliable will be discussed in detail while others, adequately described in the literature, are listed in the bibliography.

1. **The Neutral Red Test** as described by Middlebrook and Dubos in 1948, is performed as follows: Several colonies are picked from a culture slant and added to 5 ml. of 50 percent methyl alcohol; after incubating at 37° C. for 1 hour the alcohol is decanted and the alcohol washing procedure repeated with 5 ml. of fresh 50 percent methyl alcohol. The alcohol is again decanted and the colonies resuspended in 5 ml. of fresh (not over 3 to 4 weeks old) barbital buffer (5 percent NaCl + 1 percent Na barbital) to which is added 1 ml. of aqueous neutral red (20 mg. percent); this suspension is then held at room temperature for 30 minutes before reading. Neutral red has a yellow color in this alkaline barbital buffer; however, if the dye becomes fixed by the cells, the latter acquire a red color in a yellow supernatant fluid. Dubos and Middlebrook noted that it was the virulent bacilli which bound the dye, while avirulent strains remained yellow, hence, the neutral red test is used to distinguish virulent mycobacteria from avirulent strains. As originally described, the neutral red test is rather time-consuming, requiring at least 3 hours to perform. Several modifications of the test have provided considerable savings in time. One, devised by Wayne *et al.* (see bibliography), entails

the use of the millipore filter. Another modification, performed at CDC, requires the use of Herrold's egg yolk agar, notably different from other egg media in its lack of added mineral salts and asparagine. The procedure for the CDC modified test follows. Several colonies of the organism in question are picked from Herrold's egg yolk agar and suspended in 5 ml. of the barbital buffer described above; one ml. of the aqueous neutral red is added and after standing 15 minutes at room temperature the results are read. It should be pointed out that while the neutral red test is helpful to tuberculologists, neither it nor any other **single** cytochemical test should be relied upon for final virulence determination. If there is any element of doubt about a given culture, animal pathogenicity and other confirmatory tests should be performed before final reports are issued.

2. The observation of **Cord Formation** also may provide some helpful information regarding virulence of the acid-fast bacilli. As originally described by Middlebrook *et al.*, it was noted that virulent strains of tubercle bacilli tended to orient themselves in close proximity to one another in the shape of serpentine cords. If these "cords" are carefully examined under the oil immersion lens, they are seen to be made up of individual bacilli lying side-by-side and end-to-end in parallel alignment. This phenomenon is best observed in media containing **no** tween 80 or other wetting agents, e.g., tween-less albumin broth. In this laboratory it has been possible to demonstrate beautiful cord preparations using the water of condensation from an egg slant. In the event there is no such water, 0.5 ml. of sterile saline may be added to the slant and a portion of one colony ground into this fluid; after several days in the incubator, a loopful of this added saline may be removed to a microscope slide, heat-fixed and stained by Ziehl-Neelsen or other acid-fast procedure. Upon examination under 100x magnification, the serpentine cords, if present, are clearly visible.
3. The **Niacin Test** of Konno is used to distinguish human strains, regardless of virulence, from most other acid-fast bacilli. The test is based on the fact that human strains produce more niacin than any of the other commonly encountered acid-fast bacilli. Notable exceptions to this statement are *M. microti* (vole bacillus) and *M. ulcerans*, both of which may be niacin positive. This niacin can be detected by the use of several chemicals which combine with the niacin to form a yellow color complex. It has been the experience of this laboratory that a recent modification of the niacin test, devised by Runyon, is more economical and more reliable than the original procedure of Konno. Runyon's test is performed as follows: Sterile water or saline (0.5 - 1.0 ml.) is added to the culture slant and the tube is placed so that the fluid layers over the colonies. If

niacin is present, it will be extracted in 5 or 10 minutes. A portion of the fluid (0.25 – 0.5 ml.) is removed and equal quantities of aniline* (4 percent in ethyl alcohol) and cyanogen bromide* (10 percent aqueous) are added. (Fume hood should be used for cyanogen bromide.) If niacin is present, a yellow color forms almost immediately throughout the solution. It is recommended that an alkaline germicide (cresol with NaOH) be added to check tear gas formation prior to discarding the tubes. When evaluating his modified test on cultures grown on a variety of media, Runyon found that most media gave good results, with the exception of blood agar; aqueous extracts of the latter were pink, thereby obscuring the yellow color of the niacin test. This test is very reliable in that most niacin positive strains encountered in the diagnostic laboratory can safely be labelled as human varieties. The fact that a culture is niacin negative, however, does not preclude the possibility that it is of the human variety. It should be reiterated that the niacin test is not indicative of virulence, but rather, it distinguishes human strains, **regardless of virulence**, from most other acid-fast bacilli.

4. **Catalase Test.** It is a well known fact that acid-fast bacilli produce the enzyme catalase. It has been shown that tubercle bacilli which become resistant to isoniazid will lose, or exhibit a lowering of, their catalase activity. This loss of catalase activity also has been correlated with attenuation in virulence of said microorganisms for the guinea pig. The suggested correlation between catalase activity and virulence of mycobacteria prompted a study by Schweiger *et al.* which demonstrated that it was possible to distinguish virulent from avirulent mycobacteria on the basis of catalase activity. Studies in this laboratory have shown that it is possible to subgroup the acid-fast bacilli on the basis of their catalase activity at different temperatures and pH.

In this laboratory, catalase tests are performed in two ways: 1) For determination of catalase activity at room temperature, 0.5 ml. of a 1:1 mixture of 10 percent tween 80** and 30 percent hydrogen peroxide*** is added directly to the culture slant held at room temperature. 2) To determine the effect of temperature and pH on catalase activity, several spadefuls of growth are scraped from a culture slant and added to 0.5 ml. of the appropriate buffer solution, and thence held in a water bath of proper

*These stock solutions may be prepared and stored in brown bottles in the refrigerator. These solutions will keep 3 to 4 weeks or longer. If either stock solution changes color or precipitates, it should be discarded and fresh material prepared.

**The polyoxyethylene derivative of sorbitan monooleate, procured from Atlas Powder Company, Wilmington, Delaware.

***Superoxol, Merck and Company, Inc., Rahway, New Jersey.

temperature for 20 to 30 minutes; to this bacterial suspension is then added 0.5 ml. of the tween-peroxide mixture. The catalase enzyme, if present and active, breaks down the hydrogen peroxide to water and nascent oxygen; the latter bubbles throughout the reaction mixture, and it is this bubbling that is recorded as a positive test. Reaction tubes which do not bubble immediately are observed for 20 minutes before discarding. Studies in this laboratory have revealed that the most critical temperature-pH combinations for catalase studies are room temperature and 68° C. at pH 7.

At room temperature the acid-fast bacilli are catalase positive. Those strains which possess no catalase activity at room temperature should be suspected of being isoniazid resistant strains. By suspending organisms in phosphate buffer of pH 7 and then placing them in a 68° C. water bath for 20 minutes, it is possible to selectively knock out the catalase activity of human and bovine strains regardless of virulence; at this temperature-pH combination, all other acid-fast bacilli (avian, saprophyte, and atypical) are catalase positive.

5. The observation of **acid production from carbohydrates** is very helpful in the differential typing of rapid growing acid-fast saprophytes and *M. fortuitum*. The procedures for this test are adequately described by Gordon *et al.* (bibliography). The composition of the inorganic nitrogen base medium is as follows: $(\text{NH}_4)_2\text{HPO}_4$, 1 g.; KCl, 0.2 g.; MgSO_4 , 0.2 g.; agar 15 g.; distilled water 1000 ml. pH is adjusted to 7.0 and 15 ml. of 0.04 percent brom cresol purple added. The medium is put up in 5 ml. amounts, sterilized by autoclaving (15 lb./15 min.), and prior to use 0.5 ml. of a 10 percent aqueous solution of the carbohydrate to be tested, sterilized by autoclaving, is added aseptically to each tube.

Strains of *M. phlei* are identified by acid production from glucose, mannitol and sorbitol and usually from mannose and arabinose; no acid is produced from rhamnose, inositol or dulcitol. *M. smegmatis* ferments many sugars, producing acid from glucose, rhamnose, xylose, arabinose, sorbitol, inositol, mannose, mannitol, trehalose and galactose, and usually from dulcitol. Strains of *M. fortuitum* produce acid from glucose, mannose, trehalose, and occasionally mannitol. In our laboratory, acid is frequently produced from maltose. There is, however, no acid production from arabinose, sorbitol, inositol or dulcitol.

It can be seen that these sugar reactions are of considerable help in typing rapid growing acid-fast bacilli, and this typing is

especially important in view of the fact that certain rapid growing bacilli are in fact, not saprophytes, but agents of disease in man.

There are other cytochemical tests that have been proposed for use in typing and virulence-testing of acid-fast bacilli. Many of these tests are referred to in the bibliography but are not described in detail in the context either because they are no longer considered reliable or because they haven't been tested on sufficient numbers of cultures to warrant their inclusion in routine studies.

Methods for Testing Sensitivity of Tubercle Bacilli to Therapeutic Agents

Sensitivity tests may be performed either by the direct method, using as inoculum the treated and concentrated primary clinical material such as sputum, or by the indirect method in which a portion of a previously isolated pure culture of the organism is the inoculum. The direct method has the advantage of saving time and of inoculating the test medium with a bacterial population in which the proportion of resistant and susceptible organisms is that existing in the exudate sampled. The exudate must contain, however, an adequate number of organisms so that clear and reliable differences between the growth appearing on the drug-containing tubes and the control tubes can be perceived. There should be at least 50 colonies on each of the control tubes. Furthermore, the organisms in this inoculum of treated clinical material must be evenly dispersed.

If the organisms present in the direct inoculum of processed clinical material are so few as to render doubtful the significance of differences between the tests and the controls, the test must be repeated using a pure culture of the bacilli isolated on a drug-free medium. The pure culture or "indirect" method allows the preparation of a standardized inoculum.

If the medium chosen for this work is one requiring inspissation and heat sensitive antibiotics are to be used, it becomes necessary to add the antibiotic, before inspissation, in sufficient quantity to allow for deterioration due to heat and at the same time insure the final desired effective quantity in the finished medium. Accuracy is difficult under these conditions and this laboratory has accordingly been accustomed for some time to use a medium in which inspissation is not required. Herrold's egg-yolk medium is used in this laboratory. If the specimen to be examined is one of primary clinical material, using the direct method, two additional control tubes of Lowenstein-Jensen medium without antibiotic are included in the series. Other media not requiring inspissation have been used with equally good results, e.g., the 7-H9 and 7-H10 media of Middlebrook (see appendix for formulae).

A. Suggested Procedures for Several of the More Commonly Used Antituberculous Drugs.

1. Streptomycin (SM):

a. Pathological specimens — clinical material.

- 1) Decontaminate and concentrate the clinical specimen or sample by the usual NaOH method.

- 2) Using aseptic technique, mix the sediment well with 1.0 ml. of sterile distilled water. The amount of water will vary with the number of tubes to be inoculated. Inoculate 0.1 ml. of this treated and diluted sediment onto each tube of medium. Each set of media contains: 2 tubes of Herrold's egg-yolk agar medium containing 10 and 100 mcg., respectively, of streptomycin per ml. of medium, 2 control tubes of Herrold's medium without antibiotic, and 2 control tubes of Lowenstein-Jensen medium without antibiotic.

b. Cultures

If repeated tests of the antibiotic sensitivity of cultures of tubercle bacilli are to be comparable, it appears desirable that the inocula be standardized. A variety of methods is available for the standardization of suspensions of tubercle bacilli. One method which combines simplicity with a fair degree of accuracy is that involving the use of the Hopkins vaccine centrifuge tube. The technique for this procedure is given under Preparation of Cultures for Typing by Animal Inoculations, Steps 1 - 9. (see page 30).

- 1) From the standardized suspension prepare a culture dilution containing 10^{-2} mg./ml.
- 2) Using aseptic technique, mix well with a pipette and inoculate 0.1 ml. of the 10^{-2} suspension onto each tube of medium. The inoculum would be 10^{-3} mg. or .001 mg. onto each tube. Each set of medium tubes contains: 2 tubes of Herrold's medium containing 10 and 100 mcg. respectively, of streptomycin per ml. of medium, and 2 control tubes of Herrold's medium without antibiotic. When pure cultures are used, it does not appear necessary to employ the more sensitive Lowenstein-Jensen medium for control.

c. Incubation

After the tubes have been inoculated, incubate at 37° C., leaving the inoculated tubes in the slanted position for 2 weeks in order that growth may not be confined to the bottom of the slant surface. Examine at the end of 2 weeks and each week thereafter until the cultures are 4 weeks old.

d. Reading and Reporting Results

The reporting of the results of the sensitivity tests is based upon the following reading procedures. (Table VI).

TABLE VI
INTERPRETATION OF DRUG SENSITIVITY TESTS

		Growth in Tubes	Report
Controls	10 mcg./ml.	100 mcg./ml.	
Growth	No Growth	No Growth	Sensitive to 10 mcg./ml.
Growth	Growth (equal to controls)	No Growth	Resistant in 10 mcg./ml. Sensitive to 100 mcg./ml.
Growth	Growth (equal to controls)	Growth (equal to controls)	Resistant in 100 mcg./ml.
Growth	Growth (less than controls)	No Growth	Partially resistant in 10 mcg./ml. Sensitive to 100 mcg./ml.
Growth	Growth (equal to controls)	Growth (less than controls)	Resistant in 10 mcg./ml. Partially resistant in 100 mcg./ml.

Although the written reports consist of appropriate phrases, selected from column four of the above table, the laboratory is advised to keep a record of the data upon which the reports have been based. A useful supplement to this record may be made according to the following schedule:

If less than 25 percent of the surface is covered with growth, the colonies are actually counted.

If 25 to 50 percent of the surface is covered with growth, growth is recorded as 2+.

If 50 to 75 percent of the surface is covered with growth, growth is recorded as 3+.

If 75 to 100 percent of the surface is covered with growth, growth recorded as 4+.

2. Para-Aminosalicylic Acid (PAS):

The method of testing, reading, recording, and reporting for sensitivity to PAS is the same as described above for streptomycin with the exception that the concentrations of sodium para aminosalicylic acid to be added to the medium are .001 mg./ml., .01 mg./ml., and 0.1 mg./ml., respectively. Included in each test are the usual control tubes of medium containing no drug.

3. Isoniazid (INH):

The method of testing, reading, recording, and reporting for sensitivity to isoniazid is the same as that described for streptomycin and for PAS. The concentrations of isoniazid used are 0.2 mcg./ml., 1 mcg./ml., 5 mcg./ml. of the finished medium. For cultures known or suspected to be resistant, a tube containing 10 mcg./ml. of medium may be included in the test. The usual control tubes without the drug are included in the test.

4. Viomycin:

The method of testing, reading, recording, and reporting for sensitivity to viomycin is the same in principle as that used for the foregoing therapeutic agents. The concentrations used in the finished medium are 10 mcg./ml., 50 mcg./ml., and 100 mcg./ml., respectively. Each test includes the usual tubes of control media without the antibiotic.

5. Pyrazinamide (PZA):

The method of testing, reading, recording, and reporting of sensitivity to PZA is the same as that used for other drugs. The concentrations suggested in the Veterans Administration conference on chemotherapy of tuberculosis (1956) are 10 mcg./ml., 20 mcg./ml., 40 mcg./ml., 100 mcg./ml., and 500 mcg./ml. of media. Solid media at pH 5.25 is recommended.

6. Cycloserine:

The preceding methods of testing apply also to cycloserine, using the following drug concentrations: 5 mcg./ml., 10 mcg./ml., 20 mcg./ml., and 50 mcg./ml. of media.

It is recommended that all the above-named drug dilutions be made up just prior to incorporation in the medium; this is especially true for cycloserine which is unstable in solution. It is further recommended that cycloserine media be kept NOT longer than two weeks, due to its rapid deterioration and loss of potency.

B. Preparation of Medium and Dilutions of Therapeutic Agents

1. Modified Herrold's Egg-Yolk Agar medium.

a. Agar Base:

Difco dehydrated nutrient agar 23 gm.

The above-named nutrient agar contains:

Bacto-beef extract 3 gm.

Bacto-peptone 5 gm.

Bacto-agar 15 gm.

Glycerol (reagent grade)	10 ml.
Distilled water	750 ml.
Malachite green (2 percent aqueous)	10 ml.

- b) Dissolve ingredients by heating to 100° C., and distribute in 150 ml. quantities in 250 ml. flasks.
- c) Autoclave for 15 minutes at 15 lbs. (121° C.)
- d) Cool to 47° C.
- e) Add antibiotic or drug dilution just **before** adding the egg-yolk.
- f) Add 50 ml. of egg yolk per flask just before tubing.* Mix well by rotating the flask.
- g) Tube medium aseptically, 3 to 4 ml. per tube, and slant the tubes before the medium solidifies.
- h) Incubate the tubes for 48 hours to test for sterility.

2. Preparations of Antibiotic or Drug Dilutions.

The first or pilot dilution of the therapeutic agent is made in distilled water and is sterilized by filtration through a bacterial filter. Subsequent dilutions are made aseptically in sterile distilled water. It has been found that media containing the antibiotics streptomycin or viomycin will keep for at least 8 weeks at 5° C.

a. Preparation of **Streptomycin** Dilutions:

- 1) Dissolve 0.2 gm. of streptomycin in 10 ml. of distilled water. Filter.
- 2) Dilute 1 ml. of 1) with 9 ml. of sterile distilled water.

When added to 200 ml. of the finished medium

- 1 ml. of 1) gives 100 mcg./ml. of medium.
- 1 ml. of 2) gives 10 mcg./ ml. of medium.

b. Preparation of Sodium **PAS** Dilutions:

- 1) Dissolve 0.6 gm. of PAS in 3 ml. of distilled water. Filter.
- 2) Dilute 1 ml. of 1) with 9 ml. sterile distilled water.
- 3) Dilute 1 ml. of 2) with 9 ml. sterile distilled water.
- 4) Dilute 1 ml. of 3) with 9 ml. sterile distilled water.

*Scrub fresh eggs with soap and water. Rinse. Place in 70 percent alcohol for 15 minutes. Separate the egg yolks and whites aseptically. Mix the egg yolks until they are homogeneous. Note that the egg yolk is added *after* media has been sterilized. Therefore rigid aseptic precautions are necessary.

When added to 200 ml. of the finished medium

- 1 ml. 1) gives 1 mg./ml. (100 mg. percent)
- 1 ml. 2) gives 0.1 mg./ml. (10 mg. percent)
- 1 ml. 3) gives 0.01 mg./ml. (1 mg. percent)
- 1 ml. 4) gives 0.001 mg./ml. (0.1 mg. percent)

c. Preparation of **Isoniazid** Dilutions:

- 1) Dissolve 10 gm. of isoniazid in 10 ml. of distilled water. Filter.
- 2) Dilute 1 ml. of 1) with 4 ml. of sterile distilled water.
- 3) Dilute 1 ml. of 2) with 4 ml. of sterile distilled water.

When added to 200 ml. of finished medium

- 1 ml. 1) gives 5 mcg./ml.
- 1 ml. 2) gives 1 mcg./ml.
- 1 ml. 3) gives 0.2 mcg./ml.

d. Preparations of **Viomycin** Dilutions:

- 1) Dissolve 0.2 gm. of viomycin in 10 ml. of distilled water. Filter.
- 2) Dilute 1 ml. of 1) with 9 ml. of sterile distilled water.

When added to 200 ml. of the finished medium

- 1 ml. 1) gives 100 mcg./ml. of medium
- 0.5 ml. 1) gives 50 mcg./ml. of medium
- 1 ml. 2) gives 10 mcg./ml. of medium

e. Preparation of **Pyrazinamide** Dilutions:

- 1) Dissolve 1 gm. of Pyrazinamide in 10 ml. of distilled water at about 60° C. Filter.
- 2) Dilute 1 ml. of 1) with 4 ml. of sterile distilled water.
- 3) Dilute 1 ml. of 2) with 1.5 ml. of sterile distilled water.
- 4) Dilute 1 ml. of 2) with 4.0 ml. of sterile distilled water.

When added to 200 ml. of the finished medium

- 1 ml. 1) gives 500 mcg./ml. of medium
- 1 ml. 2) gives 100 mcg./ml. of medium
- 1 ml. 3) gives 40 mcg./ml. of medium
- 1 ml. 4) gives 20 mcg./ml. of medium
- 0.5 ml. 4) gives 10 mcg./ml. of medium

f. Preparation of **Cycloserine** Dilutions:

- 1) Dissolve 0.1 gm. of cycloserine in 10 ml. of distilled water. Filter.

- 2) Dilute 2 ml. of 1) with 3 ml. of sterile distilled water.
- 3) Dilute 1 ml. of 1) with 4 ml. of sterile distilled water.
- 4) Dilute 1 ml. of 1) with 9 ml. of sterile distilled water.

When added to 200 ml. of the finished medium

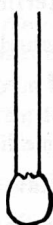
- 1 ml. 1) gives 50 mcg./ml. of medium
- 1 ml. 2) gives 20 mcg./ml. of medium
- 1 ml. 3) gives 10 mcg./ml. of medium
- 1 ml. 4) gives 5 mcg./ml. of medium

Proper dilutions of any other drug may be readily calculated in a similar manner.

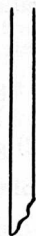
C. Inoculating "Spade:"

A satisfactory harvesting spade may be devised by flattening the end of a short inoculating needle of stiff wire with hammer and anvil.

SPADE — 18 gauge wire (nichrome)



Front View



Side View

Appendix

A. Stains and Reagents

1. Ziehl-Neelsen Reagents:

a. Carbol Fuchsin:

Saturated solution of basic fuchsin (10 gm. basic fuchsin in 100 ml. of 95 percent ethyl alcohol)	10 ml.
5 percent aqueous solution of phenol	90 ml.

b. Acid Alcohol:

HCL (concentrated)	3 ml.
Ethyl alcohol 95 percent	97 ml.

c. Counter Stain:

Methylene Blue	0.3 gm.
Distilled water	100 ml.

2. Phenol Red Indicator (0.4 percent):

Stock solution: Dissolve 0.1 gm. of solid phenol red in 25 ml. N/20 NaOH. This may be stored in the dark.

3. 4 Percent NaOH Digestant:

Dissolve 40 gm. of NaOH in 1000 ml. distilled water. Add 1 ml. of phenol red indicator stock solution to every 99 ml. of NaOH digestant. This solution should be freshly prepared at least once a month. The digestant should be sterilized. This gives a 0.004 percent concentration of phenol red.

4. 2 N HCl:

Dilute 167.2 ml. concentrated HCl up to 1000 ml. with distilled water. Distribute in 2 ml. quantities in screw-cap test tubes and sterilize.

5. Physiological Saline:

Dissolve 8.5 gm. NaCl in 1000 ml. distilled water.

B. Laboratory Equipment*

1. Inspissators:

- Inspissator – Levin Model – constant temperature at 50° C. to 80° C. Holds 144 tubes – twice a day. Size: 30" x 14" x 10". Distributor: Will Corporation; Price: \$375.00 (as of 1958).

*This does not necessarily constitute an endorsement of these products by the U. S. Public Health Service. Prices are current and are subject to change.

- b. Inspissator – Coagulator “Elconap” #7530 – automatic electric, jacketed, constant temperature at 50° – 90° C. Size: 29" x 22" x 12". Holds 288 to 384 tubes twice a day. Distributor: Standard Scientific Supply Company, 34 W. 4th Street, New York 12, New York. Price: \$396.00 to \$528.00 (1952).
- c. U.S.P.H.S. inspissator – not commercial – holds 130 tubes per hour. The cost of materials and labor is from \$500.000 to \$700.00 (as of 1954).
- d. Several laboratories are using the usual hot air sterilizer (size: 24" x 17" x 18" with 4" insulation) as an inspissator by placing a large pan of water on the bottom of the sterilizer and heating the sterilizer to 89° C. A large tray with a layer of towels on the bottom is filled with one layer of slanted egg medium culture tubes (this tray holds approximately 90 tubes). The tray of tubes is placed in the hot air sterilizer 8 inches above the pan of water, and allowed to heat for 40 minutes. The temperature of the oven drops to 32° C. when the tubes are first placed in the oven, but it gradually rises and remains at 89° C. for the last 10 minutes of inspissation. Using this method, good results in the texture, moisture content, sterility, and consistency of the medium have been obtained. Only a few tubes near the sides of the oven, where the temperature is irregular will need to be discarded. This procedure may be satisfactory, but an inspissator is preferable.

2. Paint Shaker:

Paint conditioner, laboratory model, Red Devil #34.

Holds ¼-pint to 1-quart can.

No attachments or adjustments.

Obtainable from Red Devil Tools, Irvington, New Jersey.

Price: \$60.00 (1954).

3. Paint-Shaker Can:

The can itself is 6" in diameter and 6¼" in height. The removable inside rack, for holding 26 tubes (16 mm. diam.), has two shelves. Each shelf is 5-7/8" in diameter and the entire rack is 5-1/8" in height. The can and rack are constructed of 16-gauge metal, as is the removable lid. A disc of the same material, 4-3/8" in diameter is spot-welded to both lid and bottom in such a manner as to accommodate the adjustable clamps of the paint-shaker. Circular sponge rubber pads should be inserted above and below the rack in order to minimize breakage. (Can specially constructed)

4. Splash Proof Can:

Can, stainless steel, rustproof; 6" in diameter x 3" in height. Can has a splash-proof lid made to fit 1" down on can. Lid constructed of stainless steel and hole in center 1¼" in diameter having a stainless steel funnel, 5" at top diameter tapered to 1¼" at bottom welded to center hole of lid. (Can specially constructed)

5. Discard Can:

Can constructed of #34 gauge stainless steel, to be rustproof and water-tight to withstand 250° to 300° F. Outside dimensions 9" x 10" long x 5" deep. Top hinged on piano hinge to overhang sides by a minimum of ½". All seams soldered with 50-50 solder and edges are finished smooth. (Can specially constructed)

6. Pipette Discard Trays:

Covered catheter trays. A. S. Aloe Company, St. Louis. (Hospital Supply Catalogue) or American Hospital Supply Corporation.

7. Slide Warmer:

Slide Warming Table – Ranson Electric (Cat. No. 19646). Can be bought from Will Corporation, Rochester 3, New York.

8. Cornwall Pipetting Unit:

No. 1251 – containing cornwall syringe, double valve, ¾" stainless cannula, 20" amber rubber tubing with metal sinker. Can be obtained from Becton, Dickinson and Company, Rutherford, New Jersey.

9. Caulfield Pipettor:

Pipette control safety bulb – Aloe No. 69300, St. Louis Missouri.

10. Hand Lens:

Magnifiers, Coddington, Folding (Cat. No. 17541) 10X or 14X magnification: Can be obtained from Will Corporation, Rochester 3, New York, or Hastings Triple Aplanot, 10X or 14X, A. S. Aloe Company, St. Louis, Missouri

11. Luer-Lok Syringes (Becton-Dickenson):

These syringes prevent accidental separation of syringe and needle. Any laboratory supply company.

12. Culture Tubes:

Kimball Brand – screw cap – 20 x 150 mm. Size D
Aloe No. 78290 or Kimball #45066.

13. Centrifuge Tubes:

Bacteriological culture tubes, screw capped (Kimball No. 45066) 125 mm. in length, 16 mm. outside diameter. Can be obtained from Will Corporation, Rochester 3, New York.

14. Avian Tuberculin:

Can be obtained from veterinary supply house.

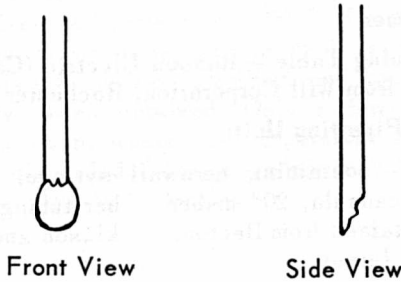
15. Potato Flour:

Can be obtained from J. M. Henson Bakery Supplies, 500 Stewart Ave. S.W., Atlanta, Georgia, or any bakery supply company.

16. Spade:

A spade may be made by flattening the end of a nichrome wire (18 gauge). The flattened area should be 5 to 10 mm. long by 0.5 mm. thick.

SPADE - 18 gauge (Nichrome)



C. Culture Media

1. Modified Lowenstein's Medium:

Salt Solution:

Monopotassium Phosphate (anhydrous)	2.4 gm.
Magnesium sulfate 7H ₂ O	0.24 gm.
Magnesium citrate	0.6 gm.
Asparagine	3.6 gm.
Glycerol (reagent grade)	12.0 ml.
Distilled water	600.0 ml.

Potato Flour	30.0 gm.
Homogenized whole eggs	1000.0 ml.
Malachite green, 2 percent aqueous solution	20.0 ml.

30 gm. of potato flour is added to the flask of salt solution and the mixture is autoclaved at 121° C. for 30 minutes.

Fresh eggs, not more than 1 week old, are employed and cleaned by vigorous scrubbing in an approximately 5 percent soap solution. They are left in the soap solution for 30 minutes, and are then placed in running cold water, until the water becomes perfectly clear. They are then immersed in 70 percent alcohol for 15 minutes. They are broken into a sterile flask, homogenized completely by shaking, and filtered through 4 layers of sterile gauze.

One liter of homogenized whole eggs is added to one flask of the potato flour-salt solution, which has been cooled to room temperature, and to this is added 20 ml. of malachite green. The medium is thoroughly mixed.

The medium is tubed by means of a sterile aspirator bottle, or funnel, with bell attachment, or similar tubing device. Between 5 and 6 ml. is delivered into 150 mm. pyrex test tubes, and these tubes are solidified by inspissation at 85° C. for 50 minutes. The medium is then checked for sterility by incubation at 37° C. for 48 hours.

It is advisable to keep the medium stored in a refrigerator. This will keep for at least 1 month in the refrigerator, if the tubes are tightly closed to prevent evaporation.

2. Petragani Medium (Frobisher Modification):

MIXTURE A

Milk	225.0 ml.
Potato starch	9.0 gm.
Peptone	1.5 gm.
Diced potato	150.0 gm.

Heat in double boiler 1 hour, stirring constantly.

MIXTURE B

Eggs	8.0 (whole)
Glycerol (reagent grade)	18.0 ml.
2 percent aqueous malachite green	15.0 ml.

Mix well.

MIXTURE C

Dextrose	1.5 gm.
Asparagine	1.5 gm.
Water	50.0 ml.

Warm until dissolved.

Mix A, B, and C in a sterile flask and strain through one layer of gauze. Tube and sterilize in slanting position. Sterilize by inspissation for 1 hour at 80° C.

3. Trudeau Committee Medium (A.T.S.)

Egg yolk 500.0 ml.
Potato flour water (2 percent glycerine) 500.0 ml.

- a. The potato flour water is made by adding 20 gm. potato flour to 500 ml. of 2 volume percent glycerol water in a flask. Heat to boiling with constant stirring. Cool to 50° C.
- b. Egg yolk: Fresh hen eggs are carefully cleaned with wet gauze, rinsed in alcohol, and flamed. The egg white and yolks are separated. A proportion of one whole egg to eleven egg yolks is used and 500 ml. of this combination is prepared.
- c. The 500 ml. of egg yolks are poured into the 500 ml. of potato flour water and to this is added 20 ml. of 1 percent malachite green solution in 50 percent alcohol. All ingredients are thoroughly mixed and tubed.
- d. The medium is coagulated in a slanted position and sterilized by a single one-hour inspissation at 90° C.

4. Peizer's Medium

a. Agar Base:

Bacto beef extract	3.0	gm.
Casein hydrolysate (Difco Casamino Acids)	10.0	gm.
Potato starch	15.0	gm.
Bacto asparagin	3.0	gm.
Dipotassium phosphate	3.5	gm.
Ferric ammonium citrate	0.1	gm.
Citric acid	0.1	gm.
Magnesium sulphate	0.015	gm.
Bacto agar	15.0	gm.
Distilled water	1,000.0	ml.

Add all the ingredients except the starch and agar to 480 ml. of distilled water, cover, and boil slowly for 15 minutes over a small bunsen flame. Dissolve the potato starch in 50 ml. of cold distilled water and stir slowly into the boiling solution. Continue stirring and boiling the solution for approximately 3 minutes to cook the starch. Add distilled water to bring the volume up to 500 ml. and place the covered container of starch mixture in the Arnold sterilizer to steam for 1 hour. At the same time melt the agar-agar which has been added to 500 ml. of distilled water in the Arnold sterilizer

for 1 hour. Next, mix the starch solution and the melted agar. Dispense in convenient amounts and sterilize in the autoclave for 10 minutes at 15 lbs.

One liter of this agar base, melted and cooled to 55° C. may be enriched with either one of the two following enrichment mixtures:

b. Enrichment Mixture:

- 1) 10 egg yolks (separated aseptically)
25 ml. sterile saline
40 ml. sterile glycerol (reagent grade)
13 ml. sterile 1 percent malachite green
1 ml. sterile 20 percent dextrose

Shake the yolks well with the saline before adding the glycerol and the malachite green. The enrichment mixture must be well shaken before adding to the melted agar which has previously been cooled to 55° C.

or

- 2) 5 sterile egg yolks (separated aseptically)
30 ml. sterile sodium oleate
36 ml. sterile 1 percent malachite green

Shake well to mix before adding to the melted agar, previously cooled to 55° C.

The enriched agar is tubed with aseptic precautions in screw-cap tubes in about 8 ml. quantities. The tubes are then slanted, leaving a butt of at least 1 inch. The tubes of medium are incubated for 24 hours to test for sterility.

5. Dubos-Middlebrook Media (Tween-Albumin Broth and Oleic Acid-Albumin Agar):

a. Basal Medium:

Potassium acid phosphate (KH_2PO_4) 1.0 gm.
Dibasic sodium phosphate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) 6.3 gm.
(or 2.5 gm. of the anhydrous Na_2HPO_4)

Asparagine (for liquid media) 2.0 gm.
(for solid media) 1.0 gm.

Heat the above in 100 ml. of distilled water to dissolve

ADD:

Distilled water 850 ml.

Enzymatic digest of	
casein or Casitone (for liquid media)	2 gm.
(for solid media)	1 gm.
Ferric Ammonium citrate (for liquid media)	0.05 gm.
(for solid media)	0.005 gm.
Magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	0.01 gm.
(1 ml. of 1% stock solution	
in distilled water)	
Calcium chloride (CaCl_2)	0.0005 gm.
(1 ml. of 0.05% stock solution	
in distilled water)	
Zinc sulfate (ZnSO_4)	0.0001 gm.
(1 ml. of 0.01% stock solution	
in distilled water)	
Copper sulfate (CuSO_4)	0.0001 gm.
(1 ml. of 0.01% stock solution	
in distilled water)	

Adjust pH to 6.5-6.8 with HCl

b. For liquid Tween-albumin media

Basal Medium	900 ml.
Tween 80	0.5 ml.
(or 5 ml. of 10% stock solution	
in distilled water)	

Autoclave, cool to 56° C.

ADD:

Bovine albumin fraction V	5 gm.
(100 ml. of 5% stock in 0.85%	
saline neutralized with NaOH	
and sterilized by Seitz filtration)	
Glucose	5 gm.
(10 ml. of 50% stock in distilled	
water; sterilize in autoclave,	
121° C./15 min.)	

Dispense as desired.

c. For Oleic acid-albumin agar

Use only:

- 1 gm. of asparagine
- 1 gm. of enzymatic digest of casein
- 0.005 gm. of ferric ammonium citrate

Basal medium 900 ml.
Agar 11.0 gm.

Autoclave, cool to 56° C:

ADD: 100 ml. of the following oleic acid albumin complex.

- (1) Dissolve 0.12 ml. (0.1 gm.) of oleic acid in 10 ml. of N/20 NaOH by shaking with rotary motion in small flask.
- (2) Add 5 ml. of this to 95 ml. of **neutral** 5 percent solution of bovine albumin fraction V in 0.85 percent saline.
- (3) Sterilize by filtration through bacteriological filters, either glass or Seitz.

Dispense as desired.

6. Tarshis Blood Agar

Plain Agar 1.5 gm.
Glycerol (analytical grade) 1.0 ml.
Human bank blood (with ACD solution*) 30.0 ml.
Distilled water 69.0 ml.
Penicillin (50 to 100 units/ml.)
Final pH 6.8

Agar is dissolved in glycerol-water by heating.
Autoclave at 121° C./15 min. Cool to 45° C. and
add penicillin and blood.

*ACD solution has the following composition:

Citric acid. 0.5 gm.)
Sodium citrate 1.37 gm.) Per 100 ml. of trans-
Glucose 2.45 gm.) fusion solution.

7. Middlebrooks 7-H3 Medium

KH₂PO₄ 1.0 gm.
l - glutamate (Na salt) 0.5 gm.

*Glycerol (reagent grade)	2.0	ml.
Ferric ammonium citrate	0.1	gm.
CaCl ₂ .2H ₂ O	0.0005	gm.
CuSO ₄ .5H ₂ O	0.001	gm.
Ca pantothenate	1.0	mgm.
Na ₂ HPO ₄ .12H ₂ O	6.3	gm.
Ammonium sulfate	0.5	gm.
Na ₃ citrate.2H ₂ O	0.1	gm.
MgSO ₄ .7H ₂ O	0.05	gm.
ZnSO ₄ .7H ₂ O	0.001	gm.
Pyridoxine.HCl	1.0	mgm.
Biotin	0.5	mgm.
Vitamin free casein hydrolysate	0.1	gm.

*Omit glycerol if Tween 80 is used in liquid

Dissolve in 100 ml. of distilled water. Adjust to pH 6.6.

a. For liquid medium:

Before autoclaving, add Tween 80 in final concentration of 0.05 percent and after autoclaving (121° C./15 min.) cool to 56° C. and add bovine albumin fraction V in final concentration of 0.5 percent.

b. For solid medium:

Before autoclaving, add agar in final concentration of 1.5 percent and 1 mcg./ml. of malachite green (Coleman and Bell). After autoclaving add oleic acid-albumin complex in final concentration of 0.5 percent.

Oleic acid albumin made as follows: Dissolve 0.12 ml. of oleic acid in 10 ml. of N/20 NaOH by shaking with rotary motion in small flask. Add 5 ml. of this to 95 ml. of neutral 5 percent solution of bovine albumin in 0.85 percent saline. Sterilize by filtration.

After autoclaving, add to **ALL** media 0.2 percent final concentration of glucose from a sterile concentrated stock solution.

8. Middlebrook's 7-H9 Oleic Acid-Albumin Agar

KH ₂ PO ₄	1.0	gm.
Na ₂ HPO ₄ .12H ₂ O	6.3	gm.

l- glutamate (Na salt)	0.5	gm.
(20 ml. of 2.5 percent solution)		
Ammonium sulfate	0.5	gm.
(20 ml. of 2.5 percent solution)		
Glycerol (reagent grade)	2.0	ml.
(omit if Tween is used in inocula)		
Na ₃ citrate.2H ₂ O	0.1	gm.
(1 ml. of 10 percent solution)		
Fe ammonium citrate (green)	0.04	gm.
(0.4 ml. of 10 percent solution)		
MgSO ₄ .7H ₂ O	0.05	gm.
(5 ml. of 1 percent solution)		
CaCl ₂	0.0005	gm.
(0.5 ml. of 0.1 percent solution)		
ZnSO ₄ .7H ₂ O	0.001	gm.
(1 ml. of 0.1 percent solution)		
CuSO ₄ .5H ₂ O	0.001	gm.
(1 ml. of 0.1 percent solution)		
Pyridoxine HCl	1.0	mgm.
(1 ml. of 0.1 percent solution)		
Biotin	0.5	mg.
(0.25 ml. of a 2 mg./ml. solution)		

Dissolve above, in order given, in 900 ml. distilled water.

Adjust pH to 6.6 with 10 percent HCl (about 5 ml./liter of base medium)

For inhibition of contaminants add 1 mcg./ml. of malachite green oxalate.

Add purified agar (Baltimore Biological or Difco) to 1.5 percent.

Autoclave

Cool to 45°-50° C. and add:

Oleic acid-albumin complex to 0.5 percent
 Glucose to 0.2 percent
 Catalase to 3 mcg./ml.

9. Middlebrook's 7H-10 Medium.

Base solids:

MgSO₄.7H₂O 0.5 gm.

Fe ammonium citrate	0.4 gm.
Na ₃ citrate.2H ₂ O	4.0 gm.
Ammonium sulfate	5.0 gm.
L-glutamic acid (Na salt)	5.0 gm.
Na ₂ HPO ₄ (anhydrous)	15.0 gm.
KH ₂ PO ₄ (anhydrous)	15.0 gm.
	<hr/>
	45 gm.

Store above at room temperature after mixing and indicate on label that 0.45 gm. will make up to 100 ml. with distilled water at pH 6.6, if 1.5 gm. Baltimore Biological agar is used/100 ml.

Heat-labile solution (filter-sterilized) -1 tube (13.5 ml.) to be added to each 100 ml. of above agar base **after** autoclaving agar base:

Oleic acid-albumin complex (5 percent) . . .	10.0 ml.
Glycerol	0.5 ml.
50 percent glucose	0.4 ml.
Pyridoxine-HCl (100 mcg./ml.)	1.0 ml.
Biotin (50 mcg./ml.)	1.0 ml.
Malachite green (100 mcg./ml.)	1.0 ml.
Catalase, technical (1000 mcg./ml.)	0.3 ml.

Bibliography

General Reference Books

- American College of Chest Physicians. *Fundamentals of Pulmonary Tuberculosis*. Springfield, Ill., Charles C. Thomas Co., 1949
- Calmette, A.: *Tubercle Bacillus Infection and Tuberculosis in Man and Animals*. Baltimore, Williams and Wilkins Co., 1923.
- Canetti, G.: *The Tubercle Bacillus in the Pulmonary Lesions of Man*. New York, Springer Publishing Company, Inc., 1955.
- Ciba Foundation Symposium on Experimental Tuberculosis, *Bacillus and Host*. Boston, Little, Brown and Co., 1955.
- Darzens, E.: *The Bacteriology of Tuberculosis*. Minneapolis, University of Minnesota Press, 1958.
- Drea, W. F., and Andrejew: *The Metabolism of the Tubercle Bacillus*. Springfield, Ill., Charles C. Thomas Co., 1953.
- Finch, W. E.: *Disinfectants, Their Values and Uses*. New York, The MacMillan Co., 1958.
- Francis, J.: *Tuberculosis in Animals and Man*. London, Cassell and Company, Lmtd., 1958.
- Long, E. R.: *The Chemistry and Chemotherapy of Tuberculosis*. Baltimore, The Williams and Wilkins Co., 1958.
- Pinner, M.: *Pulmonary Tuberculosis in the Adult*. Springfield, Ill., Charles C. Thomas, Co., 1945.
- Pottenger, F. M.: *Tuberculosis*. St. Louis, C. V. Mosby Co., 1948.
- Rich, A. R.: *The Pathogenesis of Tuberculosis*. Springfield, Ill., Charles C. Thomas Co., 1946.
- Soltys, M. S.; Hill, C. S. St.; and Ansell, I.: *Tubercle Bacillus and Laboratory Methods in Tuberculosis*. Edinburgh & London, E. & S. Livingston Ltd., 1952. (Distributed in U. S. A. By Williams & Wilkins Company, Baltimore, Md.)
- Wells and Long: *The Chemistry of Tuberculosis*. Baltimore, Williams and Wilkins Co., 1932.
- Willis, H. S., and Cummings, M. M.: *Diagnostic and Experimental Methods in Tuberculosis*. Springfield, Ill., Charles C. Thomas Co., 1952.

Digestion, Decontamination and Concentration

- Balmer, H. F., and Arneson, G. A.: A combined concentration method for tubercle bacilli. *Amer. J. Clin. Path.* 17, 71, 1947.
- Beattie, M.: Cultivation of *Mycobacterium tuberculosis*. A comparison of hydrochloric acid and trisodium phosphate in the preparation of sputum and the use of fluid Dubos-Davis medium and Petraghani medium. *J. Lab. & Clin. Med.* 34(6): 733, 1949.
- Cameron, G. M., and Castles, R.: Chlorox digestion. *Am. Rev. Tuberc.* 52:530, 1935.
- Collins, C. H.: Oxalic acid and acid-iron-peroxide in the routine culture of tubercle bacilli from sputum. *Tubercle*, 33(5):149, 1952.
- Cooke, and Giordane, A. S.: Relative value of concentration methods of sputum and gastric contents for the detection of acid fast bacilli. *Am. J. Clin. Path. Tech. Sect.* pp. 15-94, 1945.
- Corper, H. J., and Nelson, C. R.: Methods of concentrating acid-fast bacilli. *Am. J. Clin. Path.* 19:269, 1949.
- Corper, H. J., and Stoner, R. E.: An improved procedure for the diagnostic culture of mammalian tubercle bacilli. *J. Lab. & Clin. Med.* 31:1364, 1946.
- Corper, H. J., and Uyei, N.: The isolation of tubercle bacilli from contaminated tuberculous materials. *Am. Rev. Tuberc.* 16:299, 1927.
- Corper, H. J., and Uyei, N.: Oxalic acid as a reagent for isolating tubercle bacilli and a study of the growth of acid-fast non-pathogens on different

- media with their reactions to chemical reagents. *J. Lab. & Clin. Med.* 15:348, 1930.
- Felsen, J.: A method for detecting sparsely distributed tubercle bacilli. *Arch. Path.* 10:110, 1930.
- Freiman, D. G., and Pinner, M.: Routine examination of sputum for acid-fast bacilli. *Am. Rev. Tuberc.* 59:449, 1949.
- Gifford, D.; McKenley, F.; and Hunter, C. A.: A comparative study of methods of isolating *Mycobacterium tuberculosis*. *Am. J. Pub. Health* 41:164, 1951.
- Hambleton, A.: Potential danger to laboratory workers of the trisodium phosphate method in the diagnostic culture of tubercle bacilli. *Am. J. Med. Technol.* 23:181, 1957.
- Hanks, J. H.; Clark, H. F.; and Feldman, H.: Concentration of tubercle bacilli from sputum by chemical flocculation methods. *J. Lab. & Clin. Med.* 23:736, 1938.
- Hanks, J. H., and Feldman, H. A.: Study of demonstration of tuberculosis in urine, by means of phosphate flocculation. *J. Lab. & Clin. Med.* 25:974-980, 1940.
- Hawirko, R. Z., and Murray, E. G. D.: Oil partition for the collection of small numbers of tubercle bacilli from aqueous suspensions. *Canad. J. Pub. Health* 45:208, 1954.
- Holloway, J. B., and Cummings, M. M.: An evaluation of a method of obtaining gastric washings. *Am. Rev. Tuberc.* 60:228, 1949.
- Kelso, R. E., and Galbraith, T. W.: A rapid simple technic for detection of acid-fast bacilli in urine. *Am. J. Clin. Path. Tech. Suppl.* 7:8-11, 1943.
- Keningale, J. A.: The problem of contamination in routine cultural methods for *M. tuberculosis*. *The Bulletin of the Institute of Medical Laboratory Technology (London)* 14(10):159, 1949.
- Kibler, P., and Lundholm, R.: Culture of tubercle bacilli from specimens of gastric juice. *Am. J. Clin. Path.*, 19:685, 1949.
- Klein, G. C., et al.: Efficacy of centrifugation as a method of concentrating tubercle bacilli. *Am. J. Clin. Path.* 22:581-585, 1952.
- Lockard, C. D.: Recovery of *Mycobacterium tuberculosis* from sputum or gastric washings. *Am. J. Clin. Path.* 17:91, 1947.
- Mitchell, R. B.; Jefferies, M.; and Stucker, C. L.: The cultural diagnosis of tuberculosis in a public health laboratory. *Pub. Health Lab.* 6:32, 1948.
- Mullahy, M. E.: Detection of acid-fast bacilli. Use of sodium hydroxide and trisodium phosphate as digestants in preparing specimens. *Am. J. Clin. Path.* 20(7):672, 1950.
- Palen, M. I.; Wong, H. Y.; and Leifson, E.: Detection of *Mycobacterium tuberculosis* in sputum by pepsin digestion and interface concentration with pentane. *Am. Rev. Tuberc.* 75:148, 1957.
- Patterson, R. A.; Thompson, T. L.; and Larsen, D. H.: The use of zephiran in the isolation of *M. tuberculosis*. *Am. Rev. Tuberc.* 74:284, 1956.
- Patterson, R. A.: A new method for the isolation of *M. tuberculosis*. *Am. J. Pub. Health* 46:1429, 1956.
- Peizer, L. R.; Chaves, A. D.; and Widelock, D.: A trisodium phosphate transport-digestion method of processing sputum and gastric specimens for the detection of *Mycobacterium tuberculosis*. *Am. Rev. Tuberc.* 70:366, 1954.
- Rappaport, F., and Rosenknopf, D.: Improved concentration methods for bacteria, including tubercle bacilli. *Am. J. Clin. Path.* 18:579, 1948.
- Roberts, E. G.; Wallace, J. L.; and Erlick, H.: Methods for isolating tubercle bacilli. *Am. Rev. Tuberc.* 61(4):563, 1950.
- Robinson, L., and Stovall, W. D.: Factors influencing the demonstration of tubercle bacilli by concentration methods. *J. Lab. & Clin. Med.* 27:84, 1941.
- Saxholm, R.: Experiments with a new culture method for tubercle bacilli. *Am. Rev. Tuberc.* 69(2):305, 1954.
- Saxholm, R.: Further experiments with combinations of pancreatin and quaternary ammonium compounds for cultivation of *Mycobacterium tuberculosis*. *Am. Rev. Tuberc.* 72:98, 1955.

- Saxholm, R.: Pretreatment of sputum with enzymes and quaternary ammonium compounds for slide cultivation of tubercle bacilli. *Acta path. et microbiol. scandinav.* 37:132, 1955.
- Saxholm, R.: Cultivation of *M. tuberculosis* from urine and gastric lavage by the pancreatin-quaternary ammonium compound method. *Am. Rev. Tuberc.* 74:616, 1956.
- Schain, P.; Magdalin, S.; and Russo, A.: A comparative study of several concentration methods. *Quar. Bull. Sea. View Hosp.* 7, 99, 1942.
- Schain, P. S.; Magdalin, S.; and Russo, A.: Sputum examination. A comparative study of the chlorox and Tergitol-Javelle water concentration methods. *Am. Rev. Tuberc.* 57(6):640, 1948.
- Schwartz, V.: The action of gastric contents on tubercle bacilli. *Am. Rev. Tuberc.* 58:123, 1948.
- Sirsi, M.: A study on the methods of isolation and culture of *Mycobacterium tuberculosis*. *Current Sci.* 19(10):316, 1950.
- Spendlove, G. A.; Cummings, M. M.; and Patnode, R. A.: Toxicity of sputum digestants to tubercle bacilli in water and sputum. *Am. Rev. Tuberc.* 60:628, 1949.
- Steenken, W., and Smith, M. M.: Culture of tubercle bacilli. *Am. Rev. Tuberc.* 43:2, 1941.
- Steenken, W., and Smith, M. M.: A shaking machine and containers for shaking sputums and other body fluids during the process of homogenization. *J. Lab. & Clin. Med.* 27:1582, 1942.
- Steenken, W.: Demonstration of tubercle bacilli. *Am. Rev. Tuberc.* 42:543, 1940.
- Tarshis, M. S., and Lewis, W. G.: Use of chlorox and trisodium phosphate in the demonstration of acid-fast bacilli in sputum. *Am. J. Clin. Path.* 19:693, 1949.
- Tonge, J. I., and Hughes, P. G.: A comparative study of laryngeal swabs and gastric lavage in the detection of tubercle bacilli. *Am. Rev. Tuberc.* 73:930, 1956.
- Uhlenhuth and Xylander: Antiformin, ein bakterienauflösendes Desinfektionsmittel. *Berl. Klin. Wchnschr.* 45:134, 1908.
- Van Vranken, M.: Diagnostic culture of tubercle bacilli. *Am. Rev. Tuberc.* 55:374, 1947.
- Webb, C. R., *et al.*: The efficiency of centrifugation as a means of concentrating tubercle bacilli from sputum. *Am. Rev. Tuberc.* 76:899-901, 1957.
- Yegian, D., and Budd, V.: Toxic effect of sodium hydroxide on tubercle bacilli. *Am. J. Clin. Path.* 22(5):456, 1952.
- Roth, W., and Birkhauser, H.: Die Pufferung des Magensaftes bei der Untersuchung auf Tuberkelbazillen. *Schweizerische Med. Wochenschrift* 83:240, 1953.

Microscopy and Staining

- Alexander-Jackson, E.: A differential triple stain for demonstrating and studying non-acid-fast forms of the tubercle bacillus in sputum, tissue, and body fluids. *Science* 99:307, 1944.
- American Trudeau Committee: Fluorescent microscopy. *Am. Rev. Tuberc.* 50:465, 1944.
- Berg, J. W.: Chemistry of acid-fastness. *Proc. Soc. Exper. Biol. & Med.* 84:196, 1953.
- Berg, J. W.: The dual nature of acid-fastness. *Yale J. Biol. & Med.* 26:215, 1953.
- Briggs, G. O. A., and Jennison, M. H.: Fluorescence microscopy and the modified Ziehl-Neelsen technique. *Tubercle*, 29:252, 1948.
- Cooper, F. B.: A modification of the Ziehl-Neelsen staining method for tubercle bacilli. *Arch. Path. & Lab. Med.* 2:382, 1926.
- Darrow, M. A.: Staining the tubercle organism in sputum smears. *Stain Tech.* 24:93, 1949.
- Ebina, T., *et al.*: Morphological changes of tubercle bacilli caused by staining procedures. *Nature* 180:42-43, 1957.

- Grasham, C.: Fluorescent microscopy. *Am. J. Clin. Path.* 9:39, 1945.
- Gross, M.: Rapid staining of acid-fast bacteria. *Am. J. Clin. Path.* 22:1034, 1952.
- Hallberg, V.: New method of staining tubercle bacilli (Nachtblau). *Acta med. scandinav.* P. 1, Supplement 46, 1939.
- Hanks, J. H.: Retention and differentiation of carbol-fuchsin-stained mycobacteria in diagnostic films. *Am. Rev. Tuberc.* 74:597, 1956.
- Hanks, J. H.: Retention and differentiation of mycobacteria in tissue sections. *Am. Rev. Tuberc.* 74:608, 1956.
- Koch, R.: Die Aetiologie der Tuberculose. *Berlin Klin. Wchenschr.* 19:221, 1882.
- Kuroda, O.: A new staining method for tubercle bacilli. *J. Japan M. A.* 28:232, 1952. (*Am. Rev. Tuberc.* 67(5):113, 1954).
- Lack, C. H., and Tanner, F.: The significance of pleomorphism in *Mycobacterium tuberculosis* var. *hominis*. *J. Gen. Microbiol.* 8:18, 1953.
- Lack, C. H.: The malachite green-safranin stain. *Tubercle* 39:125-126, 1958.
- Lamanna, C.: The nature of acid-fast stain. *J. Bact.* 52:99, 1946.
- Lee, H. I.: Comparison of procedures for staining tubercle bacilli in fluorescence microscopy. *J. Lab. & Clin. Med.* 29:218, 1944.
- Lind, H. E.: Limitations of fluorescent microscopy for the detection of acid-fast bacilli. *Am. J. Clin. Path.* 19:72, 1949.
- Massen, W., and Knothe, H.: The staining of tubercle bacilli in sputum with soda fuchsin. *Deutsche med. Wchenschr.* 74:153, 1949.
- Matsuoka, T., and Nakajima, M.: The new method of staining tubercle bacillus and its biological and clinical significances. *Ann. of Tuberc.* 6:33-39, 1955.
- Matthaei, E.: Simplified fluorescence microscopy of tubercle bacilli. *J. Gen. Microbiol.* 4:393, 1950.
- Moran, T. J.; Radcliffe, M. L.; and Tevault, I. H.: Rapid method of staining tubercle bacilli with Tergitol #7. *Am. J. Clin. Path.* 17:75, 1947.
- Muller, H. E., and Chernock, R. L.: A rapid staining technique for acid-fast organisms. *J. Lab. & Clin. Med.* 30:169, 1945.
- Murohashi, T., and Yoshida, K.: Improvement of malachite green-fuchsin staining. *Acta. tuberc. scandinav.* 36:195-98, 1958.
- Pottenger, J. E.: Controlled staining of *Mycobacterium tuberculosis*. *Am. Rev. Tuberc.* 45:497, 1942.
- Pottenger, J. E.: Present practice in staining *Mycobacterium tuberculosis* in representative institutions. *Am. Rev. Tuberc.* 45:568, 1942.
- Pottenger, J. E.: Counterstains in demonstrating *Mycobacterium tuberculosis* in sputum. *Am. Rev. Tuberc.* 45:558, 1942.
- Richards, O. W., and Kline, E. K.: Demonstration of the tubercle bacilli by fluorescent microscopy. *Am. Rev. Tuberc.* 44:255, 1941.
- Schulte, T. H.: Zur Tuberkelbazillen Färbung. *Deutsche med. Wchenschr.* XVI: 1225, 1920.
- Thompson, L.: Comparison of carbol fuchsin with the fluorescent dye auramine for the demonstration of acid-fast bacteria. *Proc. Staff Meeting of Mayo Clin.* 16:673, 1941.
- Tomasello, S. A., and Altabach, H.: Staining tubercle bacilli in tissue and smear, employing aerosol O. T. *Am. J. Clin. Path.* 17:73, 1947.
- Wollenman, O. J.; Montgomery, P. O. B.; and Foard, Z. B.: Acid-fast non-tubercle bacilli in paraffin. *Am. J. Clin. Path.* 21:295.
- Yegian, D., and Baisden, L.: Factors affecting and beading of the tubercle bacillus by the Ziehl-Neelsen technique. *J. Bact.* 44:667, 1942.
- Yegian, D., and Kurung, J.: Morphology of the *Mycobacterium tuberculosis*: A study of the artefacts produced in smears. *Am. Rev. Tuberc.* 56:36, 1947.
- Yegian, D., and Vanderlinde, R.: The nature of acid-fastness. *J. Bact.* 54: 777, 1947.
- Yegian, D., and Porter, K. R.: Some artefacts encountered in stained preparations of tubercle bacilli. I. Non-acid-fast forms arising from mechanical treatment. *J. Bact.* 48:83, 1944.
- Yegian, D., and Budd, V.: Ziehl-Neelsen Technique - staining properties modified by different preparations of basic fuchsin. *Am. Rev. Tuberc.* 48:54, 1943.

Ziehl, F.: Zur Färbung des Tuberkelbazillus. Deutsch. med. wchnschr. 33: 451, 1882.

Culture Media

- American Trudeau Society: Report of the Committee on Evaluation of Laboratory Procedures. Am. Rev. Tuberc. LIV:4, 1946.
- Berry, J. W., and Lowry, H.: A slide culture method for the early detection and observation of growth of the tubercle bacilli. Am. Rev. Tuberc. 60:51, 1949.
- Boissevain, C. H.: Growth promotion of the tubercle bacillus by serum albumen. Proc. Soc. Exper. Biol. & Med. 44:110, 1940.
- Clark, M. E.: Human plasma and blood agar for cultivation of tubercle bacilli. Am. J. Clin. Path. 23:671, 1953.
- Colwell, C. A., and Hess, A. R.: The effect of inspissation and storage on isoniazid concentrations in culture media. Am. Rev. Tuberc. 75:678, 1957.
- Corper, H. J.: The cultivation of recently isolated and laboratory strains of human tubercle bacilli on artificial media. Am. Rev. Tuberc. 3:461, 1919.
- Corper, H. J., and Cohn, M. L.: Combination egg media for the diagnostic culture of tubercle bacilli. Am. Rev. Tuberc. LIII:575-582, 1946.
- Corper, H. J., and Cohn, M. L.: Media for tubercle bacilli: An evaluation of different media for diagnostic cultures of tubercle bacilli. Am. Rev. Tuberc. 46:560, 1942.
- Corper, H. J., and Cohn, M. L.: Nutrient quality of eggs for growing tubercle bacilli. Am. J. Hyg. 18:1-25, 1933.
- Davies, R.: The influence of haemoglobin and ferrous ammonium sulphate on the growth of the tubercle bacilli. J. Path. & Bact. 45:773, 1937.
- Dubos, R. J., and Middlebrook, G.: Media for tubercle bacillus. Am. Rev. Tuberc. LVI:334, 1947.
- Dunphy, D. L., and Fousek, M. D.: A simple medium for growth of tubercle bacilli. Yale J. Biol. & Med. 19:1013, 1947.
- Evans, B., and Hanks, J.: Growth of small numbers of acid-fast bacteria in blood and serum. Proc. Soc. Exper. Biol. & Med. 40:112, 1939.
- Fallin, J. R., et al.: Toxic effect of penicillin on the culture of tubercle bacilli. Am. J. Clin. Path. 22:703, 1952.
- Frobisher, M., Jr.: Fundamentals of bacteriology. W. B. Saunders Company, 1948. p. 658. (Petragnani's Medium as modified by Frobisher.)
- Gale, D.: The inspissation of egg media for the cultivation of tubercle bacilli. Am. Rev. Tuberc. 73:139, 1956.
- Herrold, R. D.: Egg yolk agar medium for growth of tubercle bacilli. J. Infec. Dis. 48:236, 1931.
- Hirsch, J. G.: Charcoal media for the cultivation of tubercle bacilli. Am. Rev. Tuberc. 70:955, 1954.
- Hirsch, J. G.: Studies on egg yolk growth factors for tubercle bacilli. Am. Rev. Tuberc. 70:977, 1954.
- Hirsch, J. G.: The use of charcoal in diluents for tubercle bacilli. Am. Rev. Tuberc. 70:989, 1954.
- Hirsch, J. G.: The use of Triton WR 1339 and of malachite green in charcoal media for tubercle bacilli. Am. Rev. Tuberc. 71:894, 1955.
- Holm, J., and Lester, V.: Diagnostic demonstration of tubercle bacilli. Acta tuberc. scandinav. XVI:2, 1941.
- Hudgins, A. K., and Patnode, R. A.: Effect of age of eggs on sensitivity of modified Lowenstein medium. Am. J. Clin. Path. 22:809, 1952.
- Kelly, R. G., and Murphy, E. A.: Lowenstein's medium. Am. Rev. Tuberc. 49: 110, 1944.
- Kenny, M.; Johnson, P. M.; and Goldman, M.: A comparative study of three egg yolk media in the isolation of *M. tuberculosis*. Am. Rev. Tuberc. 72:863, 1955.
- Kirschner, O.: Die Leistungsfähigkeit der Tiefenkultur des Tuberkelbazillus bei Verwendung besonders geeigneter flüssiger Nährböden. Zent. F. Bakt. (Abst. 1) 124:403, 1932.

- Lowenstein, E.: Die Methodik der Reinkultur von Tuberkelbazillen aus dem Blute. Deutsche med. Wchenschr. LVI:1010, Juni 13, 1930.
- Lowenstein, E.: Die Zuchtung der Tuberkelbazillen aus dem Stromenden Blute. Zent. f. Bakt. (Abst. 1) 120:127, 1931.
- MacNabb, A. L.: Cultural methods of isolation of tubercle bacilli. Am. J. Pub. Health, 26:619, 1936.
- McCarter, J. R., and Kanne, E. M.: Egg mediums for the isolation of all three types of tubercle bacilli. J. Infec. Dis. 71:102, 1942.
- Minami, K.: Bactericidal action of oleic acid for tubercle bacilli. I and II. J. Bact. 73:338-352, 1957.
- Peizer, L., and Schecter, C.: A new medium for bacteriologic diagnosis of tuberculosis. Am. J. Clin. Path. 20:682, 1950.
- Reinisch, E.: Blood medium for routine cultivation of tubercle bacilli. Am. J. Clin. Path. 23:929, 1953.
- Sasano, K. T., and Medlar, E. M.: Egg-yolk-potato medium: Its efficacy for demonstrating small numbers of tubercle bacilli. Am. Rev. Tuberc. 48:297, 1943.
- Schwabacher, Herta: A comparison of different media for the growth of the tubercle bacillus. Tubercle 18:199, 1937.
- Smith, J. W.; Humiston, J.; Criger, W. P.; and Kirby, W. M.: Evaluation of Dubos' solid medium containing penicillin in isolation of tubercle bacilli. Proc. Soc. Exp. Biol. & Med. 70:589, 1949.
- Tarshis, M. S., and Frisch, W.: Blood media for the cultivation of *Mycobacterium tuberculosis*. Am. J. Clin. Path. 21:101, 1950.
- Tarshis, M. S.; Weed, W. A.; Kinsella, P. C.; Parker, M. V.; and Dunham, W. B.: Further experience with a new blood medium for the cultivation of *Mycobacterium tuberculosis*. Am. J. Pub. Health 45:1157, 1955.
- Townsend, S. M.: Elimination of pre-cleaning cage-laid hens' eggs in preparation of egg fluid for tuberculosis bacteriologic media. Am. Rev. Tuberc. 79:677, 1959.
- Wong, S., and Weinzirl, J.: An inexpensive synthetic medium for growing *Mycobacterium tuberculosis*. Am. Rev. Tuberc. 33:577, 1936.
- Woolley, J. S., and Petrick, F. G.: A potato-egg medium for the isolation of tubercle bacilli. Am. Rev. Tuberc. 24:596, 1931.
- Yamane, I.: A crystalline substance isolated from egg yolk which promotes growth of a minute inoculum of human tubercle bacilli. Nature 179:45-6, 1957.
- Zacharias, S. J.: Isolation of *Mycobacterium tuberculosis* using blood and plasma media. Am. J. Med. Tech. July-Aug. 1958, pp. 208-210.

Animals

- Boden, S. V., and Andersen, M. E.: Diet and experimental tuberculosis in the guinea pig. The importance of the source of ascorbic acid. Acta path. et microbiol. scandinav. 39:107, 1956.
- Calmette, A.: Tubercle bacillus infection and tuberculosis in man and animals. Chapters XX-XXIX, Translations by Soper, W. B., and Smith, G. H.: Williams and Wilkins Co., Baltimore, 1923.
- Eggerth, A. H.; Drescher, E.; and McOsker, V. C.: Infection of chick and duck embryos with tubercle bacilli. Am. Rev. Tuberc. 57:632, 1948.
- Feldman, W. H., and Magath, T. B.: The reliability of guinea pig inoculation in the diagnosis of tuberculosis. Am. Rev. Tuberc. 24:312, 1931.
- Feldman, W. H.: A scheme for numerical recording of tuberculous changes in experimentally infected guinea pigs. Am. Rev. Tuberc. 48:248, 1943.
- Griffith, A. S.: Further experiments on the golden hamster (*Cricetus auratus*) with tubercle bacilli and the vole strain of acid-fast bacillus (Wells). J. Hyg. 41:260, 1941.
- Griffith, A. S.: The relative susceptibility of the field vole to the bovine, human and avian types of tubercle bacilli and to the vole strain of acid-fast bacillus (Wells, 1937). J. Hyg. 39:244, 1939.
- Hoyt, A., et al.: Sex differences of normal and immunized mice in resistance to experimental tuberculosis. Am. Rev. Tuberc. 75:618, 1957.

- Korns, J. H., and Lu, G. Y. C.: The diagnosis of tuberculosis by means of the hamster. *Am. Rev. Tuberc.* 17:279, 1928.
- Korns, J. H., and Lu, G. Y. C.: The use of the hamster in the diagnosis of certain forms of tuberculosis. *China M. J.* 41:234, 1927.
- Lack, C. H.: The pathogenesis of tuberculosis as shown in studies of omental spreads. *Am. Rev. Tuberc.* 73:362, 1956.
- Lange, B., and Lydtin, K.: Intracutaneous virulence testing in the guinea pig. *Zent. f. Bakt. CVIII*:22, 1928.
- Lester, V.: Saprophytic acid-fast bacilli as a source of error in diagnostic work. *Acta tuberc. scandinav.* XIII:251, 1939.
- McNelly, E., and Riddell, W. A.: Use of egg embryos in the culture of *M. tuberculosis*. *Am. J. Pub. Health* 39:1463, 1949.
- Moore, M.: The chorioallantoic membrane of chick embryos and its response to inoculation with some mycobacteria. *Am. J. Path.* 18:827, 1942.
- Nugar, J.: The golden hamster as a test animal for tubercular infection. *Nature*, 150:432, 1942.
- Patnode, R. A.; Cummings, M.M.; and Spendlove, G. A.: The adaptability of mice to the laboratory diagnosis of tuberculosis. *J. Lab. & Clin. Med.* 34: 1081, 1949.
- Pierce, C.; Dubos, R.; and Schaeffer: Multiplication and survival of tubercle bacilli in the organs of mice. *J. Exper. Med.* 97:189, 1953.
- Viallier, J., and Lager, S.: Sur les caracteres distinctifs de mycobacterium tuberculosis var. bovis. *Comp. Rend. Séances Soc. Biol.* 149:118, 1955.
- Youmans, G. P., and Youmans, A. S.: Response of mice to standard infecting dose of *Mycobacterium tuberculosis* var. hominis. *Proc. Soc. Exper. Biol. & Med.* 90:238, 1955.

Therapeutic Agents

- Abraham, E. P.: The nature of drugfastness. *Tubercle* 31:146, 1950.
- Albert, A.: Mode of action of isoniazid. *Nature* 177:525, 1956.
- Barclay, W. R., et al.: Mode of action of isoniazid. *Am. Rev. Tuberc.* 67:490, 1953.
- Barclay, W. R., and Russe, H.: The *in vitro* action of cycloserine on *M. tuberculosis*. *Am. Rev. Tuberc.* 72:236, 1955.
- Barry, V. C. et al.: Hincinstarch in the treatment of pulmonary tuberculosis. *Am. Rev. Tuberc.* 73:219, 1956.
- Barry, V. C., and Edward, D. W.: The metabolic products of hincinstarch. *Am. Rev. Tuberc.* 74:798, 1956.
- Brennan, A. J., and Wichelhausen, R. M.: Streptomycin resistant tubercle bacilli. Isolation of resistant organisms from pleural fluid prior to institution of streptomycin therapy. *J.A.M.A.* 140:1275, 1949.
- Colwell, C. A.; Pitner, G.; and Moravec, M.: The significance of results of *in vitro* drug-sensitivity tests with *Mycobacterium tuberculosis*. *Am. Rev. Tuberc.* 63:679, 1951.
- Committee on Chemotherapy and Antibiotics. The use of PAS in tuberculosis. *Dis. Chest.* 16:904-906, 1949.
- Cummings, M. M., et al.: Effects of cycloserine on *Mycobacterium tuberculosis in vitro*. *Antibiotics & Chemother.* 4:198, 1955.
- Delaude, A.; Karlson, A. G.; Carr, D. T.; Feldman, W. H.; and Pfuetze, K. H.: Increase in resistance of tubercle bacilli to sodium P-aminosalicylic acid: Observations on cultures isolated from patients during chemotherapy. *Proc. Staff Meetings Mayo Clinic* 24:341-346, 1949.
- Desbordes, J., and Paraf, M.: A new, entirely synthetic antibiotic which is active against the tubercle bacillus (action of para-amino-salicylic acid in tuberculosis). *La Semaine des Hopitaux*, 25:296-300, 1949.
- Doane, E. A., and Bogen, E.: Streptomycin dependent tubercle bacilli. *Am. Rev. Tuberc.* 64:192, 1951.
- Editorial: Rapid appraisal of sensitivity of tubercle bacilli to streptomycin. *Lancet*, I:786-787, May 7, 1949.

- Epstein, I. G., *et al.*: The treatment of human pulmonary tuberculosis with cycloserine: progress report. *Dis. Chest* 29:241, 1956.
- Ferebee, S. H., and Palmer, C. E.: Prevention of experimental tuberculosis with isoniazid. *Am. Rev. Tuberc.* 73:1, 1956.
- Fisher, M. W.: Sensitivity of tubercle bacilli to streptomycin: An *in vitro* study of some factors affecting results in various test media. *Am. Rev. Tuberc.* 57:58-62, 1948.
- Fisher, M. W.: Streptomycin resistant tubercle bacilli: Their development during streptomycin therapy of pulmonary tuberculosis. *Am. Rev. Tuberc.* 57:53-57, 1948.
- Fisher, M. W.: The antagonism of the tuberculostatic action of isoniazid by hemin. *Am. Rev. Tuberc.* 69:469, 1954.
- Grunberg, E., and Blencowe, W.: The influence of pyridoxine on the *in vitro* antituberculous activity of isoniazid. *Am. Rev. Tuberc.* 71:898, 1955.
- Harris, H. W., *et al.*: Comparison of isoniazid concentrations in the blood of people of Japanese and European descent. *Am. Rev. Tuberc.* 78:944, 1958.
- Heller, A., *et al.*: Studies with C¹⁴ labeled para-aminosalicylic acid and isoniazid. *Am. Rev. Tuberc.* 75:71, 1957.
- Hines, R. A.: Peripheral neuritis following isoniazid therapy. *J.A.M.A.* 159:1197, 1955.
- Hobby, G. L., and Dougherty, N.: Isolation of a streptomycin resistant organism capable of utilizing streptomycin for growth. *Proc. Soc. Exper. Biol. & Med.* 69:544-548, 1948.
- Hobby, G., *et al.*: Late emergence of *M. tuberculosis* in liquid cultures of pulmonary lesions resected from humans. *Am. Rev. Tuberc.* 70:191, 1954.
- Howard, W. L.; Maresh, F.; Mueller, E. E.; Yannitellim, S. A.; and Woodruff, C. E.: The role of pulmonary cavitation in the development of bacterial resistance to streptomycin. *Am. Rev. Tuberc.* 59:391, 1949.
- Karlson, A. G., and Needham, G. M.: Determination of streptomycin sensitivity of tubercle bacilli by use of egg-yolk agar medium. *Proc. of Staff Meetings of Mayo Clinic* 23:401, 1948.
- Karlson, A. G.; Delaude, A.; Carrl, D. T.; Pfuetze, K. H.; and Feldman, W. H.: The occurrence of tubercle bacilli resistant to para-aminosalicylic acid (PAS). *Dis. Chest* 16:667, 1949.
- Kendig, I. V., *et al.*: Psychological side effects induced by cycloserine in the treatment of pulmonary tuberculosis. *Am. Rev. Tuberc.* 73:438, 1956.
- Leifheit, H. C., and Smith, E. R. B.: Quantitative estimation of isonicotinic acid hydrazide and p-aminosalicylic acid in blood serum. *Am. J. Clin. Path.* 31:142, 1959.
- Maher, J. R.: Mode of action of isoniazid. *Am. Rev. Tuberc.* 75:517, 1956.
- Mitchison, D. A.: A comparison of techniques for measuring the isoniazid resistance of tubercle bacilli. *J. Clin. Path.* 6:118, 1953.
- Neilands, J. B.: Metal and hydrogen-ion bonding properties of cycloserine. *Arch. Biochem.* 62:151, 1956.
- Poole, N. F., and Meyer, A. E.: Comparison of new chemical method of determining isonicotinoyl hydrazide in serum with microbiological assay. *Proc. Soc. Exper. Biol. & Med.* 98:375, 1958.
- Reiss, J., and Townsend, S. M.: The rising incidence of isoniazid-resistance: its clinical significance. *Dis. Chest* 32:274, 1957.
- Tucker, W. B.: An account of the cooperative investigation of the Veterans Administration, Army and Navy. Three papers. I. *Am. Rev. Tuberc.* 72:718, 1955. II. *Am. Rev. Tuberc.* 72:733, 1955. III. *Ibid.* 72:756, 1955.
- U. S. Public Health Service Tuberculosis Prophylaxis Trial: Prophylactic effects of isoniazid on primary tuberculosis in children. *Am. Rev. Tuberc.* 76:942, 1957.
- Weiss, P. J.: A new colormetric test for the estimation of dihydrostreptomycin. *Antibiotic & Chemother.* 6:653, 1956.
- Weiss, W., *et al.*: Impact of isoniazid on tuberculous meningitis. *J.A.M.A.* 164:947, 1957.

- Williston, E. H., and Youmans, G. P.: Streptomycin resistant strains of tubercle bacilli: Production of streptomycin resistance *in vitro*. Am. Rev. Tuberc. 55:536-539, 1947.
- Williston, E. H., and Youmans, G. P.: Factors affecting the sensitivity *in vitro* of tubercle bacilli to streptomycin. Am. Rev. Tuberc. 59:336, 1949.
- Yegian, D., *et al.*: Para-aminosalicylic acid in the sputum. II. Elimination of its effect on culture for tubercle bacilli. Am. Rev. Tuberc. 71:860, 1955.
- Youmans, G. P.; Williston, E. H.; and Osborne, R. R.: Occurrence of streptomycin resistant tubercle bacilli in mice treated with streptomycin. Proc. Soc. Exper. Biol. & Med. 70:36-37, 1949.

Virulence and Variation

- Beaver, P. W., and Bayne-Jones, S.: Mycobacterium Ryan strain, isolated from pleural exudate. J. Infect. Dis. 49:399, 1931.
- Bloch, H., and Noll, H.: Studies on the virulence of tubercle bacilli. The effect of cord factor on murine tuberculosis. Brit. J. Exper. Path. 36:8, 1955.
- Branch, A. S.: Study of acid-fast organisms other than mammalian tubercle bacilli isolated from disease in man; occurrence of avian tubercle bacillus infection of man and diagnosis of avirulent avian tubercle bacilli. Tubercle 14:337, 1933.
- Buhler, V. B., and Pollak, A.: Human infection with atypical acid-fast organisms. Am. J. Clin. Path. 23:363, 1953.
- Hauduroy, P.: La virulence des Mycobacteries. Rev. immunol. 18:62, 1954.
- Karlson, A. G., and Ikemi, Y.: Comparison of the virulence for mice and guinea pigs of an isoniazid-sensitive tubercle bacillus and its isoniazid-resistant variant. Proc. Staff Meetings Mayo Clinic 29:119, 1954.
- Middlebrook, G., and Cohn, M. L.: Some observations on the pathogenicity of isoniazid-resistant variants of tubercle bacilli. Science 118:297, 1953.
- Morse, W. C.; Weiser, O. L.; Kuhns, D. M.; Fusillo, M.: Study of the virulence of isoniazid-resistant tubercle bacilli in guinea pigs and mice. Am. Rev. Tuberc. 69:464, 1954.
- Norden, A., and Linell, F.: A new type of pathogenic mycobacterium. Nature 168:826, 1951.
- Pinner, M.: Atypical acid-fast microorganisms; chromogenic acid-fast bacilli from human beings. Am. Rev. Tuberc. 32:424, 1935.
- Raffel, S.: Pathogenicity of the tubercle bacillus. Proc. Soc. Exper. Biol. & Med. 64:507, 1947.
- Smithburn, K. C.: Virulence of tubercle bacilli. Its variations attendant on animal passage. Am. Rev. Tuberc. 39:116, 1939.
- Tarshis, M. S., and Frisch, A. W.: Chromogenic acid-fast bacilli from human sources. Am. Rev. Tuberc. 65:278, 1952.
- Yegian, D., and Budd, V.: Certain non-pathogenic mycobacteria as a source of error in diagnosis of drug-susceptibility tests. Am. Rev. Tuberc. 68:557, 1953.

B. C. G.

- American Trudeau Society: A. T. S. States policy on B. C. G. Bull. N. T. A. March 1948.
- Aronson, J., and Palmer, C. E.: Experience with B. C. G. in the control of tuberculosis among North American Indians. Pub. Health Rep. 61:802, 1946.
- Birkhaug, K.: A detachable spring actuated multiple puncture apparatus for B. C. G. vaccination. Am. J. Clin. Path. 19:492, 1949.
- Birkhaug, K.: B. C. G. and the newer epidemiology of tuberculosis. Bull. N. J. Acad. Med. 24:411, 1948.
- Boquet, A.: The pathogenic properties of B. C. G. Am. Rev. Tuberc. 24:764, 1931.
- Calwell, H. G.: Fatal case of tuberculosis produced by B. C. G. Am. Rev. Tuberc. 73:301, 1956.
- Coello, G.: B. C. G. vaccination (a review). Indian J. of Med. Sci. 2:755, 1948.

- Cohn, M. L.; Davis, C. L.; and Middlebrook, G.: Airborne immunization against tuberculosis. *Science*, 128:1282, 1958.
- Dahlstrom, G.: The future of BCG vaccination. *Acta tuberc. scandinav.* 32: 250, 1956.
- Dubos, R. J., *et al.*: Differential characteristics *in vitro* and *in vivo* of several substrains of B. C. G. IV. Immunizing effectiveness. *Am. Rev. Tuberc.* 74: 699, 1956.
- Dubos, R. J., *et al.*: Report of *ad hoc* advisory committee on BCG to the Surgeon General of the United States Public Health Service. *Am. Rev. Tuberc.* 76: 726, 1957.
- Editorial: BCG vaccination against tuberculosis. *J.A.M.A.* June 1957.
- Federal Security Agency: Selected references to articles on BCG. *F.S.A. Bulletin.*
- Flakmer, S., *et al.*: Fatal tuberculosis from BCG vaccination. *Acta Pediat.* 44: 219, 1955.
- Hollstrom E., and Hard, S.: A fatality from BCG vaccination. *Acta dermat. venereol.* 33:159, 1953.
- Hyge, T. V.: The efficacy of BCG vaccination. *Acta tuberc. scandinav.* 32: (89), 1956
- Levine, M. I.: An evaluation of the use of BCG in the prevention of tuberculosis in infants and children. *Am. J. Pub. Health* 37:1089, 1947.
- Medical Advisory Committee of Research Foundation: Why have we not accepted BCG vaccination? *J. A. M. A.* 164:951, 1957.
- Meyer, J.: A fatal case of tuberculosis produced by BCG. *Am. Rev. Tuberc.* 70: 402, 1954.
- Rosenthal, S. R.: The preservation of BCG by freeze-drying. *Acta tuberc. scandinav.* 26:106, 1952.
- Ibid.*: Standardization and efficacy of BCG vaccination against tuberculosis. Twenty year study: a critical evaluation. *J.A.M.A.* 157:801, 1955.
- Tuberculosis Program, Public Health Service, U.S.A.: Experimental studies of vaccination, allergy, and immunity in tuberculosis. Papers I-III, *Bull. World Health Org.* 12:13-62, 1955; Papers IV-VI, *Am. J. Hyg.* 62:168-199, 1955.
- U. S. Public Health Service, Tuberculosis Control Division: Story of B. C. G. *Int. Med. Digest*, May 1948.
- Wallgren, A. J.: BCG: Past, present, and future. *Am. Rev. Tuberc.* 76:715, 1957.

Tuberculin Test

- Chesrow, E. J., *et al.*: Tuberculin testing of the aged. *Dis. Chest.* 32:2, 1957.
- Choremis, C., *et al.*: Effects of isoniazid on allergy. *Am. Rev. Tuberc. Supplement*, August 1956, 197-208.
- Corper, H. J., and Clark, C.: Autolytic tuberculin. *Am. Rev. Tuberc.* 54:401, 1946.
- Holm, J., and Lind, P.: Standardization of tuberculin. *Pub. Health Rep.* 62: 188, 1947.
- Jensen, K. A., and Lind, P.: Specificity of purified tuberculins. *Acta tuberc. scandinav.* 17:37, 1943.
- Low, E.: Mantoux and Heaf multiple puncture tuberculin tests: comparison in BCG vaccinated and unvaccinated subjects. *Tubercle* 37:102, 1956.
- Palitz, L. S., and Aronsohn, M. H.: Tuberculin sensitivity in the aged. *Am. Rev. Tuberc.* 75:461, 1957.
- Pepys, J.: The relationship of nonspecific and specific factors in the tuberculin reaction. *Am. Rev. Tuberc.* 71:49, 1955.
- Raffel, S.: The relationship of acquired resistance, allergy, antibodies, and tissue reactivities to components of the tubercle bacillus. *Am. Rev. Tuberc.* 54:564, 1946.
- Raffel, S.: Chemical factors involved in the induction of infectious allergy. *Experientia* 6:410, 1950.
- Schwartz, S., *et al.*: A tuberculin survey in a metropolitan district and its relation to morbidity and mortality. *Dis. Chest* 28:21, 1955.
- Seibert, F. B., and Dufour, E.: A study of certain problems in the use of standard tuberculin fractionation of P.P.D. *Am. Rev. Tuberc.* 58:363, 1948.
- Seideman, R. M.: A comparative study of O.T. and P.P.D. *Am. J. Hyg.* 30:1, 1939.
- Woodruff, C. E.: Quantitative tuberculin test. *Am. Rev. Tuberc.* 55:488, 1947.

Biochemistry

- Advances in carbohydrate chemistry: Vol. 3 edited by Pigman, W. W. and Wofom, M. L., New York, Academic Press, Inc., 1946.
- Anderson, R. J.: The chemistry of the lipids of tubercle bacilli. Harvey Lectures 35:271, 1940.
- Anderson, R. J.: The chemistry of the lipids of the tubercle bacillus. Yale J. Biol. & Med. 15:311, 1943.
- Anderson, R. J.: Introduction to bacteriological chemistry. Baltimore, Williams and Wilkins, 1946.
- Asselineau, J.: Lipids du bacille tuberculeux. Prog. Explor. Tuberc. 5:1, New York, S. Karger, Bale, 1952.
- Asselineau, J., and Lederer, E.: Sur la constitution du "cord factor" isolé d'une souche humaine de bacille tuberculeux. Biochim. et biophys. acta 17:161, 1955.
- Bloch, H., *et al.*: A toxic lipid component of the tubercle bacillus ("Cord Factor"). I. Isolation from petroleum ether extracts of young bacterial cultures. Am. Rev. Tuberc. 67:629, 1953; Noll, H., and Bloch, H.: *Ibid*, II. Occurrence in chloroform extracts of young and older bacterial cultures. Am. Rev. Tuberc. 67:828, 1953. Asselineau, J., *et al.*: *Ibid*, III. Occurrence and distribution in various bacterial extracts. Am. Rev. Tuberc. 67:853, 1953.
- Bloch, H., *et al.*: Constituents of a "toxic-lipid" obtained from *Mycobacterium tuberculosis*. Biochim. et biophys. acta 23:312, 1957.
- Foldes, J.: The nature of specific polysaccharides of tubercle bacilli. Acta mycol. Acad. Scient. Hung. 2:297-313, 1955.
- Ginsberg, B., *et al.*: Amino acid composition of Mycobacteria. Arch. Biochem. 60:164, 1956.
- Goldman, D. S.: Enzyme systems in the mycobacteria. I. The isocitric dehydrogenase. J. Bact. 71:732, 1956.
- Ibid.*: II. The malic dehydrogenase. J. Bact. 72:401, 1956.
- Ibid.*: III. The condensing enzyme. J. Bact. 73:602, 1957.
- Malek, I., and Sterzl, J.: Les substances nucleaires du bacille tuberculeux. Comptes Rendes des Séances de la Société de Biologie 142:1053, 1948.
- Menzel, A. E. O., and Heidelberger, M.: Protein fractions of the human strain, H-37, of tubercle bacillus. J. Biol. Chem. 124:89, 1938.
- Noll, H., and Bloch, H.: Studies on the chemistry of the cord factor of *Mycobacterium tuberculosis*. J. Biol. Chem. 214:251, 1955.
- Noll, H., *et al.*: The chemical structure of the cord factor of *Mycobacterium tuberculosis*. Biochim. et biophys. acta 20:299, 1956.
- Seibert, F.: The chemical composition of the active principle of tuberculin and the isolation in crystalline form and identification of the active principle of tuberculin. Am. Rev. Tuberc. 17:402, 1928.
- Seibert, F.: A history of the development of P.P.D. Am. Rev. Tuberc. 44:1, 1941.
- Seibert, F.: The chemistry of tuberculin. Chem. Rev. 34:107, 1944.
- Seibert, F. B.: Constituents of mycobacteria. Ann. Rev. Microbiol. 4:35, 1950.
- Seibert, F. B., *et al.*: Isolation, identification and classification of proteins of tuberculin and the tubercle bacillus. Am. Rev. Tuberc. 71:704, 1955.
- Stacey, M., *et al.*: Polysaccharide complexes isolated from *M. tuberculosis* (human strain). Biochim. et biophys. acta 7:146, 1951.
- Steenken, W.: The isolation of purified protein derivatives and carbohydrates from tuberculins and their biological properties. J. Biol. Chem. 141:91, 1941.

Hematology

- Allgower, M., and Bloch, H.: The effect of tubercle bacilli on the migration of phagocytes *in vitro*. Am. Rev. Tuberc. 59:562, 1949.
- Bloch, H.: The relationship between phagocytic cells and human tubercle bacilli. (Good references) Am. Rev. Tuberc. 58:662, 1948.
- Brocard, H.; Grivaux, M.; and Basset, G.: Haematological tests of progress during the treatment of certain forms of pulmonary tuberculosis with streptomycin. Paris Med. 39:118, 1949.
- Favour, C.; Fremont-Smith, P.; and Miller, J. M.: Factors affecting *in vitro* cytotoxicity of W.B.C. by tuberculin. Am. Rev. Tuberc. 60:212, 1949.

- Gilliland, I. C., *et al.*: Serum proteins in pulmonary tuberculosis. *Brit. M. J.* p. 1460, 1956.
- Heilman, D. H.; Feldman, W. H.; and Mann, F.: Specific cytotoxic action of tuberculin. *Am. Rev. Tuberc.* 52:1945.
- Hotopp, M., and Kahn, M. C.: The fate of phagocytized acid-fast bacteria as determined by the single cell method. *J. Infect. Dis.* 58:324, 1936.
- Sabin, F. R., *et al.*: Studies on tuberculosis. *J. Exper. Med.* 52:1-151, 1930 (Supp. No. 3).

Serology

- Caulfield, A. H. W.: The tuberculo-complement fixation and inhibition test. *Am. Rev. Tuberc.* 11:509, 1925.
- Dozier, S. M., *et al.*: Demonstration of circulating antibodies to *M. tuberculosis* in humans. I. *Am. Rev. Tuberc.* 75:949, 1957. II. Fusillo, *et al.*, *Am. Rev. Tuberc.* 75:954, 1957.
- Freund, G., and Walter, A.: Saprophytic acid-fast bacilli and paraffin oil as adjuvants in immunization. *Proc. Soc. Biol. & Med.* 56:47, 1944.
- Gerstl, B., *et al.*: Detection of apparently absent circulating antibodies in tuberculous sera. *Am. Rev. Tuberc.* 72:345, 1955.
- Heidelberger, M.: Chemical aspects of the precipitin and agglutination reaction. *Chem. Rev.* 24:323, 1939.
- Labzoffsky, N. A.: Experiences with a new antigen for a complement fixation test in tuberculosis. *Canad. J. Microbiol.* 1:794, 1955.
- Labzoffsky, N. A., *et al.*: Complement fixing antibodies in tuberculous guinea pigs. *Canad. J. Microbiol.* 2:461, 1956.
- Lagercrantz, R.: Immunological studies of mycobacteria. (two papers) *Acta path. et microbiol. scandinav.* 38:416-432, 1956.
- Lewis, P. A.: The complement fixation reaction as applied to tuberculosis. *Am. Rev. Tuberc.* 3:129, 1919-20.
- Middlebrook, G., and Dubos, R.: Specific serum agglutination of erythrocytes sensitized with extracts of tubercle bacilli. *J. Exper. Med.* 88:521, 1948.
- Parlett, R. C., *et al.*: The detection of antibodies in the serum of tuberculous patients by an agar double-diffusion precipitation technique. *Am. Rev. Tuberc.* 77:462, 1958.
- Rheins, M., *et al.*: Serologic investigations of mycobacterial antigens. (Five papers) I. *Am. Rev. Tuberc.* 73:563, 1956. II. *Ibid.*, 73:571, 1956. III. *Ibid.*, 74:756, 1956. IV. *Ibid.*, 74:764, 1956. V. *Ibid.*, 75:958, 1957.
- Rheins, M., *et al.*: Tuberculous antibodies demonstrated by agar diffusion. I. Specificity and incidence of agar-diffusion antibodies in rabbit sera. *Am. Rev. Tuberc.* 74:229, 1956; Burrell, R. G., *et al.* *Ibid.* II. Further characterization of these antibodies and their distribution in human tuberculous sera. *Am. Rev. Tuberc.* 74:239, 1956.
- Sasana, K. T.: Further studies on the complement fixation test for tuberculosis. *Am. Rev. Tuberc.* 9:136, 1924.
- Seibert, F. B., *et al.*: The significance of antibodies to tuberculoprotein and polysaccharide in resistance to tuberculosis. *Am. Rev. Tuberc.* 73:547, 1956.

Clinical Laboratory Tests

- Gleich, M.: Levison test. *Am. J. Dis. Child.* XLIII:1077, 1932.
- Levy, J. F.: Biochemical studies on tuberculosis. VI. The diagnostic significance of the nucleic acid test in tuberculosis. *J. Phil. Med. Assn.* 25:75-81, 1949.
- Mendel, L.: Daranyi reaction. *Jahrb. Kinderheilk.* CVI:15, 1924.
- Seibert, F. B.; Pfaff, M. L.; and Seibert, M. V.: A serum polysaccharide in tuberculosis and carcinoma. *Arch. Biochem.* 18:279, 1948.
- Seibert, F. B.; Seibert, M. V.; Atno, J.; and Campbell, H. W.: Variations in protein and polysaccharide content of sera in the chronic diseases tuberculosis, sarcoidosis and carcinoma. *J. Clin. Invest.* 26:90, 1947.

- Tanner, F. H., and Tolman, P. J.: The Weltman serum coagulation test. *Am. J. Clin. Path.* 11:528, 1941.
- Wuhrmann, F. H., and Wunderly, C. H.: The cadmium reaction. *J. Lab. & Clin. Med.* 34:1162, 1942.

Disinfectants

- Frobisher, M., Jr., and Sommermeyer, L.: A study of the effect of alcohols on tubercle bacilli and other bacteria in sputum. *Am. Rev. Tuberc.* 68:419, 1953.
- Frobisher, M. Jr., *et al.*: Studies on disinfection of clinical thermometers. I. Oral thermometers. *App. Microbi.*, 1:187, 1953.
- Green, L.: The effect of triethyleneglycol upon *Mycobacteria*. *Tuberkulosearzt* 6:177, 1952.
- Hirsch, J. G.: The resistance of tubercle bacilli to the bactericidal action of benzalkonium chloride (Zephiran). *Am. Rev. Tuberc.* 70:312, 1954.
- Lawrence, C. A., and Grover, A. A.: Hospital sterilizing solutions. *Bull. Am. Soc. Hosp. Pharm.* 8:224, 1951.
- Ritter, H. W.: The germicidal effect of a quaternary ammonium compound (cetylpyridinium chloride) on *Mycobacterium tuberculosis*. *App. Microb.* 4:114, 1956.
- Smith, C. R.: Alcohol as a disinfectant against the tubercle bacillus. *Pub. Health Rep.* 62:1285, 1947.
- Smith, C. R.: The bactericidal effect of surface active agents on tubercle bacilli. *Pub. Health Rep.* 65:1588, 1950.
- Wolinsky, E., *et al.*: Tuberculocidal activity of clorpactin, a new chlorine compound. *Antibiot. Med.* 1:382, 1955.

Special Techniques

- Cooke, G. M., *et al.*: Staining of bacterial colonies on the millipore filter to improve contrast. *Stain Tech.* 32:63, 1957.
- Coster, J. F., and Manten, A.: A simple technique for the differentiation of tubercle bacilli and saprophytic mycobacteria. *Am. Rev. Tuberc.* 74:958, 1956.
- Crowle, A. J.: Lung density as a measure of tuberculous involvement in mice. *Am. Rev. Tuberc.* 77:681, 1958.
- Dubos, R. J., and Middlebrook, G.: Cytochemical reaction of virulent tubercle bacilli. *Am. Rev. Tuberc.* 58:698, 1948.
- Fenner, F.: The enumeration of viable tubercle bacilli by surface plate counts. *Am. Rev. Tuberc.* 64:353, 1951.
- Froman, S., *et al.*: Bacteriophage active against virulent *Mycobacterium tuberculosis*. I. Isolation and activity. *Am. J. Pub. Health* 44:1326, 1954.
- Froman, S., *et al.*: Bacteriophage susceptibility and cultural characteristics of BCG and other tubercle bacilli. *Dis. Chest* 28:377, 1955.
- Goldman, E. C., and Goldman, D. S.: Cording and neutral red reaction of two avirulent strains of mycobacterium. *Am. Rev. Tuberc.* 73:674, 1956.
- Haley, L. D., and Arch, R.: Use of millipore membrane filter in the diagnostic tuberculosis laboratory. *Am. J. Clin. Path* 27:117, 1957.
- Hauduroy, P., and Pasternak-Gallia, Y.: Sur une réaction cytochimique en rapport avec la virulence des mycobactéries. *Ann. Inst. Pasteur* 77:91, 1949.
- Jones, W. D., Jr.: Simple method for maintaining stock cultures of mycobacterium species. *Am. J. Clin. Path.* 27:363, 1957.
- Koch-Weser, D., and Ebert, R. H.: Tetrazolium reduction by tubercle bacilli. *J. Lab. Clin. Med.* 46:608, 1955.
- Konno, K.: New chemical method to differentiate human-type tubercle bacilli from other mycobacteria. *Science* 124:985, 1956.
- Krasnow, I., *et al.*: A microcolonial test for the recognition of virulent mycobacteria. *Am. Rev. Tuberc.* 71:361, 1955.
- Lack, C. H.: The pathogenesis of tuberculosis as shown in studies of omental spreads. *Am. Rev. Tuberc.* 73:362, 1956.
- Melvin, I. G., and Vandiviere, H. Mac.: The titration of cord formation as related to virulence of mycobacteria. *Am. Rev. Tuberc.* 78:83, 1958.

- Middlebrook, G.; Dubos, R. J.; and Pierce, C. H.: Virulence and morphological characteristics of mammalian tubercle bacilli. *J. Exper. Med.* 86:175, 1947.
- Morse, W. C.; Dail, M. C.; and Olitzky, I.: A study of the neutral red reaction for determining the virulence of mycobacteria. *Am. J. Pub. Health* 43:36, 1953.
- Parlett, R., and Youmans, G. P.: Antigenic relationships between mycobacteria as determined by agar diffusion precipitin techniques. *Am. Rev. Tuberc.* 73:673, 1956.
- Ibid.*: Antigenic relationships between ninety-eight strains of mycobacteria using gel-diffusion precipitation techniques. *Am. Rev. Tuberc.* 77:450, 1958.
- Runyon, E. H., *et al.*: Distinguishing mycobacteria by the niacin test. *Am. Rev. Tuberc.* 79:663, 1959.
- Schweiger, O., *et al.*: Studies on the catalase enzyme of mycobacteria. I. Correlation between catalase activity and virulence of mycobacteria under certain experimental conditions. *Am. Rev. Tuberc.* 77:146, 1958.
- Viallier, J.; Kalb, J. C.; and Tiguad, J.: Sur la coloration par le rouge neutre du bacille tuberculeux. *Compt. Rend. Soc. de Biol.* 144:1513, 1950.
- Will, D. W., *et al.*: Cultural characteristics and drug and bacteriophage resistance of avian tubercle bacilli. *Am. Rev. Tuberc.* 76:435, 1957.
- Wilson, F. J., *et al.*: Use of oxidation-reduction dyes in the determination of virulence of mycobacteria *in vitro*. *Am. Rev. Tuberc.* 65:187, 1952.
- Wrinkle, C. K., and Patnode, R. A.: A test tube modification of the oxidation-reduction dye test for the determination of virulence of mycobacteria *in vitro*. *Am. Rev. Tuberc.* 66:99, 1952.

Atypical Acid-Fast Bacilli

- Buhler, V. B., and Pollak, A.: Human infection with atypical acid-fast organisms. *Am. J. Clin. Path.* 23:363, 1953.
- Crow, H. E., *et al.*: A limited clinical, pathologic, and epidemiologic study of patients with pulmonary lesions associated with atypical acid-fast bacilli in the sputum. *Am. Rev. Tuberc.* 75:199, 1957.
- Gastambide-Odier, M. M., *et al.*: Comparison of the native lipids of atypical acid-fast bacilli with the lipids of known mycobacterial types. *Am. Rev. Tuberc.* 75:843, 1957.
- Gastambide-Odier, M. M., and Smith, D. W.: A rapid means of differentiating atypical acid-fast bacilli and avian tubercle bacilli from other mycobacteria. *Am. Rev. Tuberc.* 77:662, 1958.
- Gordon, R. E., and Smith, M. M.: Rapidly growing, acid-fast bacteria. II. Species' description of *Mycobacterium fortuitum* Cruz. *J. Bact.* 69:502, 1955.
- Huppert, M., *et al.*: Characterization of atypical mycobacteria and of *Nocardia* species isolated from clinical specimens. II. Procedure for differentiating between acid-fast microorganisms. *Am. Rev. Tuberc.* 76:468, 1957.
- Keltz, H., *et al.*: A characterization of atypical acid-fast bacilli obtained from patients with pulmonary tuberculosis. *Dis. Chest* 34:368, 1958.
- Koch, M. L., *et al.*: The selective activity of fluid thioglycollate medium for group differentiation of atypical chromogenic mycobacteria, *Mycobacterium tuberculosis*, and saprophytic mycobacteria. *Am. Rev. Tuberc.* 77:356, 1958.
- Konno, K., *et al.*: Differentiation of human tubercle bacilli from atypical acid-fast bacilli. I. and II. *Am. Rev. Tuberc.* 77:669, 1958.
- Kushner, D. S., *et al.*: Atypical acid-fast bacilli. II. *Mycobacterium fortuitum*: Bacteriologic characteristics and pathogenicity for laboratory animals. *Am. Rev. Tuberc.* 76:108, 1957.
- McMillen, S., and Kushner, D. S.: Atypical acid-fast bacilli. I. A cultural scheme for rapid identification. *Am. Rev. Tuberc.* 76:103, 1957.
- Pollak, A., and Buhler, V. B.: The cultural characteristics and animal pathogenicity of an atypical acid-fast organism which causes human disease. *Am. Rev. Tuberc.* 71:74, 1955.
- Runyon, E. H.: Anonymous mycobacteria in pulmonary disease. *Med. Clinics N. America* 43:273, 1959.

- Tarshis, M. S.: Preliminary observations on the development of atypical (chromogenic) variants of the H37Rv strain of *M. tuberculosis* under the influence of streptomycin and isoniazid *in vitro*. *Am. Rev. Tuberc.* 78:921, 1958.
- Timpe, A., and Runyon, E. H.: The relationship of "atypical" acid-fast bacteria to human disease. *J. Lab. Clin. Med.* 44:202, 1954.
- Wayne, L. G., *et al.*: Characterization of atypical mycobacteria and of *Nocardia* species isolated from clinical specimens. *Am. Rev. Tuberc.* 76:451, 1957.
- Wayne, L. G.: Quantitative aspects of neutral red reactions of typical and atypical mycobacteria. *Am. Rev. Tuberc.* 79:526, 1959.
- Wolinsky, E., *et al.*: Atypical chromogenic mycobacteria associated with pulmonary disease. *Am. Rev. Tuberc.* 75:180, 1957.
- Wolinsky, E., *et al.*: Drug susceptibilities of 20 "atypical" as compared with 19 selected strains of mycobacteria. *Am. Rev. Tuberc.* 76:497, 1957.
- Wood, L. E., *et al.*: Human infections with the "yellow" acid-fast bacillus. *Am. Rev. Tuberc.* 73:917, 1956.

