U. S. NAVY

NATIONAL NAVAL MEDICAL CENTER

NAVAL MEDICAL SCHOOL

BACTERIOLOGY

(Large)

PREFACE

The purpose of this manual is to provide assistance to both laboratory technician and clinician in the study of the etiology of pathological processes falling within the general scope of bacteriology. Whereas most specimens are considered from the point of view of bacterial infection, the rickettsiae, viruses, and fungi must also be borne in mind and measures for their detection carried out when indicated. Such measures are described in this manual. The preparation of media and stains and their use as well as other technical information of value to the bacteriologist have been included.

The methods presented herein are those considered reliable and practical. Their application must depend on the facilities available, the qualifications of the technician or laboratory officer and the advisability of their execution.

CONTENTS

I INTRODUCTION

The bacteriologist plays an important part in medical diagnosis and, with the increased use of chemotherapeutic agents in infectious diseases, has assumed an important position in modern therapeutic procedures. The value of the bacteriological laboratory rests upon the close cooperation between the bacteriologist and the clinician, and in order to achieve the best result, the bacteriologist must have a clinical viewpoint and the clinician must be familiar with the advantages and shortcomings of laboratory technique in order to interpret laboratory reports for their fullest value.

The validity of bacteriological tests depends as fully upon the correct technique by which the material is collected as upon the laboratory examination. The collected sample should represent the actual substance from the lesion, as far as possible uncontaminated by extraneous material. The containers should be suitable for the material and should be thoroughly cleaned and sterilized. When material is scanty it should be collected upon sterile cotton swabs. Fluids, depending upon the amount, should be collected in sterile tubes or bottles. Aseptic collections at times may be facilitated by the use of syringes. Examination of the specimen should be made as soon as possible after collection. Specimens which dry rapidly or are susceptible to change should be delivered at once to the laboratory and a knowledge of the proper temperature at which specimens should be held while awaiting examination is essential. Material for shipment through the mail requires careful packing in containers to prevent loss from breakage or leakage, and must conform with postal regulations.

For bacteriological study of autopsy material, collection should be made as early as possible to avoid contamination through the dissemination of intestinal and other extraneous organisms. The use of sharp, jagged-edged pipettes for obtaining blood and fluids from the various organs and vessels facilitates the collection of specimens. Except where the outside of a tissue is known not to be contaminated, the surface of the tissue from which the culture is taken should first be seared with a hot spatula or knife blade.

Ordinary microscopic examination of material in suspension, as urine, gives general information about the cellular and bacterial content. For the examination of fixed bacteria, material is spread in a thin film on a slide or coverglass, dried in air, fixed and stained by one of the methods described in the appendix. The laboratory diagnosis of certain diseases depends almost entirely upon direct smear examination, owing to the difficulty of culturing the causative organisms. In other diseases it forms a valuable adjunct to cultural methods. Indirect evidence of the presence of certain diseases may also be obtained from the smear by studying the type of cells and other structures in (1) sputum, (2) spinal fluid, (3) transudates and exudates, and (4) urine.

In order to determine the species of organisms present in a given specimen, pure cultures must be obtained for detailed study. Certain organisms may require the addition of enriching substances to the basic culture media (Appendix), while for others modification of the usual incubation procedures must be made (e.g. anaerobic bacteria). After isolation of pure cultures is effected, the organism may be differentiated by cultural, morphological, biochemical, serological and pathogenic characteristics.

II THE TREATMENT OF SPECIMENS FOR DETECTION AND ISOLATION OF INFECTIOUS AGENTS

In culturing specimens the bacteriologist should know what type of organism is expected in order that suitable culture media may be inoculated and the proper incubation procedures followed. Table I presents the sources from which the common pathogenic bacteria may be isolated, and Table II indicates briefly the methods employed for the diagnosis of infections caused by these organisms.

BLOOD - To obtain a sample of blood for bacteriological examination the following apparatus is required: a sterile venepuncture needle, a sterile 10 or 20 cc. syringe, a citrate bottle or flask (prepared by placing 0.5 cc. of a 10 per cent solution of sodium citrate in the bottle or flask and sterilizing with dry heat), a tourniquet, and iodine and alcohol for disinfection of the skin. Ten or twelve cc. of blood is removed from the median basilic vein, or other suitable or available vein, and mixed with the solid citrate in the bottle by gentle rotation in order to prevent clotting. One cc. of blood is added to each of two sterile Petri dishes and then to each plate 12 or 15 cc. of melted basic medium, cooled to about 44-46 degrees C. is added. After thorough mixing, the plates are allowed to harden and then inverted and placed in the incubator. The melted agar must be cooled to 44-46 degrees C. in order not to coagulate or brown the blood or to injure any organisms which may be present. Five cc. of the blood are added to a bottle containing 50-60 cc. of basic broth medium (containing 0.005 per cent p-aminobenzoic acid and 0.1 per cent glucose), and three cc. of blood are added to a tube (200 x 25 mm.) containing 40 cc. of the same medium enriched with 0.1 per cent agar. The latter medium should be heated in a boiling water bath for 10-15 minutes and then cooled to 40 degrees C. before use in order to remove any dissolved oxygen. All cultures are labelled with the date and the name of the patient and placed in the incubator (37 degrees C.). Most blood cultures should be examined daily for five days after which they may be discarded, but blood cultures from cases of rheumatic fever, bacterial endocarditis, brucellosis and tularemia should be held for three or four weeks. When the blood broth cultures are cloudy, Gram stained smears should be made and a blood agar plate streaked. Since pneumococci in broth may autolyze very easily, there should be no delay in examining a flask of broth if growth is suspected.

If Neisserian infection (gonococcus, meningococcus) is suspected, proceed in the usual manner except for the use of a liter Florence flask instead of a bottle for the 50-60 cc. broth quantity. This flask and the pour plates should be incubated under about 10 per cent carbon dioxide. After one, two and three days of incubation the plates and broth cultures should be examined for growth. The broth should be streaked onto freshly prepared chocolate agar plates which, with the original cultures, should be incubated in a sealed container containing about 10 per cent carbon dioxide. Due to the debris present in the broth cultures and the heavy red background in the Gram stained smears it is difficult to detect the gram negative diplococci. It is, therefore, advisable to subculture the blood-broth at each examination. If a container for the flask is not available the cotton stopper may be pushed down into the neck of the Florence flask and carbon dioxide added by the method of Shaughnessy (Appendix) or by setting fire to the cotton stopper, pushing it down into the neck of the flask and quickly closing the flask with a tightly fitting rubber stopper. The "candle-jar technique" (Appendix) may be used to provide a suitable carbon dioxide tension for the plates.

TABLE 1.

SOURCES FOR THE ISOLATION OF COMMON PATHOGENIC BACTERIA

Organism	Blood	Eye	Ear and Mastoid	Nose and Throat	Sputum and Lungs	Mouth and Teeth	Urine or Genital Discharge	Feces	Gastro-intestinal tract or Gall Bladder	Skin	Pus, Wounds, or Lesions	Transudates or Exudates	Spinal Fluid
Staphylococci	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptococci-hemolytic	+	+	+	+	+	+	+	+	+	+	+	+	+
Streptococci-viridans	+	+	+	+	+	+	+		R	+	+	+	R
D. pneumoniae	+	+	+	+	+	+	+				+	+	+
N. intracellularis	+			+									+
N. gonorrheae	R	+					+						R
N. catarrhalis				+	+		+						
C. diphtheriae		+	+	+							R		
M. tuberculosis					+		+	+	+	R		+	+
M. leprae				+						+	+		
B. anthracis	+				+			+		+			R
C. tetani								+			+		
C. welchii	+							+			+		
Eb. typhosa	+				R		+	+	+		R		R
S. paratyphi	+						+	+	+				
S. schottmulleri	+						+	+	+				

TABLE 1 (Continued)

SOURCES FOR THE ISOLATION OF COMMON PATHOGENIC BACTERIA

Organism	Blood	Eye	Ear and Mastoid	Nose and Throat	Sputum and Lungs	Mouth and Teeth	Urine or Genital Discharge	Feces	Gastro-intestinal tract or Gall Bladder	Skin	Pus, Wounds, or Lesions	Transudates or Exudates	Spinal Fluid
E. coli	+						+	+	+		+		
S. dysenteriae								+					
K. pneumoniae	+		+	+	+	+	+				+	+	
P. pestis	+				+						+		
P. tularensis	+	+									+		
H. influenzae	+	+	+	+	+	+					+	+	+
H. pertussis					+								
H. lacunatus		+											
H. ducreyi											+		
A. mallei	R			+							+		
B. abortus	+						+	+	+		+		
B. melitensis	+						+	+	+		+		
P. aeruginosa	+		+	+				+	+		+	+	
V. comma								+	+				
A. bovis					+						+		

R = Rare.

TABLE 2

METHODS FOR THE DIAGNOSIS OF INFECTIONS CAUSED BY THE COMMON PATHOGENIC BACTERIA

Bacteria	Smear	Culture	Serological				
			Agglutination	Precipitation	Alexin-fixation	Skin	Animal
Staphylococci	+	+					
Streptococci-hemolytic	+	+				+	
Streptococci-viridans	+	+					
D. pneumoniae	+	+	+	+			+
N. intracellularis	+	+	+				
N. gonorrheae	+	+			+		
N. catarrhalis		+					
C. diphtheriae	+	+					+
M. tuberculosis	+	+				+	+
M. leprae	+						
B. anthracis	+	+		+			+
C. tetani	+	+					+
C. welchii	+	+					+
Eb. typhosa		+	+				
S. paratyphi		+	+				
S. schottmulleri		+	+				
E. coli		+					
S. dysenteriae		+	+				
K. pneumoniae	+	+	+	+			
P. pestis	+	+	+				+

TABLE 2 (Continued)

METHODS FOR THE DIAGNOSIS OF INFECTIONS CAUSED BY THE COMMON PATHOGENIC BACTERIA

Bacteria	Smear	Culture	Serological				Animal
			Agglutination	Precipitation	Alexin-fixation	Skin	
P. tularensis		+	+				+
H. influenzae	+	+					
H. pertussis	+	+					
H. lacunatus	+						
H. ducreyi	+	+				+	
A. mallei	+	+	+	+	+	+	+
B. abortus		+	+	+		+	+
B. melitensis		+	+	+		+	+
P. aeruginosa		+					
V. comma	+	+	+				+
A. bovis	+	+					

Blood cultures from suspected cases of brucellosis are handled in the usual manner with incubation of the pour plates in the candle-jar and with the broth cultures under approximately 10 per cent carbon dioxide. After incubation for four days the broth cultures should be shaken thoroughly and 0.5 cc. streaked on the basic blood agar plate. This plating procedure should be carried out every fourth day for 18 days and the plates incubated in the candle-jar for at least five days. Any of the citrated blood that is left over may also be incubated and streaked onto the basic medium at three day intervals until completely used.

When colonies on the solid medium first become visible to the naked eye, they appear as minute, transparent, colorless drops on the surface. Since Brucella will not grow on dried agar, it is advisable to make transplants to fresh, moist media at two to three day intervals. This procedure may be followed even when no growth is visible to the naked eye. Microscopic examination of the early growth reveals small gram-negative bodies, and a person not familiar with the growth habits of Brucella may not identify the organisms. However, after transferring to fresh blood agar and incubation for 24-48 hours the colonies are larger and the organism is more definitely the gram-negative coccobacillus.

The colonies of Brucella in a poured plate, containing blood, resemble those of Streptococcus viridans, if such colonies are deep in the medium. After further incubation, especially if growing on the surface, the colony appearance is more closely that of E. typhosa.

If growth is obtained, tubes of dextrose, lactose, maltose, mannite, sucrose, and plain broth are inoculated. A non-motile, gram-negative coccobacillus which ferments none of the carbohydrates and is agglutinated by antibrucella serum is diagnostic of the Brucella group. The species may be identified by studies outlined in the section on the Brucella group. (See Index).

The culture of the blood for Brucella may be supplemented by the inoculation of 2 cc. amounts of the citrated blood into the peritoneal cavity of each of two guinea pigs. The animals are kept for three months if necessary before they are killed. The spleen, liver, peritoneal fluid and lymph nodes (if enlarged) are cultured for Brucella.

In any attempt to isolate members of the Brucella group the necessity for cultivation for several weeks should be expected, and the very young early forms, on the plate cultures, should be looked for carefully.

From suspected cases of tularemia, 0.5 - 1.0 cc. quantities of the patient's blood are inoculated onto ten or twelve glucose-cystine blood agar slants (Appendix) and onto one plain tryptose agar slant, (which serves as a control) and incubated for from 4 - 10 days, or until growth is observed.

If anaerobes are suspected a duplicate pour plate may be incubated in the anaerobic jar (Appendix) and Brewer's sodium thioglycollate medium may be used in addition to the media used routinely. If available, Brewer's anaerobic agar and Petri dish cover may be used (See Appendix - Techniques and Procedures).

If infection by H. influenzae (Pfeiffer's bacillus) is suspected the routine procedure is followed. If there is any sign of growth, chocolate or "combination" (see Appendix Medium II) blood agar plates are streaked. Minute colonies appear in 24-48 hours, and may sometimes show a satellite type of growth around colonies of staphylococci present as contaminants or purposely inoculated onto the chocolate agar, for the demonstration of the satellite colony type of growth.

THROAT, NASAL ACCESSORY SINUSSS, EAR, NOSE, MASTOID AND SPUTUM - Specimens from these locations should be streaked on blood agar plates, Loeffler's slants, and blood agar tellurite plates (if available) and incubated in the candle-jar. Smears should be made and studied by Gram's stain and any other staining methods that appear indicated or that are needed to demonstrate organisms indicated by the clinician. The Loeffler's slant should be examined as soon as possible (10 or 12 hours of incubation is about the minimum incubation time) and the tellurite plate after 18 - 24 hours of incubation. The Loeffler's slant growth and suspicious colonies on the tellurite plate should be smeared and stained with Neisser's methylene blue, Beck's, or some other suitable stain.

H. influenzae should always be looked for in these cultures by staining by Gram's method any minute colonies growing as satellites near other bacteria. Some hemolytic strains of the Hemophilus group (e.g. Hemophilus hemolyticus, sometimes called "Bacillus X") may be confused with hemolytic streptococcus colonies.

If Vincent's infection is believed to be present a direct smear should be made, fixed and stained with carbol fuchsin (15 seconds) or methylene blue (at least three minutes) and the spirochetes and fusiform bacilli looked for.

Tonsil and adenoid tissue should be removed from the specimen bottle and immersed in boiling water for 10 seconds. The tissue is placed in a sterile mortar, 2 to 3 cc. of sterile saline and a small amount of sterile sand is added and the tissue ground thoroughly with a sterile pestle. The material is then streaked onto a blood agar plate in the routine manner.

If a fungus or actinomyces infection is suspected Sabouraud's medium and plain or blood agar should be inoculated as indicated in the section on fungi.

For the detection of H. pertussis infection (whooping cough) special cough plates are prepared from glycerine-potato agar containing 30-50 per cent blood. The plate is held a few inches from the mouth of the patient, who should be made to cough directly onto the plate. The plates should be incubated aerobically at 37° C. in a moist atmosphere. Colonies of H. pertussis appear in about 2-5 days as small, grey-white, hemispherical, opaque colonies resembling mercury droplets. The organisms are hemolytic, but due to the large amount of blood in the medium, the area of hemolysis may be indistinct.

EYE - On requests for bacteriological study of eye conditions the laboratory must make careful study of smears and cultures of material taken from the conjunctival sac, stain smears by Gram and any other stains when indicated or requested and inoculate blood agar and chocolate agar plates. Should no organisms be

seen on smear and none isolated on culture, scrapings from the conjunctiva should be studied by Rice's stain (Appendix) for inclusion bodies of trachoma, infectious blenorrhea of the new-born and swimming-pool blenorrhea (all virus diseases). Smears of the conjunctival sac exudate should also be studied by Wright's blood stain for eosinophiles, the finding of which may be suggestive of an allergic condition. All plates should be incubated in the candle-jar. Where culture facilities are not available very valuable information may be obtained from the stained smears. The direct smear should not be relied upon for the diagnosis of diphtheritic, staphylococcic or streptococcic infection since diphtheroids and non-pathogenic staphylococci and micrococci are quite frequently found in the normal eye.

CEREBRO-SPINAL FLUID - At the time of collection of the spinal fluid a chocolate agar slant and a blood or "combination" blood agar slant should each be inoculated at the bedside from the puncture needle (very important in the case of the meningococcus) with about one-half cc. of the spinal fluid. It is important that media be warmed before being used. Reduced-tension-cultures should be made of these slants.

It is recommended that spinal fluid be collected in three or four clean, dry, sterile test tubes, labelled in the order of collection, and immediately plugged with sterile stoppers. By having the fluid divided in three or four portions, one may be used for culture, one for cell count and increased globulin determination, another for chloride and sugar studies, for Kahn or Wassermann or for any other purpose that may be desired. If not divided in this manner, a small quantity of the spinal fluid should be removed aseptically for the cell count, and after centrifugation, the sediment smeared for stain and culture and the supernatant used for other studies.

Warmed blood agar and chocolate agar or "combination" blood agar plates should be streaked with the sediment routinely and incubated as soon as possible in the candle-jar. A Gram stain on the sediment may permit an early diagnosis of the infection. If pneumococci are present they may be typed directly by the Quellung method. H. influenzae, if present usually will belong to type b and may similarly be identified by a direct Quellung test.

If a fungus infection is suspected as a result of the microscopic examination of the sediment, the clinical history, or other evidence, Sabouraud's slants should be inoculated for aerobic and anaerobic incubation at 37° C. and for aerobic incubation at room temperature.

If no growth is obtained aerobically after 24-48 hours although organisms were found on microscopic examination, a new specimen of spinal fluid, if obtainable, should be cultured anaerobically and a tube of semisolid agar inoculated for the cultivation of micro-aerophilic organisms.

Where pleocytosis (increase in cell count) is present, but no bacteria are found, a sugar and chloride determination will help in the decision as to whether a bacterial meningitis exists. This would be indicated by a sugar below 50 mgm. per cent, or by a low chloride, suggesting tuberculosis.

If tuberculosis is suspected, one of the concentration procedures of Hanks may

be conveniently employed for detecting their presence (See Appendix). The concentrated material may be used for microscopic examination, for culture on special media, (See Appendix), or for injection into guinea pigs. These concentration procedures may be used with fluids in which a fibrin clot or pellicle has already formed and, it is believed, will increase the likelihood of finding organisms if they are present.

When a fluid showing lymphocytosis and no bacteria is encountered in a patient having evidence of meningeal irritation and a history of previous respiratory infection, the virus of lymphocytic chorio-meningitis must also be considered. In this event blood serum should be collected during the illness and again six weeks following recovery and sent in as collected to the Naval Medical School for study to determine the presence of protective antibodies against this virus.

PLEURAL, PERICARDIAL, ASCITIC AND JOINT FLUIDS - Transudates and exudates should be centrifuged, the supernatant fluid decanted, the sediment smeared and stained in the routine manner by Gram's technique, and a blood agar plate streaked and incubated in the candle-jar. If the presence of the gonococcus is suspected a reduced tension chocolate blood agar slant should be streaked as well as chocolate agar plates. If members of the coli-typhoid group of bacteria are present, Mac Conkey's, Endo's medium or eosin-methylene blue agar plates should be streaked. If the specimen is foul-smelling it is advisable to culture for anaerobic bacteria by inoculating a tube of Brewer's medium. Since the latter medium does not give isolated colonies it is of little use in separating the anaerobic from other bacteria which may be present. To accomplish this, plates must be streaked and must be incubated anaerobically (See Appendix). To prevent clotting of the above specimens they may be taken in the citrate bottles employed in culturing of blood.

WOUNDS, ULCERS AND CHRONIC SINUS TRACTS - Specimens from surgical and traumatic wounds should be studied by stained smears and cultures on blood agar plates and on Mac Conkey's, Endo's or eosin-methylene blue agar. When the presence of anaerobes is suspected a blood agar plate should be incubated anaerobically as well as aerobically. If sporulating anaerobic bacteria may be present, a tube of sterile, whole milk should be heated in a boiling water bath for 10-15 minutes and rapidly cooled. It should be inoculated with the specimen and heated for 10 minutes at 80 degrees C., rapidly cooled and incubated. A stormy fermentation is indicative of the presence of Cl. welchii. The detection of anaerobic or microaerophilic bacteria in wounds may facilitate considerably the choice of proper chemotherapeutic agent (e.g. zinc peroxide). Material from ulcers and chronic sinus tracts should be studied by smears stained by Gram and acid-fast methods and cultured for aerobic and anaerobic bacteria and fungi.

GASTRO-INTESTINAL TRACT - If the fecal specimen is solid, a small portion (about the size of a pea) should be emulsified in about 5 cc. of sterile broth, peptone, or saline. A small loopful, taken from the upper layer of the suspension is streaked on MacConkey's, Endo's or eosin-methylene-blue plates and several loopfuls on Difco S-S agar or B-B-L desoxycholate-citrate agar, both of which facilitate the isolation of E. typhosa and the Salmonella and Shigella groups. A blood agar plate should also be streaked to determine the presence of staphylococci and streptococci. If E. typhosa is being looked for, Difco bismuth-sulfite medium should

be streaked with a small portion of the solid stool. A tube of tetrathionate broth (See Appendix) or Leifson's selenite-F enrichment medium, 5-7 centimeters in depth, may be inoculated with a small portion of the original stool specimen. The selenite broth culture is incubated for 18 hours and then streaked on Difco S-S or bismuth-sulfite agars which are examined for typical typhoid colonies after 18-24 hours of incubation. It is imperative that the selenite-F broth be subcultured not later than 18-20 hours after inoculation because after this period of time Esch. coli rapidly overgrows all other organisms. The advantages of this enrichment medium, however, are negligible if a good bismuth-sulfite plating medium is available.

Bismuth-sulfite agar markedly inhibits most strains of Esch.coli and allied species and favors the development of nearly all typhoid bacilli and members of the Salmonella group. Desoxycholate-citrate agar also inhibits micro-organisms of the coliform group and favors especially the isolation of the Flexner and Newcastle types of dysentery bacilli. The growth of some strains of Shigella sonnei is restricted on this medium and it is therefore essential to include a medium that will differentiate but not restrict growth such as Mac Conkey's, Endo's or eosin-methylene-blue agar plates. For a detailed description of the use of Bismuth-sulfite agar and the appearance of colonies on this medium, see the section on media.

Colonies of E. typhosa, dysenteriae, paradysenteriae and members of the Salmonella group are translucent and usually gray or colorless on MacConkey's, eosin-methylene-blue agar and Endo's media, and colorless or delicate and pink on desoxycholate-citrate agar. The colonies of coli and allied species are opaque, deep pink or red and may have a metallic luster on Endo's medium while on eosin-methylene-blue agar they are gray with a blue or black center. On MacConkey's agar, isolated colonies of coliform bacteria are brick red in color and may be surrounded by a zone of precipitated bile. Typhoid colonies are uncolored and transparent and when growing in proximity to coli colonies, they have the appearance of clearing the areas of precipitated bile. Colonies of dysentery and paratyphoid organisms are similar to those of typhoid. This medium is felt to be the least restrictive of these differential media. Suspicious colonies may be inoculated into Kligler's iron agar (or Russell double sugar agar), stabbing the butt to the bottom of the tube and streaking the surface of the slant (See Appendix).

For the isolation of Vibrio comma from the intestinal contents of cholera cases or carriers, alkaline peptone broth (pH 8.4) is used. A tube of this medium is inoculated with the "rice-water" stool or a small portion of feces from a suspected carrier and incubated for 6-8 hours. Smears from the surface layer often show the organisms in a practically pure state. They may be tested with a specific agglutinating serum or cultured on the various media, as outlined in the section on cholera, for final identification and differentiation from the paracholera and other Vibrios (Mackie and McCartney, page 401). If desired, special plating media such as Dieudonne's or Aronson's (Appendix) may be utilized for the isolation and cultivation of this organism.

Isolation of M. tuberculosis from the stool is inconclusive in respect to the localization of the lesion. The feces may be digested with antiformin and then smeared for microscopic examination, cultured on suitable media or, best of all, injected into guinea pigs. (Appendix for methods). Because tubercle bacilli are frequently swallowed by individuals having tuberculous pulmonary lesions, the presence of these organisms in the feces does not necessarily mean infection of the intestinal tract. Occasionally, recovery of the tubercle bacillus from gastric washings is the only means by which a bacteriological diagnosis of tuberculosis of the respiratory tract can be established. Inoculation into guinea pigs of centrifuged fasting gastric contents is probably the most sensitive method for the diagnosis of pulmonary tuberculosis.

Cultures of duodenal or gall-bladder drainage material will, on occasion determine a typhoid carrier state where the stool cultures are negative. Other organisms also may be isolated from the biliary secretions.

GENITO-URINARY TRACT - Urine specimens should be centrifuged at high speed (about 3000 r.p.m.) for 10 minutes, the supernatant fluid discarded and the sediment used to streak a blood agar and an Endo, MacConkey or eosin-methylene blue plate. The usual smear should be made for a Gram stain.

When the patient is receiving sulfanilamide or its derivatives the cultures should be incubated for four or five days before being reported as negative.

Smear examination of discharges from the genital tract is frequently used in diagnosis. Prostatic, urethral, vaginal and cervical smears are stained by Gram's method and examined for leukocytes and bacteria, particularly gram-negative intracellular diplococci having the morphology of N. gonorrhoea. Sometimes in the pre-acute and chronic stages of gonorrhoea the organisms may be found only extracellularly. Due to possible presence of the non-pathogenic M. smegmae on the genitals the finding of acid-fast rods in these specimens is inconclusive for tuberculosis. Animal inoculation must be resorted to for the identification of acid-fast rods as M. tuberculosis. The precipitation concentration procedure of Hanks for the detection of acid-fast organisms may be used with considerable advantage on 24 hour urine specimens (See Appendix).

In acute cases of gonococcal infection, specimens of exudate for examination by either the smear or culture method are generally taken from the urethra or cervix. Prostatic secretions and urine may also be submitted in chronic cases. Other sources of gonococcal pus are the Bartholin's glands, Skene's ducts, the fallopian tubes, pelvic lesions and rectal discharges.

In reporting the findings in a smear examination, the laboratory worker should report only what is observed in the smear, i.e., intracellular gram-negative diplococci having the morphology of gonococci were found, or were not found. If the organisms are extracellular they should be reported as such. The report should also include a statement concerning the number of pus cells present (many, few or none).

To culture the gonococcus it is essential that the specimen be kept moist

and that it be cultured soon after procurement. The pus at the meatus is taken by the use of a sterile cotton swab and should be streaked immediately on suitable media. Chocolate agar plates and the "combination" blood agar plate should be streaked and incubated in the candle-jar. To insure the presence of moisture a thin layer of moist cotton may be placed in the bottom of the jar. A reduced tension slant of the chocolate or combination blood agar media containing 1:5,000,000 crystal violet should also be streaked. After 24-48 hours incubation the plates are inspected for the presence of colonies of gonococci. Typically such colonies are convex, transparent, from 1-3 mm. in diameter with undulated margins. By their transparency and undulating margins they can usually be differentiated from colonies of streptococci and diphtheroids which they otherwise simulate to a certain extent. Verification of the colony selection is made by staining by Gram's method.

When no typical gonococcus colonies can be detected by direct inspection, the culture may be subjected to the oxidase test. This test is based on the production of the enzyme oxidase by the organisms belonging to the genus Neisseria. The oxidase changes the color of the indicator used in the test (dimethyl paraphenylene diamine hydrochloride) so that gonococcus colonies turn pink, maroon, and finally black (See Appendix for technique of test). If subcultures are to be made for further identification, the colonies should be picked as soon as they become pink, because the dye component is toxic for the gonococcus. When oxidation has progressed until the colony is black, the cells are usually dead and subcultures fail to grow. The dye does not interfere with subsequent Gram stains. In medico-legal procedures or in instances where the bacteriological findings do not agree with the history and clinical status of the patient it may be necessary to confirm the isolation by sugar fermentation studies.

For techniques for the delayed culture of the gonococcus see section on techniques and procedures.

Lesions on the genitalia which are suspected of being of a primary syphilitic nature should be gently washed with sterile physiological saline. Any blood that collects should be absorbed with sterile gauze. The tissues at the base of the lesion should then be compressed gently until a drop of clear serum or plasma collects on the surface. A modified glass syringe for making suction over the lesion is very helpful here. (See Appendix). The drop is touched with a clean cover slip which is placed on a thin glass slide. The preparation is then examined for Treponema pallidum by means of the dark-field. The examination of two or three such moist preparations is generally advisable. If the chancre fluid cannot be examined immediately, it can be collected in sterile capillary tubes about 2 inches long, of the type often used for vaccine lymph. If the tubes are properly sealed with wax, Treponema pallidum in the chancre can usually be recognized at least for several days, although most of the motility is lost within a comparatively short time after collection. If the capillary tube is held in a horizontal position and allowed to touch the drop of fluid that has collected on the chancre, nearly all the fluid will enter the tube by capillary action. The tube is then sealed by pressing the ends into wax; the one through which the fluid entered should be closed first. While this is being done, the tube should be held in a horizontal position so that the serum or plasma will not run out.

If an antiseptic or other local treatment has been administered, a salt solution compress may be applied and the patient instructed to return on successive days for the collection and dark-field study of specimens. At least 3 consecutive daily studies should be made when the findings are negative before concluding that T. pallidum is not present in the lesion. If the regional glands are enlarged a specimen may be collected for dark-field study from one of them by injecting a small amount of sterile saline into the lymph node and then aspirating with the syringe. (See Appendix - The microscope and micrometry for the technique of dark-field examination).

Certain of the spirochetes found in nonsyphilitic lesions of the mouth resemble Treponema pallidum so closely that the results of microscopic examination of fluid containing exudate from the mouth are of questionable diagnostic significance.

Whenever examinations are made for Treponema pallidum, a specimen of the patient's blood should be collected for serological tests.

When suspicion of chancroid (soft chancre) exists, the material from the lesions should, if facilities permit, be inoculated immediately into tubes of coagulated and inactivated sterile rabbit's blood according to the method of Teague and Deibert. "A rabbit is bled from the heart with a sterile 20 cc. syringe and the blood is distributed in amounts of 1 cc. in small test tubes, a little larger than the ordinary Wassermann tube. The blood is allowed to clot at room temperature and is then heated for five minutes at 55 degrees C. It can then be preserved in the ice box or can be used immediately. Equally good results can be obtained when the tubes are kept in the ice box for 3-4 days before use without heating".

"Pieces of stiff iron wire, gauge 18, about 5-1/2 inches long, are bent upon themselves at one end for about 1/8 inch. Ten or twelve of these wires are placed in a 6 inch test tube and are heated in the dry sterilizer. The patient removes the dressing and a bit of the pus is picked up with the bent end of the wire, the latter having been first rubbed gently over the base of the ulcer or under its undermined edge. The pus is then transferred to a tube of clotted blood and distributed in the serum by passing the wire around the clot. A second tube is prepared in the same way. After 24 hours incubation at 37 degrees C. the serum around the clot is thoroughly stirred with a platinum loop and a smear is made. Examination with the oil-immersion lens shows characteristic chains of small gram-negative bacilli, sometimes in pure culture, sometimes in mixed culture. The organism is usually so characteristic that such an examination is sufficient basis for positive diagnosis. Even when antiseptic powder or ointments have been applied, repeated positive cultures have been obtained by finding a bit of pus free from drug. It is not even necessary to wash the ulcer before taking cultures".

Isolations can subsequently be made by streaking blood agar plates from the clotted blood tubes. The plates should be incubated in the candle-jar. Upon such plates isolated colonies appear, usually after 48 hours. They are small, transparent and gray and have a rather firm, finely granular consistency. The

colonies rarely grow larger than pinhead size and have no tendency to coalesce. At room temperature the cultures die out rapidly. Kept in the incubator, however, they may remain alive and virulent for a week or more.

When cultures of specimens from the genito-urinary tract fail to yield positive results on candle-jar incubation, in spite of the detection of organisms in the specimen by direct smear, the presence of anaerobic or micro-aerophilic organisms should be suspected. If the original material has been saved in the refrigerator it may be replated and incubated anaerobically, but this procedure is not entirely satisfactory as many anaerobes die on exposure to air over a period of a few hours. To save time it is advisable to incubate both aerobically and anaerobically any material which may contain anaerobic organisms. The putrid smell which commonly characterizes anaerobic infections is always an indication for an anaerobic culture. Material from cases of abortion or post-partum infections and deep traumatic and pelvic abscesses associated with genito-urinary disease should be cultured aerobically and anaerobically. Anaerobic cultures should be made by streaking a blood agar plate and incubating it preferably by Mueller's modification of Rosenthal's chromium sulfuric acid technique (Appendix) or another available anaerobic technique. By inoculating a freshly heated and cooled tube of semi-solid agar (0.3% agar) the cultivation of microaerophilic organisms is facilitated.

SPECIMENS TO BE STUDIED FOR SPIRILLUM MINUS, STREPTOBACILLUS MONILIFORMIS, ACTINOMYCES, SPIROCHETES, RICKETTSIAE, VIRUSES AND FUNGI.

RAT-BITE FEVER - is an infectious disease said to be caused by an organism resembling somewhat a spirochete, the Spirillum minus, formerly named Spirochaeta morsus muris now considered definitely not a spirochete but a small motile bacillus. The definite diagnosis of rat-bite fever should be based upon the demonstration of the Spirillum by laboratory tests. White mice and guinea pigs may be inoculated with the patient's blood, exudate from the initial lesion, serum expressed from the exanthematous patches, material aspirated from the lymph nodes, or ground-up pieces of tissue excised from lesions. These animals are very susceptible, developing infection when inoculated with very few organisms, and pass thru characteristic stages of the disease, with the spirilla in their blood. Since white mice may harbor this organism it is necessary to determine that these animals are free from Spirilla before inoculations are made and as a control to inject the same material into guinea pigs.

Examination of the blood and the exudate from lesions by darkfield illumination or by stains may be done. The organism has rarely been detected in the blood of man with certainty although it may be found in material from the lesions. For staining, Wright's and Giemsa's stains are satisfactory.

Some cases of rat-bite fever are probably due to Streptobacillus moniliformis. This organism is a gram-negative, pleomorphic bacterium, occurring as short rod-shaped forms or as elongated filaments which may show characteristic fusiform enlargements. Mice are susceptible to experimental inoculation and

develop either a rapidly fatal general infection without focal lesions or a more slowly progressive disease with swelling of the feet and multiple inflammatory lesions of joints.

In human infections the organism may be isolated by blood culture and from the joint fluid in cases with arthritis. For cultivation, 20% serum broth or 20% serum agar may be employed, using the basic medium (Appendix) as a base. In broth the organism grows in clumps and may easily be missed. When surface growth on agar is sought, the agar plates should be incubated in a jar, preferably the candle-jar. For a description of the organism, its growth requirements and cultivation, the reader is referred to the articles by F.R. Heilman (J. Infect. Disease, 1941, vol. 69, pages 32-51), and Brown, T.M. and Nunemaker, J.C. (Bull. Johns Hopkins Hosp., 1942, 201-328). The latter suggest the following procedure for cultivation of the organism: Mix gently 10 cc. of sterile 2.5% sodium citrate and 10 cc. of the patient's blood. Centrifuge at high speed for thirty minutes. Discard the supernatant. Add one or two cc. of the sedimented cells to each of two tubes of basic broth (or tryptose phosphate broth) containing 20 per cent ascitic fluid or horse serum. Incubate at 37° C. for 2 or 3 days and examine daily for growth. Streptobacillus moniliformis produces a "fluff ball" growth on the surface of the sedimented cells. If growth is not observed after three days of incubation, transfer 0.5 cc. of the original culture to fresh horse serum or ascitic fluid broth and continue this blind passage for several days.

Injection of suspected material into the foot pads and peritoneal cavity of the mouse has also been employed for the isolation of Streptobacillus moniliformis. Swelling of the feet and joints and bumps on the tail are produced in from 4 to 7 days.

ACTINOMYCES - Pus from abscesses and draining sinuses and sputum (from lungs), in cases of suspected actinomycosis, should be examined grossly for "sulphur granules" which are small, round, yellow bodies, pinhead in size. With a sterile inoculating needle separate several such granules from the rest of the material. Place a "sulphur granule" in a drop of broth on a slide and crush carefully under a cover slip. Examine under the high dry and oil immersion objectives for radiating masses of branched filaments with clubbed ends. Make a Gram-stained preparation of a crushed granule. Look for slender, gram-positive branching filaments with clubbed ends. Actinomyces cultures may be differentiated microscopically from fungi in that they form filaments which are very slender, their width being no greater than that of the average gram-positive rod-shaped bacterium. Smaller bacterial forms may also be observed when these actinomyces are examined under the microscope. The non-pathogenic aerobic actinomyces tend to produce flat, tenacious colonies with a powdery surface. While fungi are capable of growth on acid media, the actinomyces require the usual nearly neutral reaction of ordinary culture media for satisfactory growth.

For cultivation of Actinomyces bovis and Actinobacillus actinomycetemcomitans, a tiny gram-negative coccobacillus which is not seen in original preparations but which is usually found accompanying Actinomyces bovis cultures, the pus should be freed as much as possible from contaminating bacteria. Successful cultivation usually depends upon the degree of freedom from contamina-

tion with other bacteria. A "sulphur granule" should be washed in sterile saline or broth and then crushed. If the "sulphur granules" are not found cultures must nevertheless be made. A blood agar plate should be streaked and a tube of basic broth or tryptose phosphate broth containing 20 per cent serum or ascitic fluid, inoculated. All cultures should be incubated anaerobically for 4-5 days although very slow growth may be obtained under aerobic or microaerophilic conditions.

In addition to the anaerobic Actinomyces bovis there are pathogenic aerobic Actinomyces: those associated with Madura foot and acid-fast and non-acid-fast species.

Madura foot or mycetoma occurs chiefly in tropical countries and is particularly prevalent where the inhabitants go barefooted. Two clinical types of the disease have been described, the distinction being based on the color of the granules in the diseased tissue (1) white or yellow granules and (2) black granules. Infections with white and yellow granules are attributed to Actinomyces, but in some cases of "white mycetoma" may be produced by fungi. (See section on fungi).

Actinomyces madurae is readily cultivated on ordinary media under aerobic conditions. The growth is mealy, wrinkled and ordinarily creamy white in color, although at times a red color may be observed. The aerial mycelium is scant and powdery and the mycelium undergoes fragmentation in older cultures.

A number of acid-fast actinomyces have been isolated from lesions in animals and man. These species are similar in their morphological, cultural and pathogenic properties.

The most common acid-fast species infecting man is A. asteroides which produces purulogranulomatous lesions, and has been isolated from pulmonary infections and from brain abscesses. A. gypsoides, a similar-appearing species, but more actively proteolytic, has been isolated from pulmonary infections in man. In the lesions the organisms appear as rods and filaments with well-marked branching. In cultures the organisms have a bacillary or coccoid shape, are pleomorphic and show metachromatism. They are not as strongly acid-fast as the tubercle bacillus and this property tends to disappear under cultivation.

The organisms grow more readily on laboratory media and are more virulent than A. bovis. Because of the relatively slow growth and the presence of contaminating organisms, intra-abdominal inoculation of guinea pigs with the infective material furnishes the most convenient method of isolation.

Actinomyces hominis, a saprophytic non-acid-fast aerobic form present in grain and grasses, has been isolated from actinomycotic lesions in man. It is considered by some as nonpathogenic and a secondary contaminant. It resembles in growth the typical actinomycetic culture. For more detailed account of actinomyces see Topley and Wilson, Principles of Bacteriology and Immunity 1938.

In addition to the fungi and actinomyces there are actinomyces-like organisms which are usually isolated from tonsils, from lung or pleural fluid cultures or from cases of lung abscesses, bronchiectasis or pneumonia. They are frequently highly pleomorphic showing short, slender, gram-positive bacillary and coccoid forms and long filaments with irregular swellings. They are usually gram positive, non-acid-fast and nonmotile. They grow well on blood agar but not on Sabouraud's agar and some strains require aerobic and others anaerobic conditions for isolation. Examples of such organisms are Act. necrophorus, rarely encountered in human infections, and Actinomyces pseudonecrophorus which is occasionally found in puerperal sepsis. These are long, slender, filamentous, and branching, and also show short coccoid and bacillary forms. They are nonmotile and nonsporulating and are strict anaerobes, dying on exposure to air for as short a time as 40 minutes. Act. pseudonecrophorus differs from Act. necrophorus in that it fails to hemolyze blood agar plates and to ferment lactose.

Erysipelothrix rhusiopatheae is a slender, nonmotile, non-spore-forming, gram positive rod, 0.5-1.5 microns in length, occurring singly and in chains and at times showing branching. It is the causative agent of swine erysipelas, an acute infectious disease in young pigs, which is particularly prevalent in Europe. The infection is contracted by man through contact with diseased animals, and also with crabs and fish with which the erysipelothrix is associated saprophytically. Most of the cases in man in the United States occur among fish handlers and is known as erysipeloid. Ready growth is obtained on the usual culture media.

THE SPIROCHETES - (See above under genito-urinary tract specimens for examination for syphilitic infection, and also section on spirochetes. See index). The spirochetes of relapsing fever may be detected during the febrile stage of the disease in ordinary or thick films of the patient's blood, stained for a prolonged period of time by Wright's or Leishman's blood stains or by dilute carbol fuchsin. They also may be detected microscopically by means of a dark-field study of freshly prepared films of the patient's blood. If they are not found by these means, several mice should be inoculated with the blood. Within 24-48 hours the spirochetes, if present, may be found in the blood of the mouse on dark-field study of blood obtained from the mouse's tail by snipping off a small distal portion with a knife. For demonstrating them in tissue sections, silver impregnation methods (See Appendix) may be used.

The spirochetes of the genus Leptospira are slender, highly flexible filaments with closely wound regular spirals and tapering extremities often bent in the form of a hook. In addition to their morphology, the Leptospirae are characterized by their resistance to saponin and distilled water, their peculiar lashing movements and dissolution by bile salts.

Two important species of the genus Leptospira have been associated with disease in man: L. icterohemorrhagiae, the cause of infectious jaundice (Weil's disease), and L. hebdomadis, the causative agent of Japanese seven-day fever (Nanukayami).

For the diagnosis of infectious jaundice during the first week of the disease, young guinea pigs should be injected intra-abdominally with 5 cc. of the

patient's blood. In positive cases death of the guinea pig will occur in ten days with characteristic fever, jaundice, and hemorrhages in the lungs, serous membranes and muscles. The liver, lung and kidney of the guinea pig may be examined for the leptospirae. After the first week of the disease, the centrifugalized urine sediment of the patient should be examined with the darkfield. Because these leptospirae are said to have an atypical appearance in the urine, care must be taken to differentiate them from other spirochetes. If the urine is strongly acid or alkaline the leptospirae are destroyed. The urinary sediment may be injected into guinea pigs in the same manner as the blood. In the examination of rats, the kidney is macerated, examined by darkfield technique, and injected into guinea pigs.

The highly specific agglutination reaction (See section on serological methods) becomes positive after the eighth or tenth day of the disease. Agglutination in a titer of 1:300 is considered by some to be diagnostic of Weil's disease. By the end of the third week high agglutinin and lysin titers are demonstrable.

Early in the disease blood cultures may be taken using special media (Schuffner's medium) (See Appendix).

A few cases of Weil's disease appear to be caused by Leptospira canicola, a species that is normally a parasite of the dog. It closely resembles Leptospira icterohemorrhagiae but differs in its antigenic structure and in its lower virulence for guinea pigs.

In seven-day fever, which is widely distributed in Japan, injection of the patient's blood into guinea pigs results in the death of about two thirds of the animals in 6-15 days. There is enlargement of the lymphatic glands and slight jaundice, and the organisms are found in the liver. The organism is differentiated from other leptospirae by animal inoculation and by serological tests. (Ref. Topley and Wilson, 2nd Edition).

RICKETTSIAE - Specimens for the study of rickettsiae may be patient's blood, material from a skin ulcer, biopsy of hemorrhagic skin lesions, and material from autopsy. The bodies of arthropods (ticks, lice, fleas and mites) or material from an animal reservoir host may also be studied for the presence of disease-producing rickettsiae. Early inoculation of a guinea pig with the specimen is advisable. Smears for Giemsa staining, animal inoculation and tissue culture are the diagnostic bacteriological methods employed. In the rickettsial diseases of man, as with many bacterial diseases, evidence of infection is obtained by serological studies on blood serum. (See sections on rickettsiae and serological procedures for details).

VIRUSES - Material for study of the presence of viruses may be nasopharyngeal washings, feces, blood, material from skin lesions, autopsy material and the insects or animal hosts involved in the spread of the particular disease. (See section on viruses for general discussion and details as to the handling of specimens, etc.).

FUNGI - In addition to the foregoing references to fungi see section on fungi for technique of skin scrapings, study of infected hairs, etc.

SUMMARY OF PROCEDURES FOR DETECTION AND ISOLATION OF INFECTIOUS AGENTS

1. All specimens are streaked on blood plates and smeared for Gram staining. (Stools may be omitted).

2. MacConkey's, Endo's or eosin-methylene blue agar plates should also be streaked when stool, urine, bile, peritoneal fluids and other specimens of gastro-intestinal origin are to be cultured.

3. Stools should also be plated on Difco's SS agar and, if typhoid fever is suspected, on bismuth-sulfite agar.

4. Dysenteric stools may be plated on SS agar in addition to blood agar, MacConkey's, Endo's or desoxycholate-citrate agar, etc.

5. Reduced tension blood and chocolate or "combination" blood agar slants and chocolate or "combination" blood agar plates may be prepared from vaginal, urethral, eye, cerebrospinal fluids, etc., in which the presence of the gonococcus or meningococcus is suspected. The addition of 1:5,000,000 crystal violet to the media will prevent overgrowth by gram positive organisms.

6. Eye, ear, nose, throat, cerebrospinal fluids, etc., in which the presence of Pfeiffer's bacillus (Koch-Week's) (H. influenzae) is suspected, should be streaked on chocolate agar plates or better, "combination" blood agar.

7. Diphtheritic specimens are inoculated on Loeffler slants or on tellurite chocolate agar.

8. A special blood agar potato glycerine medium is required for the isolation of H. pertussis.

9. For the isolation of Rickettsiae and viruses special media containing living tissue are required. For the suitable study of these and the spirochetal organisms early animal inoculation is usually necessary.

10. Fungi are cultured on Sabouraud's medium and actinomyces on plain or blood agar.

11. All blood and chocolate blood agar plates are incubated in the "candle-jar". The candle-jar technique is used especially for cultures whose growth is dependent upon the presence of carbon dioxide.

12. Where the presence of anaerobes is suspected, plates and other cultures should be incubated in an anaerobic jar containing carbon dioxide. This may be accomplished by the use of Mueller's modification of Rosenthal's technique or by evacuation of the jar and filling it with hydrogen and about 10% carbon dioxide, etc. (See Appendix).

13. For the isolation of members of the **Brucella** group, prolonged incubation in 5 to 10% carbon dioxide and repeated transfer of early invisible growth to fresh, moist blood agar plates may be necessary.

IDENTIFICATION OF THE CAUSATIVE AGENTS OF INFECTIOUS DISEASE PROCESSES

A. BACTERIA.

B. SPIROCHAETES.

C. RICKETTSIAE.

D. VIRUSES.

E. FUNGI.

(A) THE IDENTIFICATION OF BACTERIA.

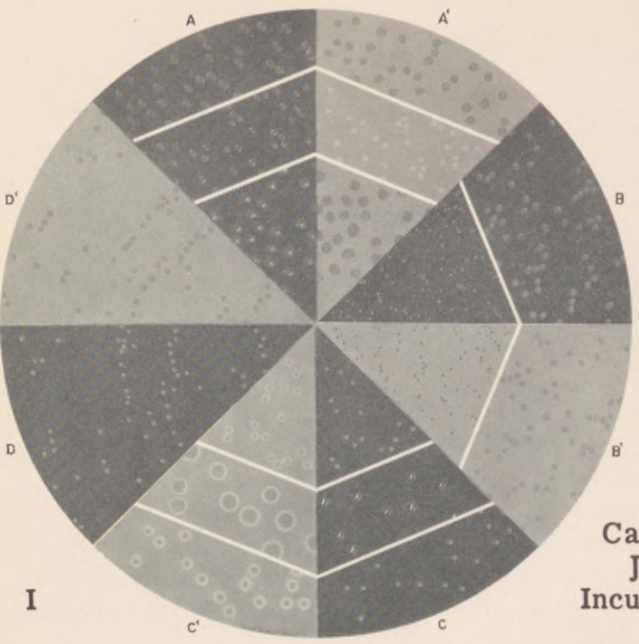
The identification of bacteria rests upon a number of characteristics - morphological, staining, serological, biochemical and pathogenic. The growth requirements and gross appearance of bacterial growth may also be employed for this purpose. To identify an unknown organism, aside from morphology and reaction to the Gram stain, such characteristics as the aerobic or anaerobic requirements of the organism, its motility or lack of motility, the presence or absence of spores and capsules, pathogenicity and acid-fastness (i.e., reaction to the Ziehl-Neelsen stain) are employed. Carbohydrate fermentation, the digestion of proteins, and reactions with specific antisera are of great importance in the differentiation of some species. The presence or absence of pigment, the type of growth on blood agar, in broth, in milk, etc., are also important differential characteristics. (See Table 2). For identification according to the most accepted classification - that described in Bergey's Manual of Determinative Bacteriology (5th Edition), a knowledge of the nomenclature employed in this scheme is essential. Having determined the characteristics described above, it is a simple matter to find the position of the organism in this accepted classification.

In the diagnostic laboratory, the bacteriologist begins his study of a specimen by microscopic examination and the streaking of plates of selected culture media. After incubation of the plates for 24 hours there is usually sufficient growth to enable him to make a tentative diagnosis from the appearance of the colony and the Gram stain. For example, Staphylococcus aureus may usually be identified by the opaque, yellow, moist, raised, smooth and convex colony it produces on the surface of agar, together with the cluster of gram-positive cocci presented in the Gram-stained smear of the colony. The presence of a member of the Eberthella-Shigella-Salmonella groups may be suspected from the type of colony produced on the differential and restrictive media used in the culturing of specimens from the gastro-intestinal tract. The type of colony and the action on blood as observed on the blood agar plate, together with the Gram stain, may in themselves be sufficient to indicate the presence of members of the group of chain forming cocci (streptococci). The failure of organisms to grow on ordinary aerobically incubated media may indicate that an anaerobe is present, etc.

Since the study of an organism is conveniently begun by a study of its morphological and staining properties, the bacteria are described in this manual in four groups set apart on the basis of morphological characteristics and reaction to the Gram stain (gram-positive cocci, gram-positive rods, etc.). Some of the genera frequently encountered in the diagnostic laboratory are divided in Table 3 according to this system of grouping, while the most frequently encountered species, their names and the diseases caused by them are presented in Table 4.

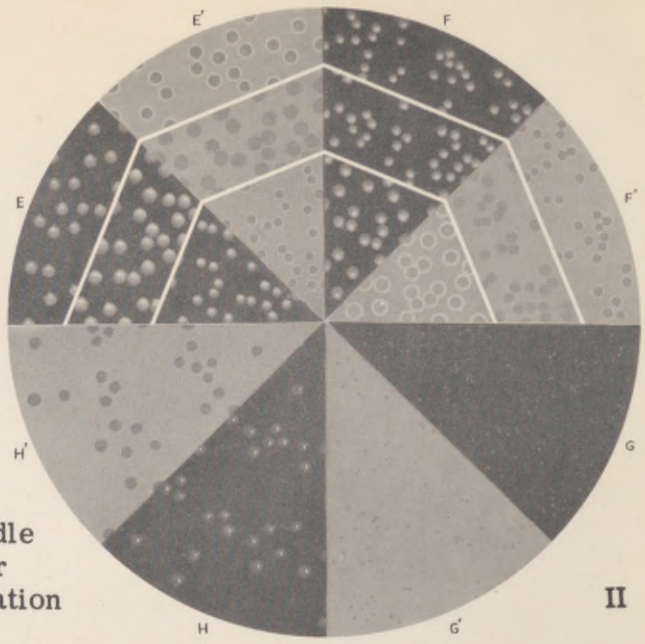
The methods for identification of the genera and species are presented in detail in the following pages for each of the four groups. In using this manual, therefore, one should place the unknown organism in one of the four groups according to its morphology and reaction to the Gram stain, and then study it following the procedures indicated for the group. It should be borne in mind, however, that whereas certain routine procedures can be laid down and followed,

COLONY MORPHOLOGY OF BACTERIA ON BLOOD AGAR



I

Candle Jar Incubation



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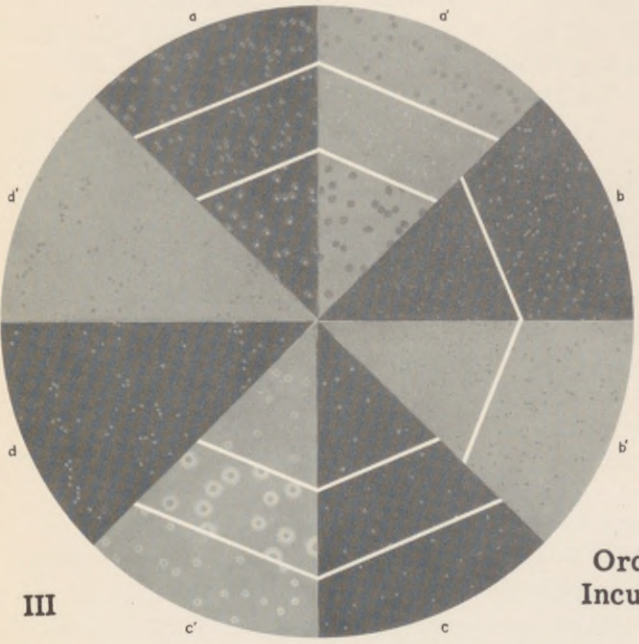
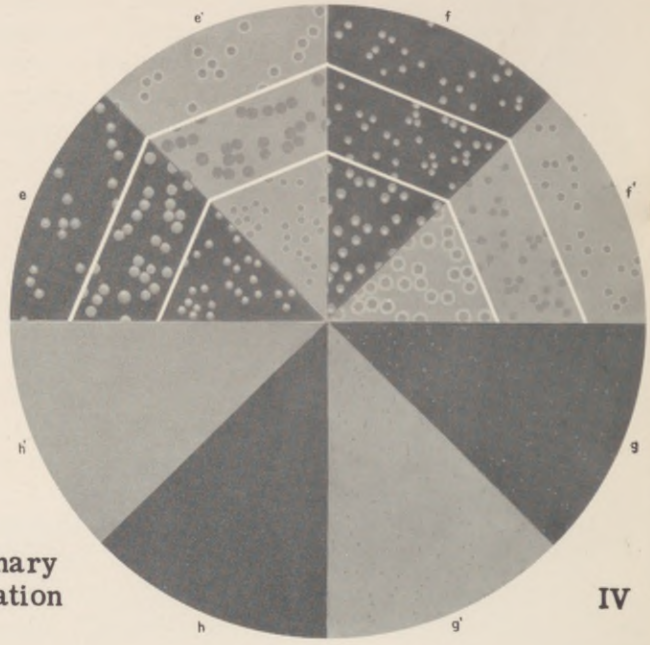
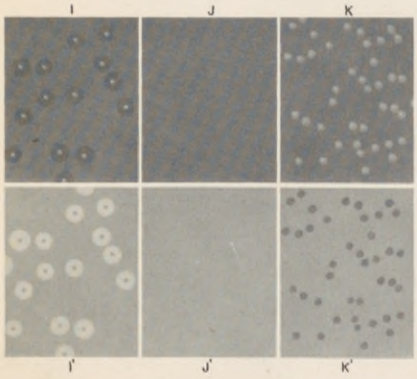


III

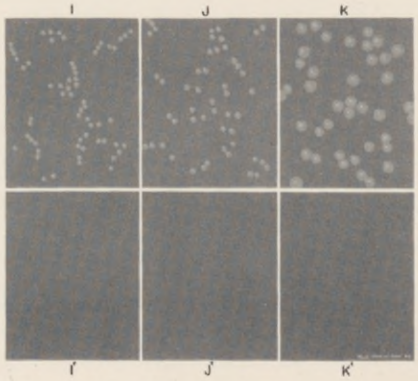
Ordinary Incubation



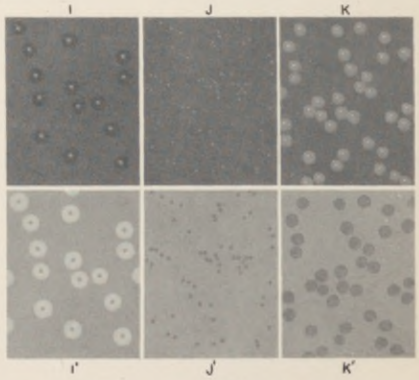
IV



Blood Agar



Chocolate Agar



Combination Agar

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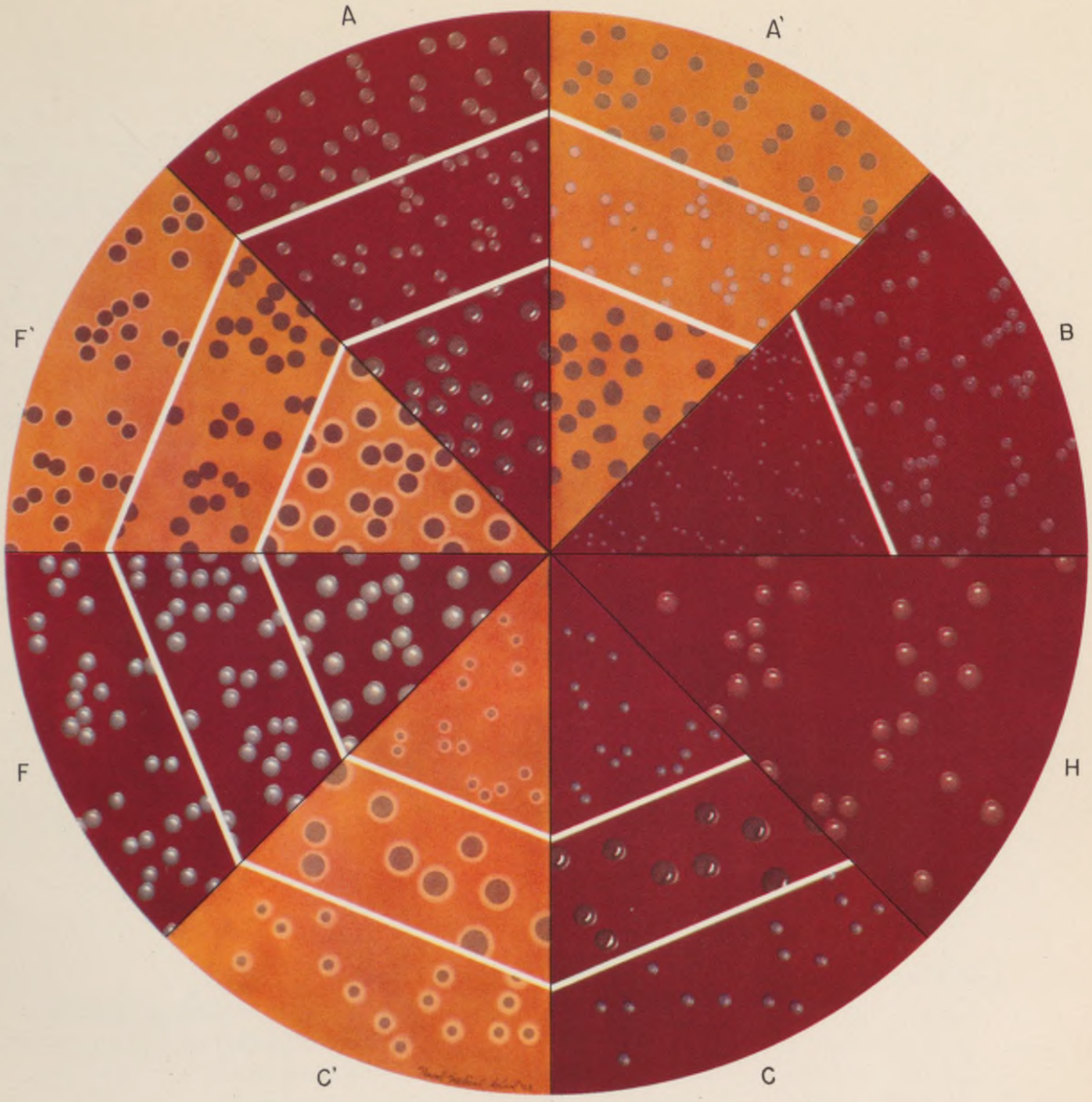
COLONY MORPHOLOGY OF BACTERIA ON BLOOD AGAR



On the opposite page are colonies by reflected light (A,B,C, etc.) and transmitted light (A',B',C', etc.), with and without candle jar incubation (small letters indicate incubation without candle jar).

The RECTANGULAR segments show three species on blood, chocolate, and "combination" agar by reflected (I,J,K) and transmitted (I',J',K') light after 24 hours of incubation in the candle jar.

The COLOR PLATE includes representative sectors selected from the black and white plate.

Key:

A-A'-a-a'	Pneumococcus (Inner segment-type III).
B-B'-b-b'	Streptococcus viridans.
C-C'-c-c'	Streptococcus hemolyticus.
D-D'-d-d'	Streptococcus (nonhemolytic).
E-E'-e-e'	Staphylococcus albus (Inner and outer segments show slight hemolysis).
F-F'-f-f'	Staphylococcus aureus (Inner and outer segments show slight hemolysis).
G-G'-g-g'	Brucella abortus.
H-H'-h-h'	Meningococcus.
I-I'	Hemolytic streptococcus.
J-J'	Hemophilus influenzae.
K-K'	Gonococcus.

one must at times be guided also by the type of specimen and the clinical and epidemiological data in the choice of procedure for the isolation and identification of organisms. Furthermore, organisms will be encountered whose identification may require studies not indicated in this manual and the use of a much more inclusive manual such as Bergey's. This will be especially true of the non-pathogens.

TABLE 3

	Gram negative		Gram positive
Coccus	(Neisseria		(Staphylococcus
			(Streptococcus
	(Escherichia	Cocci	(Sarcina
	(Salmonella		(Gaffkya
	(Eberthella		(Diplococcus
	(Shigella		
	(Klebsiella		(Corynebacterium
	(Brucella		(Mycobacterium
Rods	(Pasteurella	Rods	(Bacillus
	(Proteus		(Clostridium
	(Pseudomonas		
	(Hemophilus		
	(Malleomyces		
	(Fusobacterium		
	(Vibrio		

THE GRAM-NEGATIVE RODS

Coli-typhoid group (Escherichia, Aerobacter, Eberthella, Shigella, Salmonella) and Proteus.
(Morganella)
Slow lactose-fermenters
Alkaligenes-Pseudomonas-Serratia
Vibrio
Pasteurella
Brucella
Hemophilic bacteria
Malleomyces
Bacteroides

The presence of most of these organisms may be suspected when the source of the material studied is the gastro-intestinal tract or less frequently the genito-urinary tract. Differential plating media permit the detection of colonies of the members of the Escherichia-Aerobacter groups on one hand and of the Eberthella-Salmonella-Shigella groups on the other hand. Some highly differential and restrictive media like Bismuth-sulfite agar, SS agar, and desoxycholate-citrate agar especially favor the Eberthella-Salmonella and the Shigella groups. Desoxycholate-citrate agar restricts the growth of some strains of Shigella sonnei and it

TABLE 4

Common Name	Old Scientific Name	New Scientific Name Bergey's Manual(5th Edit.)	Human Disease Caused
Gonococcus	Micrococcus gonorrheae	Neisseria gonorrheae	Gonorrhea
Meningo-coccus	Micro. intracellularis (meningococcus)	Neiss. intracellularis	Epi. C.-S. Meningitis
----------	Micrococcus catarrh-alis	Neisseria catarrhalis	----------
Pneumococcus	Diplococcus pneumoniae	Diplococcus pneumoniae	Lobar pneumonia
Strep. hemo-lyticus	Streptococcus pyogenes	Streptococcus pyogenes †	(Wound infection, (Scarlet fever, (Erysipelis, (Sore throat.
Strep. viridans	Streptococcus viridans	Strep. salivarius *	----------
Staphylococcus	Staph. pyogenes aureus	Staphylococcus aureus	Pus infection
	Staph. pyogenes albus	Staphylococcus albus	Usually non-path.
	Staph. pyogenes citreus	Staphylococcus citreus	----------
	Micrococcus tetragenus	Gaffkya tetragena	----------
Cholera vibrio	Spirillum cholerae asiaticae	Vibrio comma	Asiatic cholera
----------	Bacillus prodigiosus	Serratia marcescens	None
----------	Bacillus pyocyaneus	Pseudomonas aeruginosa	Wound contamin-ant.
Colon bacillus	Bacillus coli-communis	Escherichia coli	----------
	Bacillus coli-communior	Escherichia communior	----------
	Citrobacter freundii	Escherichia freundii	----------
----------	Bacillus lactis aero-genes	Aerobacter aerogenes	----------
----------	Bacillus proteus vul-garis	Proteus vulgaris	----------
Paratyphoid A	Bacillus paratyphosus A	Salmonella paratyphi	Paratyphoid fever
Paratyphoid B	Bacillus paratyphosus B	Sal. schottmulleri	Paratyphoid fever
Paratyphoid C	Bacillus paratyphosus C	Sal. hirschfeldii	Food poisoning
Bacillus en-teritidis	Bacillus enteritidis	Salmonella enteritidis	Food poisoning
Bac. of hog cholera	Bacillus suipestifer	Sal. choleraesuis	Food poisoning
Mouse typhoid bacillus	Bacillus aertrycke	Sal. typhimurium	Food poisoning
Typhoid ba-cillus	Bacillus typhosus	Eberthella typhosa	Typhoid fever
Shiga's ba-cillus	Bacillus dysenteriae	Shigella dysenteriae	Dysentery (bac.)

† Type species of beta-hemolytic streptococci.
* Type species of alpha-hemolytic streptococci.

TABLE 4 (Continued).

Common Name	Old Scientific Name	New Scientific Name Bergey's Manual(5th Edit.)	Human Disease Caused
Flexner's, etc. bacilli	Bacillus dysenteriae (var.)	Shigella paradysenteriae	Dysentery
----------	----------	Shig. sp.(Newcastle type)	Dysentery
Schmitz bacillus	Bacillus dysenteriae (Schmitz)	Shigella ambigua	Dysentery
Sonne bacillus	Bac. dysenteriae Sonne	Shigella sonnei	Dysentery
----------	Bac. alkaligenes fecalis	Alcaligenes faecalis	----------
Bang's bacillus	Bacillus abortus	Brucella abortus	Undulant fever
----------	Micrococcus melitensis	Brucella melitensis	Undulant fever
----------	----------	Brucella suis	Undulant fever
Friedlander's bacillus	Bac. mucosus capsulatus	Klebsiella pneumoniae	Pneumonia
Plague bac.	Bacterium pestis	Pasteurella pestis	Bubonic plague
----------	Bacterium tularense	Pasteurella tularensis	Tularemia
Pfeiffer's, Koch-Weeks bacillus	Bacillus influenzae	Hemophilus influenzae	Resp. infect.
Bordet-Gengou bacillus	Bacillus pertussis	Hemophilus pertussis	Whooping cough
Ducrey's bac.	Bacillus ducreyii	Hemophilus ducreyii	Chancroid
Morax-Axenfeld bac.	Bacillus lucanatus	Hemophilus duplex	Conjunctivitis
Hay bacillus	Bacillus subtilis	Bacillus subtilis	Contaminant
----------	Bacillus anthracis	Bacillus anthracis	Anthrax
Welch bac.	Bac. aerog. capsulatus	Clostridium perfringens	Gas gangrene
----------	Bac. oedem. atiens	Clostridium novyi	Malig. edema
----------	Bacillus botulinus	Clostridium botulinum	Botulismus
Tetanus bac.	Bacillus tetanus	Clostridium tetani	Tetanus(lockjaw)
----------	Vib. septique	Clostridium septicum	Malig. edema
----------	Bacillus sporogenes	Clostridium sporogenes	(Found in gas gang-
----------	Bacillus histolyticus	Clostridium histolyticum	(rene.
Koch's bacillus	Bacillus tuberculosis	Mycobact. tuberculosis var: hominis var: bovis	Tuberculosis
Lepra bacillus	Bacillus leprae	Mycobacterium leprae	Leprosy
Smegma bacillus	Bacillus smegmatis	Mycobacterium smegmatis	None
Klebs-Loeffler bacillus	Bacillus diphtheriae	Coryne. diphtheriae	Diphtheria
Diphtheroids	Bacillus xerosis	Corynebacterium xerosis	----------
Pseudodiph. bacillus	Bacillus hoffmanni	Coryne. pseudodiphtheri-cum	----------
Glanders bacillus	Actinobacillus mallei	Malleomyces mallei	Glanders

TABLE 4 (Continued).

Common Name	Old Scientific Name	New Scientific Name Bergey's Manual(5th Edit.)	Human Disease Caused
----------	Spirillum obermieri	Borrelia recurrentis	Relapsing fever
----------	Spirillum vincenti	Borrelia vincentii	Vincent's angina
----------	Spirillum duttoni	Borrelia duttonii	Relapsing fever
----------	Spironema novyi	Borrelia novyii	Relapsing fever
----------	Spironema carteri	Borrelia carteri	Relapsing fever
----------	Spironema berbera	Borrelia berbera	Relapsing fever
----------	Spironema kochii	Borrelia kochii	Relapsing fever
----------	Spirochaeta refringens	Borrelia refringens	Genitalia of man
----------	Spirochaeta pallida	Treponema pallidum	Syphilis
----------	Spirochaeta pertenue	Treponema pertenue	Yaws
----------	Leptospira ictero-hemorrhagica	Leptospira icterohemorr-hagica	Weil's disease
----------	Spirochaeta hebdomadis	Leptospira hebdomadis	Seven day fever in Japan.
----------	Bacterium pneumosintes	Dialister pneumosintes	----------

is, therefore, essential to use also one of the following: MacConkey's, eosin-methylene blue agar, Endo's, or desoxycholate agar without the citrate. By using Difco's SS agar it is possible to restrict the growth of coliform organisms and yet obtain growth of the more fastidious members of the Eberthella, Salmonella, and Shigella groups. For the appearance of the colonies on these differential media see the section on the handling of specimens from the gastro-intestinal tract, and the section on media in the Appendix.

On blood agar the colonies of this group are fairly large, gray, translucent and of alpha beta or gamma type (with respect to hemolysis).

If differential plating media have been employed it is usually possible to diagnose the presence of Esch. coli from its characteristic appearance on MacConkey's, on Endo or on eosin-methylene blue agar. If it is desired to confirm the presence of this organism, sugar fermentation studies must be made. With experience, the presence of Aerobacter aerogenes, which like coli ferments lactose and consequently gives a similar color change, may be suspected from the moist and more abundant growth obtained. To differentiate this organism from Bact. coli, certain biological characteristics must be studied.

The small colorless colonies that are obtained on the differential media are suggestive of members of the Eberthella-Salmonella-Shigella groups. Sugar fermentation, motility, biological and serological characteristics are employed for their differentiation.

Since rapid bacteriological diagnosis is desired, the procedure to follow in the identification of the organism is to inoculate as soon as possible, as many types of medium that may be of help. A colony should be fished from the original plates

into a tube of peptone water and incubated at 37 degrees C. Four or five hours later, or as soon as a turbidity is evident, one drop of the peptone culture is added to the necessary media.

The sugars lactose and dextrose are of considerable value for differentiation of these organisms as indicated in Table 5.

TABLE 5.

	Lactose	Dextrose	Motility
Escherichia-Aerobacter	A + G	A + G	+ -
Salmonella	-	A + G	+
Eberthella	-	A	+
Shigella	-	A	-

A = acid formation; G = gas formation.

Thus for the confirmation of the presence of Esch. coli, inoculation of only a tube of lactose or of a tube of Russell's double sugar slant is usually sufficient. If it is desired to differentiate between Esch. coli and Aerobacter aerogenes the differential studies indicated in the following table may be made. (Table 6.)

TABLE 6.

Organism	Lactose	Dextrose	Sucrose	Mannite	V. - P.*	Methyl Red	Malonate	Gelatin	Citrate	Indole
Esch. coli communis	AG	AG	-	AG	-	+	-	-	-	+
Esch. coli communior	AG	AG	AG	AG	-	+	-	-	-	+
Aero. aerogenes	AG	AG	AG	AG	+	-	+	-	+	-
Proteus	-	AG	AG 1-5 days	-	-		+	+	+	- +
Aerobacter cloacae	AG	AG	AG	AG	+	-		+	+	

AG = Acid & Gas.
* V. - P. = Voges-Proskauer test.

Thus the use of the Voges-Proskauer test (formation of acetyl methyl carbinol), the methyl red test, the malonate test and growth on citrate may serve to differentiate between Escherichia coli and Aerobacter aerogenes. Aerobacter cloacae differs from Aerobacter aerogenes in that it slowly liquefies gelatin. The Proteus group, the motile members of which have a tendency to spread over the surface of moist agar liquefies gelatin and usually fails to attack lactose.

In routine work, for the differentiation of Aerobacter from Escherichia, the inoculation of Bacto-M.R.-V.P. medium for the performance of the Voges-Proskauer and methyl red tests usually suffices. (See Appendix).

Markedly spreading growth on moist agar, of a motile gram-negative pleomorphic rod and a musty odor permit a tentative diagnosis of the presence of Proteus. If confirmation is desired, lactose, sucrose, dextrose, mannite and gelatin may be inoculated. Fermentation of the sucrose may take as much as five days incubation. (Proteus X 19 produces indole and liquefies gelatin. Proteus XK gives a negative result with these tests).

The ability of Proteus strains to decompose urea and to form only a slight amount of gas in fermentable carbohydrates are considered two of their major characteristics (Bergey, 5th Ed.; Rustigan and Stuart, Proc. Soc. Exp. Biol. & Med., 1941, 47, 108-112; J. Bact., 1943, 45, 198-199), and according to some workers the ability to ferment mannitol should exclude organisms from this group (St. John-Brooks, R., and Rhodes, M., Third Internat. Cong. for Microbiol., Rept. of Proc., 1939, p. 167). For the detection of urea decomposition the medium and method described by Rustigan and Stuart may be employed (see section on techniques and special procedures).

If the differential plating media indicate the presence of Eberthella, Shigella or Salmonella (colorless colonies on MacConkey's, Endo's, eosin-methylene-blue, Bacto SS, desoxycholate or desoxycholate-citrate agar, or colonies resembling Eberthella typhosa on bismuth-sulfite agar) media for the differentiation of these organisms should be inoculated for the studies indicated in Table 7.

The motility and indol studies indicated in Table 7 may be made from the peptone medium. It is apparent that the formation of gas in dextrose separates the salmonellae from the eberthellae and shigellae and that the motility of the Eberthella group sets it apart from the Shigella group. Failure to ferment mannite or to produce indol sets Shigella dysenteriae (Shiga's bacillus) apart from the Shigella paradysenteriae group (the Flexner group) and from Sh. dispar, Sh. sonnei and Sh. alkalescens. Shigella sonnei like Shigella dispar ferments lactose and sucrose slowly but differs from this organism with respect to xylose fermentation and indol production. Shigella alkalescens and Shigella (sp. Newcastle type) differ from the other members of this group in fermenting dulcitol although the latter organism may vary considerably in this respect. Some strains of Sh. (sp. Newcastle type) produce small amounts of gas in dextrose and dulcitol and a variant of this organism which produces acid and traces of gas in mannite is sometimes referred to as the "Manchester bacillus". Shigella ambigua is differentiated from Sh. dysenteriae through the formation of indol. Sh. dispar and Sh. sonnei differ from the Sh. paradysenteriae group in that the former two ferment lactose and sucrose slowly,

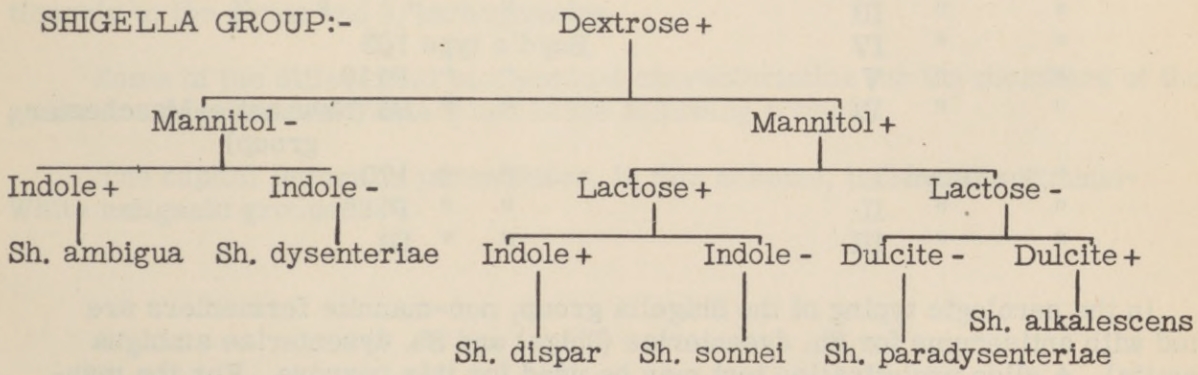
TABLE 7.

	Motility	Lactose	Dextrose	Sucrose	Mannite	Xylose	H_2S	Rhamnose	Indole	Dulcitol	Gelatin
E. typhosa	+	-	A	-	A	A/-	+		-	-	-
Sal. paratyphi (Para. A)	+	-	AG	-	AG	-	-		-	V	-
Sal. Para B group	+	-	AG	-	AG	AG	+		-	AG	-
Shig. dysenteriae (Shiga)	-	-	A	-	-	-	-	-	-	-	-
Shig. paradysenteriae (Flexner)	-	-	A	-	A	-	-	-	+	-	-
Shig. dispar	-	A 1-6 days	A	A 4-20 days	A	A	-	+	+	-	-
Shig. sonnei	-	A 2-30 days	A	A 4-32 days	A	-	-	+	-	-	-
Shig. alkalescens	-	-	A	-	A	A	-	+	+	A	-
Shig. ambigua	-	-	A	-	-	-	-	±	+	-	-
Shig. species (Newcastle Type)	-	-	A or AG	-	- or A	- or $A^{\pm}$	-	-	-	- or $A^{\pm}$ or AG(late)	-
(Manchester Type)	-	-	AG	-	AG				-	AG(late)	

A = acid.
G = gas.
V = variable.

* * * * *

while Sh. paradysenteriae do not attack these sugars. A scheme of the differential characteristics for the Shigella group is presented in the following diagram:



Shigella group.

Andrewes and Inman (Spec. Rep. Se. Med. Res. Coun., London, No. 42, 1919), studying the antigenic composition of about 200 strains of Shigella paradysenteriae, divided them into five types which they named, V, W, X, Y, and Z. While types V, W, X and Z were felt to possess type antigens of their own, Y was believed to contain a more or less equal mixture of the antigens, V, W, X, and Z with no type antigen of its own.

Boyd (Trans. Roy. Soc. Trop. Med. and Hyg., 1940, 33, 553-571) showed that more than a quarter of thousands of Sh. paradysenteriae organisms isolated in India possessed antigenic compositions different from the types described by Andrewes and Inman. Boyd also found that each member of the Andrewes and Inman types possessed a specific type antigen and a group antigen shared by all of the other types. It was found that the V, W, and Z were valid types, each having its own type antigen and varying quantities of a group antigen. Neither X nor Y were felt to be valid types - X neing considered an incomplete variant of Z and Y containing only the common group antigen. The antigen of Boyd's type 88 has been shown by Scott (Lancet, 1934, 2, 248) to be identical with that of the Newcastle and Manchester strains of dysentery bacilli.

On the basis of Boyd's suggestions a classification of the pathogenic Shigellae paradysenteriae into two groups has been established. One group contains the common paradysenteriae (Flexner) group antigen and is designated Sh. dysenteriae Flexner; the other does not contain this group antigen but possesses the paradysenteriae biochemical reactions and is designated Sh. dysenteriae Boyd. The English Army Medical Service has accepted Boyd's antigenic types for the Shigella paradysenteriae group. These types and their relationship to earlier designations are, as follows:

Serologic classification of the Sh. paradysenteriae

Boyd's new designation	Previous designation
Sh. dysenteriae Flexner I	Andrewes and Inman's V
" " " II	" " " W
" " " III	" " " Z
" " " IV	Boyd's type 103
" " " V	" " P119
" " " VI	" " 88 (Newcastle-Manchester group)
" " Boyd I	" " 170
" " " II	" " P288
" " " III	" " D1

In the serologic typing of the Shigella group, non-mannite fermenters are tested with antiserums for Sh. dysenteriae (Shiga) and Sh. dysenteriae ambigua (Schmitz). A slide agglutination test may be used for this purpose. For the mannite fermenters, preliminary testing against polyvalent sera is helpful. The English army uses three different sera for this purpose: 1) an anti-Sonne serum (see

Glynn and Starkey, J. Bact., 1939, 37, 315-331), 2) an anti Sh. Flexner I, II, and III Serum, and 3) an anti Sh. Flexner IV, V and anti Sh. Boyd I serum. The different types may be detected by using a series of monospecific serums which may be prepared by absorbing out the group agglutinins. By means of the slide agglutination test, rapid identification may be obtained. References: Boyd, J.S.K., Trans. Roy. Soc. Trop. Med. and Hyg., 1940, 33, 553-571; Weil, A.J., J. Immunol., 1943, 46, 13-46; Neter, E., Bact. Rev., 1942, 6, 1-36.

The Salmonella subcommittee of the Nomenclature Committee of the International Association of Microbiologists on the Genus Salmonella. 3rd International Cong. Microbiol. Proc.) define the genus Salmonella as follows: " A large genus of serologically related gram-negative bacilli.....showing with certain exceptions, a motile peritrichous phase in which they normally occur;.....failing to ferment sucrose or to clot milk and rarely fermenting lactose, liquefying gelatin or producing indol, they regularly attack glucose with, but occasionally without, gas production. All the known species are pathogenic for man, animals, or both." While this definition is inclusive it should be remembered that lactose fermentation, gelatin liquefaction and indol production are extremely rare characteristics of members of this genus. According to Edwards and Bruner (Univ. Kentucky Agr. Exp. St. Circular 54, 1942), any culture which ferments lactose, sucrose, salicin or adonitol, forms indol or liquefies gelatin should be excluded from the genus until antigenic analysis proves it a Salmonella. Motility, pathogenicity, hydrogen sulfide production and dulcitol fermentation, on the other hand, are properties commonly possessed by members of this genus.

Preliminary classification by cultural and biochemical studies with confirmation by antigenic analysis is generally considered the best procedure for the classification of the Salmonellae. The minor serologic relationship to the Salmonella group of various non-Salmonella organisms does not place them in the Salmonella group. Such a relationship has been found, for example, with coliform organisms which are slow lactose-fermenters. Similarly, variation in cultural and biochemical properties and the occurrence of non-pathogenic organisms having the cultural and biochemical properties of Salmonella, makes inadequate the dependence on these properties alone. Thus one should consider the following three characteristics for identification of an organism as a member of the genus Salmonella: 1) the cultural and biochemical properties indicated above as typical, 2) serologic relationship to the genus and 3) pathogenicity.

Some of the differential biochemical characteristics for the members of the genus are presented in Table 8 and in the following scheme.

The capital letters in parentheses, in this scheme, indicate Kauffmann-White antigenic groups.

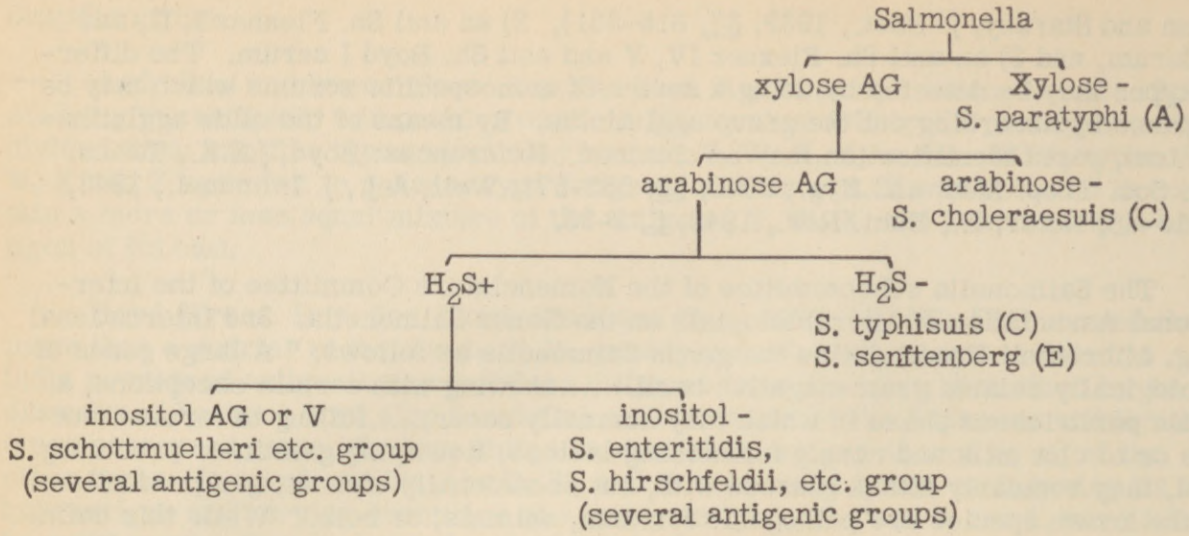


TABLE 8.

BIOCHEMICAL AND AGGLUTINATIVE CHARACTERISTICS OF THE MORE IMPORTANT SALMONELLAE

	Xylose	Arabinose	Lead Acetate (H_2S)	Inositol	Trehalose	d-Tartrate	Dextrose	Lactose	*Antigenic Group (after Bergey)
Sal. schottmuelleri (Para. B)	AG	AG	+	AG	AG	Alk	AG	-	B
Sal. typhimurium (Sal. aertrycke)	AG	AG	+	AG	AG	Acid	AG	-	B
Sal. enteritidis	AG	AG	+	-	AG	Acid	AG	-	D
Sal. choleraesuis	AG	-	-	-	-	Acid	AG	-	C
Sal. abortivoequina	AG	AG	-	-	AG	Acid	AG	-	B
Sal. hirschfeldii (Para. C)	AG	AG	+	-	V		AG	-	C
Sal. paratyphi (Para. A)	-	AG	-	-	AG	Alk	AG	-	A
Sal. typhisuis	AG	AG	-	-	AG	Alk	AG	-	C

Salmonella infections of man are of two main types: typhoid-like fevers which are occasionally called paratyphoid fever and acute gastro-enteritis which is often referred to as "Salmonella food poisoning" or "Salmonella food-infection". A third and less frequent type of infection is that seen in involvement of the urinary bladder, the pelves of the kidneys, the meninges and the appendix. The so-called paratyphoid fevers vary from a slight febrile or enteric disorder to a severe fever indistinguishable from typhoid fever. Salmonella food poisoning differs from enteric fever in its short incubation period, sudden onset, severe gastro-intestinal symptoms, marked prostration and short duration. In spite of the relatively severe symptoms, the fatality is low. The members of the genus which more commonly affect man are listed below.

S. paratyphi (Paratyphoid A) gives rise to typhoid-like infections. It is not the cause of food-poisoning.

S. Schottmuelleri (Paratyphoid B) produces a common form of typhoid-like infection in man. This organism may also cause acute gastro-enteritis.

S. hirschfeldii (Paratyphoid C) is the cause of a typhoid-like infection in man which is frequently complicated by suppurative lesions. It may also give rise to acute gastro-enteritis.

S. choleraesuis (S. suipestifer) is occasionally the cause of acute gastro-enteritis in man but much more frequently the cause of true enteric fever with invasion of the blood stream. Its natural habitat is the intestinal tract of the hog where it is found associated with hog cholera, a virus disease.

S. typhi-murium (S. aertrycke) usually produces a severe, acute gastro-enteritis, but may produce a typhoid-like infection. It porduces a natural typhoid-like disease in mice and other rodents and has also been recovered from diseased birds, pigs, and sheep.

S. enteritidis, of which there are several varieties, may produce infections in man, usually of the food-poisoning type. Rodents are probably its natural host.

It is evident from Table 8 that the latter three organisms (Salmonellae commonly involved in food poisoning) are members of different antigenic groups in the Kauffmann-White Diagnostic Scheme (see Edwards and Bruner, Univ. Ken. Agr. Sta. Circular 54, 1942, pp. 27-29; Bergey, 5th Ed., pp. 456-457). If specific agglutinating sera for these types are not immediately available, some information can be obtained with sera which are usually at hand (i.e., Salmonella schottmuelleri and Salmonella enteritidis sera). Schottmuelleri serum will cause clumping of Salmonella aertrycke and to some extent, Salmonella choleraesuis. If S. hirschfeldii serum is available, S. choleraesuis and other organisms in Group C may be agglutinated. Salmonella enteritidis serum is commonly quite specific. Thus the immediate application of these sera should be useful in determining whether the organism in question is one of the Salmonella types commonly associated with food poisoning, though it may not indicate what type it is. Salmonella typhi-murium may be differentiated from S. schottmuelleri by its action on d-tartrate (see Table 8). S. choleraesuis may also be differentiated from the other two by cultural reactions.

For reliable identification of these strains as well as other strains included in the genus Salmonella it is necessary to use antigenic analysis. For the application of this method it is necessary to understand the antigenic variations (H-O, S-R, V-W, "form" and "phase") which may occur among these organisms. One must, furthermore, have available pure "O" serums and "H" serums. The methods for preparing the antigens and serums and the techniques of Salmonella antigenic analysis are described in detail by Edwards and Bruner (1942). While the clinician may be satisfied with mere identification of an organism as a member of the Salmonella genus, for the epidemiologist, typing by methods of antigenic analysis is almost indispensible.

For a more complete description of the biochemical characteristics of the Salmonellae see Kauffmann, F., Die Bakteriologie der Salmonellagruppe, Copenhagen, Einar Munksgaard, 1941; Topley and Wilson's text, 2nd Edition, pp. 552-553; Bergey, 5th Edition. For a description of the antigenic structure and methods of typing see: "Serological identification of Salmonella cultures", Edwards, P.R. and Bruner, D.W., Univ. Ken. Agr. Exp. Sta. Circ. 54, 1942; "The state of the Salmonella problem", Bornstein, S., J. Immunol., 1943, 46, 439-496; Kauffmann, F., (see above).

Classification of an organism in the Eberthella-Shigella-Salmonella groups should be confirmed by agglutinating the organism with known antiserum if this is available. The serum is diluted to give 1/10 its titer and 1/2 cc. mixed with 1/2 cc. of a saline suspension or peptone or broth culture of the organism. One-half cc. of the suspension or culture is mixed with an equal volume of saline in order that a control on the stability of the suspension be obtained. Incubation in the 56° C. water bath for five hours followed by overnight refrigeration is sufficient. In some cases it may be necessary to transfer the culture several days before agglutination with its specific antiserum may be obtained. For routine purposes antisera for Salmonella paratyphi, Salmonella schottmuelleri, Slamonella enteritidis, Salmonella hirschfeldii, Eberthella typhosa, Shigella dysenteriae, Shigella sonnei and polyvalent Shigella paradysenteriae should be available for serological identification of organisms in this group.

For routine purposes these organisms may be differentiated through their action on lactose, dextrose, mannite, arabinose, dulcitol, inositol, and xylose and by the determination of such characteristics as motility, indol production, H_2S production, gelatin liquefaction and agglutinability by selected sera (see above). The sugars lactose and dextrose together with motility permit speedy differentiation into the Salmonella, Eberthella or Shigella groups. The use of mannite together with the study of indol formation permit separation of the Shigellae into the dysenteriae (Shiga) and paradysenteriae (Flexner) groups. Agglutinative sera are valuable for the speedy confirmation of the cultural diagnoses and for differentiation of the Salmonellae (Paratyphoid A, B, and C and enteriditis and Shigellae (Boyd's types).

The Escherichia-Aerobacter organisms may usually be differentiated by the experienced worker by the type of colony produced on Mac Conkey's, Endo's or eosin-methylene-blue agar. Most members of the Proteus group may be detected by their ability to produce a spreading growth on moist agar and the production of a

musty odor. Gelatin liquefaction, sucrose fermentation and failure to ferment mannite are common characteristics of this group of organisms.

SALMONELLA-LIKE ORGANISMS (Morganella)

There is a group of organisms the members of which resemble the Salmonellae but are indol positive and do not produce hydrogen sulfide. Unlike most members of the Proteus group they do not ferment sucrose and do not give a spreading type of growth on moist agar. These characteristics are those of the organism originally described by Castellani as Bacterium columbense and designated in Bergey's Manual (5th Ed.) as a Salmonella. Fulton, M., (J. Bact., 1943, 46, 79-82) suggests that these organisms together with Proteus morganii (Morgan's bacillus #1) be grouped in a new genus to be called Morganella. Proteus morganii ferments dextrose only with the formation of acid and gas although some strains may ferment sucrose. It produces indol and has been associated with enteritis and colitis in man and with infantile diarrhea.

SLOW LACTOSE FERMENTERS

In addition to the above there are Escherichia-Aerobacter-like organisms which ferment lactose slowly or without the production of gas. These have been called paracolon and aberrant coliform organisms by various workers. They give colorless colonies on differential plating media and when inoculated into lactose broth may require 4 or 5 days of incubation before fermentation is detected. Detection of this fermentative property may even require daily transfer in lactose broth for several days and may be accelerated by closing the test tube with a tightly fitting stopper. Some of the slow-lactose-fermenters appear to possess pathogenic powers Until these organisms are better classified they may be designated as slow-lactose-fermenting coliform organisms.

References: Stuart, Mickle, Borman, Am. J. Pub. Health, 1940, 30, 499-508;
Stuart, Wheeler, Rustigian, Zimmerman, J. Bact., 1943, 45, 101-119.

KLEBSIELLA

There is a group of organisms which grow on agar to give large, circular, convex, gray and mucoid colonies with a stringy consistency and which vary considerably in their biochemical characteristics. They resemble the Aerobacter- Escherichia group in their biochemical characteristics and are encapsulated although at times the bacteria appear to be embedded in a jelly-like substance rather than enclosed in distinct capsules. These organisms belong to the Klebsiella group in which Friedlander's bacillus is found. In Friedlander's pneumonia they may be demonstrated in the sputum. Subcutaneous inoculation of mice with one millionth of one cc. of a 24 hour broth culture will usually produce death.

The four species of pathological importance are K. pneumoniae (Friedlander's bacillus), K. granulomatis, K. rhinoscleromatis and K. ozoenae.

K. granulomatis has been found to be associated with granuloma inguinale and has been described as occurring intracellularly in the lesion as the so-called "Donovan bodies".

K. rhinoscleromatis is associated with rhinoscleroma, a chronic granulomatous condition of the nose, mouth and throat, in which the large ballooned endothelial cells are found filled with organisms. Rhinoscleroma occurs chiefly in southeastern Europe and is rarely found in the United States.

K. ozoenae is associated with ozena, a form of atrophic rhinitis having a foul odor. The discovery of the endocrine factor in the disease has relegated the organism to a minor role. The K. rhinoscleromatis and K. ozoenae strains are usually nonvirulent for laboratory animals.

Non-motile encapsulated organisms producing acid but no gas in the sugars may be members of the Klebsiella group.

ALCALIGENES and PSEUDOMONAS

When none of the test sugars are fermented, the organisms listed in the following table may be suspected. (Table 9).

TABLE 9.

	Motility	Lactose	Dextrose	Sucrose	Gelatin	Indol	Nitrates	Sodium Hippurate
Alkaligenes	+	-	-	-	-	+/-	+	-
Brucella bronchisepticum	+	-	-	-	-	-	+/-	+
Ps. aeruginosa (pyocyaneus)	+	-	A $\frac{+}{-}$	-	+			-
Br. abortus Br. melitensis	-	-	-	-	-	-	+ -	

Ps. aeruginosa (pyocyaneus) may usually be identified through its green-blue pigment and odor. Brucella bronchisepticum and alkaligenes grow more luxuriantly than do Brucella abortus and Br. melitensis and are motile.

VIBRIO

If dextrose, sucrose (occasionally lactose slowly) are fermented with production of acid and no gas, and if the organisms are actively motile, the organism should be suspected of being a Vibrio and should be studied with respect to its morphology, motility, ability to liquefy Loeffler's medium and gelatin, the production of indol and nitrosoindol (cholera red reaction) and reduction of nitrate. Indol and nitrosoindol production should be tested for after inoculating two tubes of peptone water and incubating for 48 hours. Nitrate reduction should also be tested for after 48 hours of incubation.

At present it is impossible to differentiate the members of the vibrio group with absolute reliability by means of simple biochemical or serological studies. Studies on antigenic structure show promise in this respect but are not yet complete. This group of cholera-like spirilla is a large one. Most of its members are of medical bacteriological importance chiefly because of the difficulties which they add to the task of differentiation, for while some of them merely bear a morphological resemblance to the cholera vibrio, others can be distinguished only by their serum reactions and pathogenicity for various animals. Additional difficulty, too, is contributed by the fact that within the group of true cholera organisms occasional variations in agglutinability and bacteriolytic reactions may exist.

For a discussion of this group of organisms see Topley and Wilson's Principles of Bacteriology and Immunity, second edition, pages 387-397.

PASTEURELLA

If acid but no gas is produced slowly and the organisms are non-motile, and show marked bipolar staining a member of the Pasteurella group should be suspected.

Three divisions of the Pasteurella group may be made, represented by (1) P. pestis (bubonic plague), (2) P. pseudotuberculosis (plague-like disease of rodents), (3) P. avicida (fowl cholera), the type representative of the true hemorrhagic septicemias. As a matter of convenience and because of certain cultural and pathogenic similarities, the etiological agent of tularemia while probably related to a distinct genus, serologically related to the genus Brucella, has been included tentatively in this group.

Among numerous infections in animals the best known of the hemorrhagic septicemic diseases (division 3 above) and their etiological agents are (1) chicken cholera (P. avicida), (2) swine plague (P. suilla), (3) hemorrhagic septicemia and septic pneumonia in cattle (P. boviseptica) and (4) infectious pneumonia and enteritis in sheep (P. oviseptica).

Some points of distinction between the three divisions are given in the following table: (Table 10). - next page.

TABLE 10.

	P. pestis	P. pseudotuberculosis	P. avicida
Motility	-	+	-
Indol	-	-	+
Methyl Red	+	+	-
Litmus Milk	-	alkaline	-
Bile Salt Inhibition	-	-	+
Pathogenicity to white rats	+	-	+

P. pestis is a small, thick, ovoid bacillus with rounded ends and convex sides. It is markedly pleomorphic, with vacuolated, swollen, or club-shaped forms in old lesions or cultures especially on three per cent salt agar - a point of diagnostic significance. It is non-motile, non-spore forming, encapsulated (in the animal body), gram-negative, not acid-fast, and shows polar staining. It grows well on ordinary media and on agar the colonies are small, round, transparent, colorless, umbonate, slightly granular and viscous. It is particularly pathogenic for guinea pigs and rats which are used for purposes of diagnosis and isolation. Rats inoculated subcutaneously show subcutaneous congestion, hemorrhages and adenitis of the superficial lymph glands. Death occurs in from two to eight days.

The diagnosis of plague in rats is made by the pathological findings, by the identification of the bacillus in the buboes and organs through smear or culture and by animal inoculation. Differentiation from pseudo-pestis is made by the motility and lack of pathogenicity of the latter species for white rats.

The laboratory diagnosis in man is made from the material aspirated from the bubo, by microscopical examination, culture and animal inoculation. A positive agglutinative reaction with the patient's serum is of value. In the pneumonic type the organisms may be isolated from sputum. Clinically the disease must be differentiated from typhus fever, typhoid fever, influenzae pneumonia, filarial infection and venereal buboes.

Pasteurella (Brucella) tularensis is a small, pleomorphic, non-spore-bearing, encapsulated, non-motile, gram-negative, non-acid-fast bacillus or cocco-bacillus. Bipolar staining forms may at times be noted. Primary cultures on cystine-blood-agar grow out after 4 to 7 days incubation. The colonies are usually very tiny. Smears generally show a mass of gram-negative material and usually very close study is necessary to determine the morphology of the organisms. Transfer to cystine-blood-agar slants should be made every 5 or 6 days to preserve the culture. These subcultures will yield abundant growth after 2 to 3 days of incubation.

P. tularensis may be identified by agglutination with P. tularensis antiserum. The growth from a young cystine-agar-slant culture is washed off with 5 to 6 cc. of salt solution, diluting with saline to the proper turbidity. In order to protect the laboratory worker it is advisable to use saline containing 0.5 per cent formaldehyde in making the suspension (or the salt solution suspension may be heated at 56 to 60 degrees C. for 20 to 30 minutes). This does not interfere with the agglutination reaction and is important in preventing laboratory infections. The formalized or heated suspension is set-up against P. tularensis antiserum using dilutions of serum from 1:10 to the full titer of the serum. The test should be incubated for 5 hours at 55 degrees C., refrigerated overnight and read for agglutination. (See appendix for incubation temperatures for agglutination).

Material from a primary lesion, from enlarged glands, or blood of a patient, inoculated intracutaneously, subcutaneously or intra-abdominally into a guinea pig will produce characteristic lesions. P. tularensis differs from P. pestis in its effect on inoculated animals by producing no pus at the site of inoculation, greater variability in the size of granules in the spleen, rarity of lung involvement and culturally by failure to grow on ordinary media. A series of agglutinative reactions showing an increasing titer of the patient's serum after the second week of the disease is of diagnostic value.

Because of the high degree of infectiousness of P. tularensis extreme care should be exercised in the handling of material containing this organism. Animal injection for diagnostic purposes should not be carried out unless absolutely necessary.

BRUCELLA

Colonies of Brucella are small, translucent and blue-gray in color. Smears show very short bacillary or coccoid forms staining gram-negative. Colonies suspected of being those of Brucella should be fished to slants or broth and inoculated in the candle-jar for 4 or 5 days. After a few transfers, Brucella will grow aerobically on the basic medium but three or four days incubation is usually required for growth. The cultural characteristics of Brucella may be determined by inoculating lactose, dextrose and sucrose and using the basic medium (Appendix) as a base, a deep agar (0.3%) shake tube. Peptone water should be heavily inoculated and tested for the presence of indole after a week's incubation. The sugar tube cultures may be used for motility studies.

For differentiation of the three types of Brucella, growth in shake agar, the production of H_2S, and growth on plates containing dyes are employed. An agar plate containing 1:100,000 parts basic fuchsin (Appendix) and another containing 1:200,000 thionin should be streaked with a loopful of broth culture.

Brucella ferment no sugars, are non-motile and fail to produce indole. In deep agar shake tubes, newly isolated aerobic strains will grow in a band 1 or 2 mm. below the surface of the agar while strains (newly isolated) preferring the presence of carbon dioxide in the atmosphere produce a band of colonies from 1 to 2 cm. below the surface of the medium. The three strains of Brucella may be differentiated as indicated in the following table: (Table 11).

TABLE 11.

DIFFERENTIATION OF SPECIES OF BRUCELLA

	Growth in deep agar	Growth on media containing thionin	Growth on media containing basic fuchsin	H_2S production			
				Days			
				1	2	3	4
Br. melitensis	Surface or 1-2 mm. below the surface	Good growth	Good growth	-	-	-	-
Br. abortus	1-2 cm. below the surface	No growth	Good growth	3+	2+	1+	1+
Br. suis	Surface or 1-2 mm. below the surface	Good growth	No growth	4+	4+	4+	4+

The methods to be used for determining the production of H_2S by the organisms of this group are indicated in the appendix.

HEMOPHILIC BACTERIA

Several species of bacteria of somewhat different characteristics are grouped under the generic name Hemophilus because of their dependence for growth upon some factor supplied by blood. These organisms are minute, non-motile, non-spore-forming, gram-negative, non-acid-fast, highly pleomorphic bacilli ranging from coccoid to long slender forms. The colonies are usually tiny, gray, translucent, usually non-hemolytic but occasionally beta-hemolytic in type.

H. pertussis (Bordet-Gengou bacillus) which grows as a small, rather white hemispherical, translucent, hemolytic colony (more opaque and whiter than colonies of H. influenzae) on glycerin-potato-blood agar after 2 to 4 days of incubation, is not a true hemophilic organism but is placed in this group. When first isolated H. pertussis will grow only on media containing blood but after a variable length of time on artificial media it will grow on plain media. Ultimate growth on plain media and requirement of potato-glycerin-blood agar for primary isolation, as well as restricted biochemical activities (H. pertussis does not produce indol, reduce nitrates to nitrites, or ferment dextrose) and production of alkali in broth serve to differentiate this organism from H.. influenzae. There is, besides, little if any antigenic relationship between H. pertussis and H. influenzae.

In testing Hemophilus organisms for dextrose fermentation, nitrate reduction or indol production hemopeptone base (See Appendix) should be used. For nitrate reduction studies 0.02 per cent potassium nitrate should be added. One per cent dextrose added to the hemopeptone base is used to determine dextrose fermentation. For indol production, hemopeptone not enriched with sugar should be employed. The cultures are tested for the fermentation of dextrose, production of indol and reduction of nitrates after 5 days incubation.

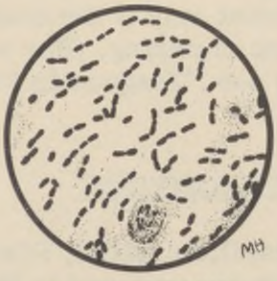
Hemophilus influenzae (Pfeiffer's bacillus) is found in the mucous membranes of the respiratory and associated tracts of man. It is considered identical with the Koch-Week's bacillus which is associated with conjunctivitis. Although usually non-hemolytic, hemolytic varieties may be encountered. The isolation of small, gram-negative, non-motile, non-spore-forming rods occurring singly, in pairs, and occasionally in long thread-like forms growing on chocolate agar but not on plain agar is usually diagnostic. On plates containing hemoglobin better growth is obtained by this organism when it is in close proximity to a colony of Staphylococcus or some other colony. This is called a satellite type of growth. A pungent, mousy odor may be evident in plates on which H. influenzae is growing.

The group of H. influenzae organisms responsible for H. influenzae meningitis is a homogeneous one and is designated type b. When responsible for a meningitis, H. influenzae may be identified directly by doing a Quellung test using type b antiserum since the pathogenic members of this group of organisms are encapsulated.

Chancroid or soft chancre, an acute destructive inflammatory lesion usually occurring on the genitalia, is caused by H. ducreyii. The detection, in the lesion, of small gram-negative rods forming chains and occurring singly, together with the growth requirements of the organism help in the diagnosis of the disease. Isolation of the organism and agglutination with specific antiserum is strong confirmatory evidence. For the isolation and growth requirements of this organism see section on handling of genito-urinary specimens.

Hemophilus lacunatus (The Morax-Axenfeld bacillus), like Ducrey's bacillus and H. pertussis, grows best on media containing fresh or heated blood, especially when first isolated. After cultivation on artificial media, however, it will grow on substrates lacking blood. This organism is associated with chronic angular conjunctivitis. In the purulent discharge from the eye it appears as a short, thick, ovoid, gram-negative, non-spore-bearing bacillus with rounded ends, usually in diplo-formation but at times singly or in chains. The bacillus can be cultivated upon alkaline media containing blood or blood serum. Upon Loeffler's blood serum, colonies appear after 24 to 36 hours as small indentations which indicate a liquefaction of the medium.

Diagnosis of a Morax-Axenfeld (H. lacunatus) conjunctivitis is easily made by smearing the pus from the eye and staining by Gram's method. (See Fig.)



H. LACUNATUS
(Morax-Axenfeld Diplobacillus)

Investigation of the growth requirements of influenza bacilli and related organisms have shown that H. influenzae requires two kinds of substances for its growth and that morphologically similar organisms can be differentiated from influenza bacilli on the basis of their need for one or the other of these substances. These substances are called the X and V growth factors and are found in blood. The former is heat stable and the latter is destroyed by heat.

Influenza bacilli and related organisms show the following differences with respect to their requirements for these growth factors. (See Table 12). In this table the plus sign indicates that the corresponding factor is essential for growth. The O sign, that the factor is not needed.

TABLE 12.

GROWTH REQUIREMENTS OF THE HEMOPHILUS GROUP

ORGANISM	X FACTOR	V FACTOR
H. influenzae	+	+
H. lacunatus	0	0
H. ducreyii	0	0
H. pertussis	0	0
H. parainfluenzae	0	+

For routine purposes the presence of H. influenzae may be diagnosed from cultural characteristics such as the improved growth on chocolate agar as compared to ordinary blood agar, failure to grow in the absence of blood, satellite type of growth around colonies of staphylococcus or other organisms and the tiny dew drop colonial morphology. The presence of capsules differentiates the pathogenic from the non-pathogenic H. influenzae.

Similarly, H. pertussis may be identified through the fact that it requires Bordet-Gengou agar for primary isolation and from the type of colonial growth obtained on this medium. The colonies are smooth, raised, glistening and pearly. Although they produce a hemolytic zone on blood agar, this hemolysis is not evident when 30 per cent blood or more is present in the medium.

A qualitative slide agglutination test may be done with the growth on the Bordet-Gengou plate. To do this, suspend the suspected colonies in a drop of saline on one end of the slide. On the opposite end of the slide mix several loopfuls of this suspension with several loopfuls of Hemophilus pertussis antiserum diluted 1:10. If the colonies are H. pertussis there will be almost immediate agglutination. The result is significant only if the control suspension without serum remains smooth and shows no clumping. (For a more complete account of whooping cough and H. pertussis see "Diagnostic Procedures and Reagents", 1941).

MALLEOMYCES

Malleomyces mallei (Actinobacillus mallei, Pfeifferella mallei), the causative organism in glanders is rarely encountered in the diagnostic laboratory.

It is a non-motile, non-spore-forming, non-capsulated, non-acid-fast, pleomorphic, gram-negative rod, staining irregularly and presenting at times a bipolar or beaded appearance.

The addition of three per cent glycerol to nutrient media enhances its rather slow growth. The most characteristic growth is obtained on potato, on which the small colonies resembling drops of honey appear in 36 to 48 hours. Later the growth takes on a reddish brown tinge and gives a pale to dark green color to the potato. On agar the brown to yellow amorphous colonies are slimy, tenacious and opaque and take on a deep brown color with age.

Since primary cultures may be difficult to obtain, it is advisable to inject some of the material to be cultured into a susceptible animal. Of the laboratory animals, the guinea pig is the most susceptible. Infection may be produced by any route of inoculation.

Laboratory diagnosis of the disease depends on (1) the identification of the organism by smear and culture, (2) animal pathogenicity, (3) mallein tests, and (4) serological (complement-fixation) tests.

Malleomyces pseudomallei (Bacillus whitmori) is responsible for a glanders-like infection (meliodosis) in rats, guinea pigs, rabbits and man in India, Federated Malay States and Indo-China. It is gram-negative and on the whole, in culture medium, it resembles M. mallei. It differs from this organism in being more actively motile and liquefying gelatin more rapidly. It is also more pathogenic for rodents than is M. mallei.

BACTEROIDES

There is a group of gram-negative anaerobic bacilli, usually of intestinal origin and frequently found in anaerobic cultures made from lung and liver abscesses, bronchiectases, puerperal sepses, etc. Colonies are small, gray and translucent and may be beta or gamma in type. Both short coccoid forms and fairly long slender bacilli may be encountered. This group of organisms has not been adequately studied and is designated Bacteroides. Weiss and Rettger (J. Bact., 1937, 33,423) feel that these organisms are the predominant organism in the adult human intestine. They propose four groups based primarily on serological and secondarily on morphological characteristics.

These organisms may be isolated on streaked blood agar plates incubated for 24-48 hours in the anaerobic jar.

GRAM-POSITIVE RODS

Corynebacterium
Bacillus
Clostridium
Mycobacterium

CORYNEBACTERIA

There is a large group of micro-organisms spoken of as diphtheroid bacilli, largely because of their morphological resemblance to the diphtheria bacillus. They are gram-positive, non-motile, often show metachromatic granules and have no spores. They form a heterogeneous group held together by morphological and superficial cultural similarity and consist largely of saprophytes and probably harmless parasites on the human and animal body. They are frequently present in the nasal mucus and in the throat. They are grouped as a separate species of the genus Corynebacterium. On blood agar, the colonies are small, gray, usually non-hemolytic, rather opaque and often quite hard.

Two members of this group which resemble C. diphtheriae are C. xerosis and C. pseudodiphthericum. They may be differentiated culturally from C. diphtheriae in that while the latter is hemolytic on blood agar the former are not. Dextrose and sucrose may also be used in basic broth to differentiate between the three organisms as indicated in the following table. (Table 13).

TABLE 13.

ORGANISM	DEXTROSE	SUCROSE	HEMOLYSIS
C. diphtheriae	A	-	Beta
C. xerosis	A	A.	None
C. pseudodiphthericum	-	-	None

However, there are diphtheroids other than C. xerosis and C. pseudodiphthericum which ferment dextrose but fail to ferment sucrose.

While our knowledge of the diphtheroid group is rather incomplete, the type species of the Corynebacterium group (C. diphtheriae) has been the subject of considerable study.

The group as a whole is endowed with a more complex morphology than that exhibited by most bacterial genera. The club form, from which the name is derived is only one of the many shapes which may be assumed by the individual cells of the type species, C. diphtheriae. The most typical form, however, and one seldom absent from 24 hour cultures grown on Loeffler's serum is that of a long, rather slender bacillus, often slightly curved, with rounded, somewhat swollen ends and sometimes with localized swellings elsewhere and staining unevenly with such stains as methylene blue, Neisser's, etc. In the same culture there may be found much shorter forms, cells which stain solidly and evenly, cells in which the irregular staining takes the form of a series of transverse bars,

CORYNEBACTERIA

* C. DIPHTHERIAE
(m.b.)

* DIPHTHEROID
(m.b.)

* C. DIPHTHERIA
(n or b)

* C. DIPHTHERIAE
(n or b)

C. DIPHTHERIA
‡
(m.b.)

* DIPHTHEROID
(n or b)

M. Hadden

m. b. = methylene blue.
n. or b. = Neisser's or Becks stain.
* = from Loeffler slant.
‡ = from Tellurite agar.

cells in which the combination of uneven staining and localized swelling gives to a single bacillus the appearance of a short chain of streptococci, and cells which display a single transverse unstained septum. If a film prepared from such a culture is stained with alkaline methylene blue or better yet with Neisser's or Beck's stain, reddish purple granules may be seen in the protoplasm of the cell. There are usually two or three such granules in a cell. When one or two are present they show a definite tendency to be situated at one or both poles. With Beck's stain these granules take a purple color while the body of the cell is brown. The presence of these granules in an elongated slightly curved rod when a 12 to 18 hour Loeffler's slant culture is examined is usually diagnostic of diphtheriae.

The arrangement of the bacilli in film preparations is also characteristic. Adjacent cells tend to lie at any angle to one another, forming a V or an L or a Y which when grouped, form clusters resembling Chinese letters or cuneiform writing. For most of the other species of diphtheroids there is much less variable morphology in smears of young cultures than there is for C. diphtheriae. A Gram's stain is sometimes helpful in differentiating between C. diphtheriae and H. influenzae.

Colonies of C. diphtheriae on blood agar plates are small and gray, resembling those of streptococci and show narrow zones of beta hemolysis.

On cystine-tellurite blood agar plates, colonies of C. diphtheriae are circular, soft and butyrous in consistency, smooth, rounded or domed although sometimes slightly conical. They vary from 0.5 to 2.5 mm. in diameter and are of a very dark slate color, almost black but not jet black. There is no hemolysis of the medium around them. Diphtheria colonies are never leathery, brittle, or membraneous in texture.

Cystine-tellurite-blood agar is not absolutely selective for Corynebacterium diphtheriae, especially with carrier cultures. Diphtheroids and staphylococci are the chief organisms which cause difficulty. Diphtheroid colonies are usually light gray in color or dark in the center and whitish or gray and definitely translucent at the periphery, especially when well isolated. When crowded they are apt to have an appearance almost indistinguishable from colonies of Corynebacterium diphtheriae. Usually, however, even when crowded they are rather flat, are of a slightly lighter gray color, and often appear green against the blood red agar. Staphylococcus colonies of some species are flatter and thinner than those of Corynebacterium diphtheriae and are of an intense glistening, jet black color. Some micrococcus and Staphylococcus aureus colonies tend to a slaty tint and often closely resemble the colonies of C. diphtheriae. Usually the colonies are large, often showing a center, or concentric rings of a lighter gray color.

While a little experience soon serves as a guide to what colonies to fish, if there is any doubt the colony should be fished.

Since the characteristic morphology of C. diphtheriae is not always found in the colonies growing on the tellurite medium, suspected colonies should be fished onto Loeffler's slants which should be incubated from 12 to 18 hours at 37 degrees C. The growth from the Loeffler's slants should be smeared and stained and, if pure cultures showing the morphology characteristic of C. diph-

theriae are obtained, fermentation studies may be made. The sugars, dextrose and sucrose, are prepared separately and in one per cent concentration in phenol red broth (tryptose or proteose #3, etc.) (See Appendix). After five days incubation the sugar tubes should be examined for evidence of fermentation. The broth used must support a satisfactory growth of the organism tested.

A culture fermenting dextrose and not sucrose in five days of incubation at 37 degrees C. may be transferred to a Loeffler's slant and after 18 to 24 hours of incubation this fresh growth may then be tested for virulence.

In the intracutaneous method for the determination of virulence, a very heavy suspension of the organisms is made by washing off the growth on the slant with 0.5 to 1 cc. of physiological salt solution. One tenth cc. of the suspension is injected intracutaneously into one shaven side (not the center) of the abdomen of a normal guinea pig. A rabbit may be used in place of the guinea pig if desired. Five or six such tests may be made on one side of a single animal. The suspension is refrigerated. Four or five hours after the test injection, the guinea pig should be given 200 units of diphtheria antitoxin intraperitoneally. Thirty minutes later, 0.1 cc. of the suspension of organisms is injected intracutaneously into the skin of the other side of the abdomen of the animal. This injection serves as a control. The guinea pig should be examined after 24, 48, and 72 hours. Erythema, edema and necrosis at the site of the test injection and the absence of such reactions on the control side characterize a positive skin test and indicate the production of toxin by the strain of C. diphtheriae.

Difficulties of interpretation may arise if, instead of pure cultures, the original growth on a Loeffler slant be suspended and used in the intracutaneous test. If such a procedure is necessary the guinea pig rather than the rabbit should be used since atypical reactions due to the presence of other organisms are less likely to occur in the former. When such an impure culture is used, if the lesion produced by the first injection (before antitoxin) is typical in character and larger than that produced by the second (after antitoxin) one may interpret this difference in size as due to the presence of toxigenic (virulent) C. diphtheriae. If the first and second injections result in lesions of the same size and if the reactions are not characteristic of that obtained with C. diphtheriae, one may assume that virulent C. diphtheriae were not present. When certain contaminating organisms such as streptococci, Ps. pyocyaneus, etc. are present both injections may produce large skin lesions. In such cases it may be necessary to first obtain the organism suspected of being C. diphtheriae in pure culture and then to repeat the virulence test.

It is important to bear in mind that in the diagnosis of diphtheria the laboratory usually plays an important but only corroboratory role. When laboratory support is needed by the physician speedy bacteriological diagnosis is of the utmost importance. For such support the microscopical demonstration of morphologically and tinctorially typical C. diphtheriae after cultivation on Loeffler's medium is usually sufficient. When mere support of the clinical diagnosis is desired, this presumptive test is usually all that need be done routinely.

On the other hand, in the enforcement of quarantine, and in studies of epidemiology, the carrier state, susceptibility, and the effects of antigens, the

laboratory may be expected to prove the presence of virulent C. diphtheriae. In epidemiological studies it may be necessary to determine the type of C. diphtheriae (gravis, mitis, intermediate). See Table 13A. For a discussion of the determination of types of C. diphtheriae see the review by McLeod in Bacteriological Reviews, 1943, 7, 1-41.

TABLE 13A

Most reliable criteria in differentiating the three types of C. diphtheriae

	MITIS	INTERMEDIUS	GRAVIS
1. Morphology	Long forms; metachromatic granules.	Barred forms often long and clubbed at ends.	Short forms tending to stain uniformly and sometimes closely resembling Hoffmann's bacillus.
2. Appearance of growth on heated blood agar.	Fairly abundant, moist, relatively smooth, semi-opaque and glistening colonies.	Flat, fine, dry, opaque, and associated with delicate olive green discoloration of medium.	Abundant, flat, dry, matted, relatively opaque.
3. Appearance of growth on special blood tellurite media.	Smooth, convex, medium-sized, with black center and semi-translucent grey periphery for first 36 hours. Finer and larger colonies.	Flat, fine, dull with black center and often small central papilla. Grey periphery with slightly raised margin. Colonies very uniform in size.	Medium to large with slight to marked radial striations and slightly to markedly indented periphery. Color varying from grey-black to black. Finer and larger colonies.
4. Consistence of colonies.	Approximately that of warm butter, colony smears under needle and forms homogeneous suspensions.	Intermediate between gravis and mitis.	Approximate to that of cold margarine, colony is pushed in front of needle and tends to fracture.
5. Appearance of growth in nutrient broth.	Heavy uniform or mixed uniform and granular turbidity. Pellicle late, soft and leaving ring on side of tube.	Finely granular turbidity, settling to leave clear supernatant.	All variations from clear fluid with marked pellicle broken by agitation to coarse flakes which settle to base of tube to slight pellicle over abundant fine turbidity mixed with granules and flakes of varying size.
6. Hemolytic activity on blood agar plates.	Distinct.	Absent.	Variable.
7. Fermentation of starch and glycogen.	Negative.	Negative.	Positive.
8. Regularity of pathogenic action in guinea-pigs.	10%-20% of non-pathogenic strains (high pathogenicity for mice).	10% non-pathogenic (low pathogenicity for mice and for spermophils).	Non-pathogenic strains extremely rare (moderate pathogenicity for mice).
9. Antigenic homogeneity or diversity.	Great diversity of antigenic groups.	Antigenically homogeneous.	Two main antigenic groups each of which has been found as an epidemic strain over wide areas.

BACILLI

There is a group of large aerobic spore-forming bacilli, which includes B. subtilis (the hay bacillus), B. cereus, B. megatherium, B. mycoides, B. anthracoides, B. anthracis, etc. Only one of this type of bacterium is known to be pathogenic, namely, Bacillus anthracis. This group is usually gram-positive, although in older cultures, gram-negative forms may be observed. The colonies are usually large, rough, dry and irregular and the organisms when present are usually contaminants. Because of their ability to produce spores these organisms are very resistant to heat and other bactericidal agents. They may be isolated when mixed with non-spore-forming organisms by subjecting the mixture to a temperature of 80 degrees C. for 10 minutes. The non-spore-forming organisms are destroyed by this treatment whereas the spores survive.

One must always be on the alert for B. anthracis because of the ease with which it may incorrectly be considered a contaminant member of the subtilis group. Culturally it is distinguished by the curled hair lock appearance of the colonies (due to the growth of the bacilli in long interwoven chains) on agar or gelatin, the inverted fir tree type of growth in gelatin stab and slow gelatin liquefaction in contrast to rapid liquefaction of gelatin by members of the subtilis group. Other aids in differentiation from these organisms are its lack of motility and the square ends of the organism. Since there are members of the subtilis group which have all or some of these cultural characteristics, the best and conclusive test is pathogenicity. Subcutaneous and intraperitoneal injection into the mouse is a convenient method of testing for pathogenicity. With a suitable anti-serum it may be possible also to demonstrate a specific protection in the mouse.

BACILLUS ANTHRACIS

SURFACE COLONY

SMEAR FROM CULTURE

SMEAR FROM MOUSE PERITONEAL FLUID

Ascoli's precipitin reaction is helpful in making a rapid diagnosis when the work has to be done with putrid material from dead animals, with tissues preserved in formalin or alcohol, or with hides. A small piece of the heavily contaminated tissue (the spleen if obtainable) is boiled in 5 to 10 cc. of saline,

cooled, filtered clear and layered on the anti-serum. A zone of precipitate occurs at the junction of the extract and anti-serum in positive tests. Suitable controls should be made at the same time. Since ordinary anti-anthrax therapeutic serum may not be potent in precipitins it may be necessary to prepare serum by immunizing rabbits against encapsulated anthrax bacilli. (Rosenberg and Romanow, Centralbl. f. Bakt, I Abt, 1929, 110,102).

THE CLOSTRIDIA

The genus Clostridium includes a large number of spore-forming, anaerobic, rod-shaped bacteria. Many of these are saprophytes, occurring in the soil, decaying animal and vegetable material and in the intestines of man and animals. Certain species, because of their ability to produce toxins are important from a medical point of view. These include Cl. botulinum which is the cause of botulism, a type of food poisoning, Cl. tetani, the cause of tetanus or lock-jaw, and a group of organisms associated with the production of gas gangrene in wounds. The best known of this last group of organisms is Cl. perfringens (welchii).

The members of this group of organisms fall into two main classes: (1) saccharolytic and (2) proteolytic. This demarcation is not a strict one since most of the organisms although belonging essentially to one class may have some of the properties of the other. However, in media containing both carbohydrates and protein, the saccharolytic organisms ferment the carbohydrate with the formation of acid and gas with practically no digestion of the protein while the proteolytic organisms digest the protein. The latter decompose meat with blackening and produce a putrefactive odor.

The following table (Table 14) presents a classification of some of the clostridia on the basis of proteolytic and saccharolytic properties.

TABLE 14

Species	Both saccharolytic and proteolytic		Saccharolytic only	Proteolytic only
	Saccharolytic predominates	Proteolytic predominates		
Cl. perfringens	+			
Cl. septique	+			
Cl. novyi	+			
Cl. botulinum		+		
Cl. chauvei	+			
Cl. fallax			+	
Cl. tertium			+	
Cl. sporogenes		+		
Cl. histolyticum		+		
Cl. tetani				+

TABLE 15.

CHARACTERISTICS OF THE MORE IMPORTANT CLOSTRIDIA

Name	Motility	Spore	Liquefaction of Gelatin	Cooked Meat Medium	Litmus Milk	Digestion of Serum	Glucose	Mannitol	Lactose	Sucrose	Starch	Exotoxin	Pathogenicity for Guinea Pig
Cl. botulinum (A,B,C)*	+	oval subterm	+	A&B gas, digestion blackened,C-	A&B ppt., digest., alk. C-	A+ B+ C-	+	-	+ -	-	-	+	+
Cl. sporogenes	+	oval subterm	+	gas, digestion, black	ppt., digestion	+	+	-	-	-	-	-	-
Cl. histolyticum	+	oval subterm	+	digestion slightly black	ppt., digestion	+	-	-	-	-	-	weak +	variable
Cl. lentoputrescens (putrificum)	+	oval term	+	gas, digestion	ppt., digestion (slow)	+	±	-	-	-	?	-	-
Cl. tetani	+	round term	+	gas	-	-	-	-	-	-	-	+	+
Cl. tetanomorphum	+	round term	+ -	gas	-	-	+	-	-	-	-	-	-
Cl. perfringens (welchii)	-	oval subterm	+	gas, pink color	acid, gas, clot. stormy fermentation	-	+	-	+	+	+	+	+
Cl. septique	+	oval subterm	+	gas, pink color	acid, clot, some gas	-	+	-	+	-	-	+	+
Cl. novyi (oedematiens)	+	oval subterm	+	gas, pink color	acid, gas, later some clot	-	+	-	-	-	+	+	+
Cl. chauvoei	+	oval subterm	+	gas, pink color	acid, clot, some gas	-	+	-	+	+	+	+	+
Cl. fallax	+	oval subterm	+	gas, pink color	acid, clot, sometimes gas	-	+	-	±	?	+	+ weak	when + freshly isolated
Cl. tertium	in young cultures +	oval term	-	gas	acid, clot	-	+	+	+	+	+	-	-

* Serological types A, B, C, each with different toxin.

For a fairly complete list of differential characteristics for some of the more important Clostridia see Table 15.

The pathogenicity of the anaerobes appears to depend almost entirely on their toxin production. Cl. tetani, for example, multiplies locally and does not invade the body. The organisms are not present in as large numbers as are found in comparable infections. Cl. botulinum is not able to grow in the tissues at all. Its pathogenicity is due to the formation of a potent toxin in foodstuffs prior to their ingestion. Cl. novyi (oedematiens) remains almost confined to the site of inoculation. Cl. perfringens (welchii) and Cl. septique do grow at the site of the wound but they become truly invasive only when the death of the animal is near. The saccharolytic pathogenic anaerobes in contrast to the proteolytic, appear to produce a toxin capable of destroying live tissue, enabling them to create for themselves a suitable medium for further growth.

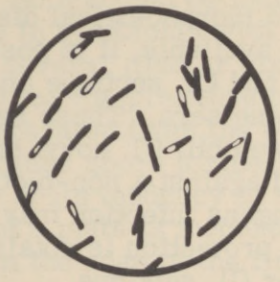
In rapid bacteriological diagnosis of infections caused by the anaerobes the following procedures should be followed. Two blood agar plates should be streaked. One should be incubated aerobically and the other anaerobically. If a mixed flora is suspected, two tubes of cooked meat medium should be inoculated. One of these should be heated for 10 minutes at 80 degrees C. in order to remove non-spore-forming organisms. Both tubes should be incubated in the anaerobic jar. Two tubes of litmus milk should be inoculated and one tube similarly subjected to a temperature of 80 degrees C. for 10 minutes. Both should be incubated in the anaerobic jar.

After 24 hours of incubation, the presence of Cl. welchii may be suspected if a large gram-positive, non-motile rod (which may or may not form spores at this time) is found in the fluid media, if there is a stormy fermentation (acid-clot and much gas formation) of the milk and if round, regular, opaque disc-like beta hemolytic colonies of gram-positive Cl. perfringens-like rods are found on the anaerobic but not on the aerobic plate.

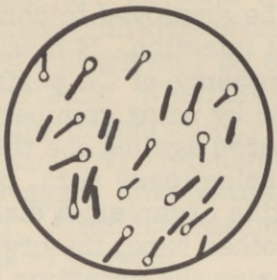
If, however, a motile drum-stick rod with the morphology of Cl. tetani is found in the fluid cultures, if there is no action on the litmus milk and if a delicate almost invisible spreading film of growth of tetanus-like organisms is present on the anaerobic but not on the aerobic plate, (the plates must be moist if the marked spreading type of growth is to be expected), the presence of Cl. tetani should be suspected. It may take 48 hours for the development of tetanus-colonies on a dry plate. It may also take 48 hours for the development of the film type of growth of tetanus (even though the plate be moist).

When there are anaerobic motile gram-positive rods which do not resemble Cl. tetani, we need be concerned chiefly with the other two pathogenic clostridia commonly found in wounds, namely: Cl. septique and Cl. novyi (oedematiens). For rapid identification of these organisms the best procedure is that of animal protection using specific antisera and preferably guinea pigs. There is another commonly found pathogenic Clostridium, Cl. chauveii, but this organism is not found in wound infection of man. It appears to be limited entirely to wound infection of animals. It is differentiated from Cl. septique, which it closely resembles,in that it never infects man, it ferments sucrose, is less pathogenic and produces less gas in animals. In smears from the livers of guinea pigs dead of Cl. septique infection, long snake-like filaments may be demonstrated. These are entirely lacking in Cl. chauveii infections. Animal protection with specific antisera may also be used to differentiate between these

CLOSTRIDIA—— THE SPORE-FORMING ANAEROBES

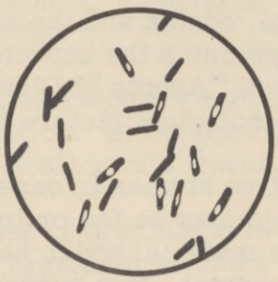


Clostridium botulinum

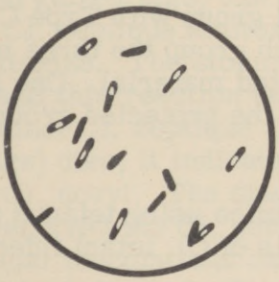


Clostridium tetani

Clostridium perfringens



Clostridium histolyticum



Clostridium Septique

Clostridium novyi

M. HADDEN

two organisms. Cl. botulinum, the only remaining pathogen of importance is, of course, not capable of growth in tissues. For rapid demonstration of the presence of botulinus in food suspected of being poisonous, protection tests using the three (A,B, and C) types of antiserum should be carried out in suitable animals (see below for method). Protection of guinea pigs with perfringens antitoxin should be employed to confirm the cultural and morphological diagnosis of Cl. welchii infection. If monovalent sera are not available, it is possible to confirm the presence of Cl. perfringens on one hand and Cl. septique and Cl. novyi on the other by using commercial anti-gas-gangrene sera. These sera are usually prepared against the three organisms: Cl. welchii, Cl. novyi and Cl. septique. If using these sera, protection is obtained against a non-motile Cl. perfringens-like organism a diagnosis of Cl. perfringens infection may be made. If on the other hand, the organism is motile and protection is obtained with the same serum the organism is either Cl. novyi or Cl. septique.

In the protection test two guinea pigs may be employed; one is injected with a relatively large dose of the antitoxic serum (several hundred units). Four hours later two or three cc. of a 24 hour culture (supernatant from a whole meat culture) is injected into one of the thigh muscles of each pig. The development of a local necrosis, edema and slight crepitation in the control but not in the protected pig in the case of Cl. perfringens or Cl. septique is diagnostic. With Cl. novyi (oedematiens) the lesions are characterized by a gelatinous exudate, edema and the absence of gas (no crepitation of the tissue). With all three organisms the unprotected pig will die in 24 to 48 hours. (See Gay and Associates "Agents of Disease and Host Resistance" 1935, for a description of the pathological picture in the guinea pig).

For protective studies in cases of botulism, mice or guinea pigs may be used. The animals may be fed or injected with extracts of the suspected food, using some of the material which has been heated in a boiling water bath for 30 minutes as a control. For purposes of treatment speed is essential in the diagnosis of botulism in man. This should therefore be made on the basis of clinical symptoms and history. The time required for bacteriological diagnosis would eliminate any possibility of successful specific treatment with antitoxin. Bengston (U.S.P.H. Serv. Pub. Health Rep. 36, 1665, 1921. U.S. Pub. Health Serv. Hyg. Lab. Bull. No. 136, 1924) recommends the following method for the rapid determination of the presence of Cl. botulinum toxin in food and for determining its type if present. A group of three mice is inoculated with Type A antitoxin, another group with Type B, and a final group with Type C antitoxin.* A fourth group receives no antitoxin. In each group the three mice are given injections of 1.0, 0.5 and 0.1 cc. of the suspected material. One or more of the mice in the unprotected group and in two of the protected groups should show characteristic symptoms in a few hours.

Protective tests may be done with guinea pigs or mice using tetanus antitoxin to confirm the cultural and morphological diagnosis of Cl. tetani infection.

*There are two more types of Cl. botulinum, namely: D and E. Type D was isolated from African cattle and E. from spoiled fish in Russia.

Because of the small number of organisms that may be present at the site of injury in a tetanus infection it is frequently difficult to isolate the organism. The tissue at the site of injury should be excised. One half should be placed in a tube of cooked meat medium and the other in a second tube of the same medium. One of these should then be subjected to a temperature of 80 degrees C. for 10 minutes. Both should be incubated in the anaerobic jar. Because Cl. tetani grows frequently in symbiosis with aerobic organisms these cultures should be incubated for several (five or six) days. The unheated culture may be examined microscopically for the presence of Cl. tetani-like organisms. When these appear they may be separated from the other non-spore-forming organisms by the heat treatment. If other spore formers are present, Cl. tetani may be isolated by taking advantage of its tendency to form a fine film over agar. The bottom of a slant or the edge of a plate is inoculated with the material. After incubation, fishings are made from the edge of the fine membraneous film of growth.

The presence of proteolytic organisms, (Cl. sporogenes, Cl. histolyticum) may be detected through their action on whole meat medium. They digest the meat producing a blackening of the medium and a foul odor.

One of these proteolytic anaerobes (Cl. histolyticum) has a high capacity for digesting tissue in the body. The injection of 1 or 2 cc. of a glucose broth culture into the thigh of a guinea pig will cause an extensive disintegration of the tissue with a denudation of the bone within 24 hours. In spite of the severe lesions, the pig may appear to be perfectly well.

The proteolytic organisms are not in themselves pathogenic but serve to complicate wounds by their intense proteolytic action. In the absence of saccharolytic clostridia they do not interfere with the healing of a wound.

A convenient way of separating the two types of organisms is by intramuscular inoculation of animals. The saccharolytic organism is more invasive and may be isolated from the deeper tissues or organs.

Henry (J. Path. & Bact., 1917, 21, 244) has devised a "filter" of protected guinea pigs by which it is possible to separate out the pathogenic organism and inject the specific serum into the patient within 48 hours. He inoculates the unknown mixed culture into cooked meat medium and incubates. The next day he inoculates the supernatant fluid into milk and injects intramuscularly into two immunized guinea pigs, one guinea pig having received a mixture of Cl. perfringens and Cl. septique antitoxins, the other a mixture of Cl. perfringens and Cl. novyi antitoxins. The stormy fermentation of milk is diagnostic for Cl. perfringens and this reaction takes place within 24 hours. If the pig that was protected against Cl. septique (The Cl. perfringens factor having been eliminated in both pigs) dies, it indicates the presence of some other pathogenic anaerobe, probably Cl. novyi. The diagnosis of Cl. novyi is further indicated if the guinea pig that received the Cl. perfringens-Cl. novyi combination of sera recovers. If the animal inoculations came out the opposite way, the presence of Cl. septique would be indicated. The pathogenic organism can usually be isolated from the heart's blood of the animal that succumbs.

In summary, for routine bacteriological diagnosis of anaerobic infection, motility, morphology of the organism, reaction in milk and cooked meat, pathogenicity and protection of experimental animals with immune sera are the im-

portant differential characteristics.

NOTE. Special precautions should be taken with cultures, equipment and animals in which these highly resistant spore-forming organisms may be present. Extreme care must be taken to prevent the contamination of the laboratory equipment. When a needle or loop is employed, heat or flame with particular care to avoid splattering. If the culture or infected material is accidentally spilled, liberal applications of 2% commercial cresol should be applied and allowed to exert its action for several hours. Contaminated apparatus should be placed in the sterilizer and precautions taken to prevent the contamination of others.

MYCOBACTERIA

Of the acid-fast rods, Mycobacterium tuberculosis is of greatest importance for the diagnostic laboratory. Demonstration of the organism by smear and culture furnishes a presumptive diagnosis which may be confirmed by animal inoculation. Such confirmation is essential when specimens from the genito-urinary tract are studied because of the presence of the non-pathogenic acid-fast M. smegmatis on the external genitals. Methods for detection of the acid-fast organisms in sputum, urine, exudates, spinal fluid and feces by smear, culture and animal inoculation are described in the Appendix. The concentration procedures described are especially helpful in direct examination by smear.

Growth on glycerol potato agar and on nutrient agar together with pathogenicity may be used to differentiate between the human, bovine, avian and cold-blooded types of tubercle bacilli as indicated in Table 16. (Saprophytic acid-fast rods grow abundantly at 20 or 37 degrees C. in seven days on nutrient agar and are usually pigmented).

TABLE 16.

	Human	Bovine	Avian	Cold-blooded
Nutrient agar	37° C. no growth 4 weeks	37° C. no growth 4 weeks	37° C. scanty growth 4 weeks	20° C. moderate growth in 7 days
Glycerol potato agar	growth luxuriant, nodular, cream colored, 4 weeks	growth scanty thin, grayish 4 weeks	growth profuse, thin, creamy 4 weeks	growth profuse, nodular, cream colored 7 days
Grows at	41 deg. C.	41 deg. C.	43 deg. C.	20 deg. C.
Susceptibility of:				
Guinea pigs	++	++	+-	-
Rabbits	+-	++	+	-
Fowls	-	-	++	-
Frogs	-	-	-	++

GRAM-POSITIVE COCCI

Staphylococcus
Gaffkya
Sarcina
Streptococcus
Diplococcus

The family micrococcaceae (Bergey, 5th edition) includes a large number of usually gram-positive spherical cells which may be facultatively saprophytic or parasitic (Micrococcus, Gaffkya, Sarcina) and the usually parasitic Staphylococcus. These organisms grow readily on most bacteriological media and are diagnosed by means of their colonial and cellular morphology. The colonies are rather large, smooth, raised, opaque, moist and may be yellow-to golden or white.

When smeared and stained, staphylococcus colonies are found to consist of clusters of gram-positive cocci. In smears of pus or of young broth cultures they are found usually in pairs rather than in clumps.

The pigmented staphylococci are usually pathogenic while the non-pigmented are usually non-pathogenic organisms. Among the latter, however, pathogenic and occasional strongly toxinogenic organisms may be found. The pigmented pathogenic organisms are designated Staph. aureus and the non-pigmented organisms Staph. albus. If there is question of pigment formation in colonies of Staphylococcus, scraping the growth off of the surface of the agar with a loop or needle will in most cases reveal the presence of this pigment. At times permitting the culture to stand at room temperature for a day will bring forth the pigment. On Loeffler's slants the detection of pigment production is especially favored.

From the number of colonies on an inoculated plate and from the presence or absence of pigment one may ordinarily be able to decide as to the role played by this group of organisms in infectious processes. If there should be a question as to the pathogenicity of a culture, a tube of mannite may be inoculated. Pathogenic strains ferment this sugar while non-pathogens do not. The coagulase test may also be used for this purpose. (See Appendix).

Several types of staphylococcal toxin have been demonstrated but there is no easily detected cultural characteristic that reliably indicates the ability to produce these toxins.

The dermonecrotic, hemolytic and lethal (for animals) toxin and the enterotoxin which is responsible for food poisoning are produced by growing the organism in shallow broth or semi-solid agar medium in closed containers containing carbon dioxide (Casman J. Bact., 1940). The filtrates are titrated against the anti-alpha hemolysin antitoxin to measure the hemolytic, dermonecrotic and mouse-lethal toxin. The toxin may be detected through its ability to lyse washed rabbit red blood corpuscles, to necrotize the skin of the guinea pig and rabbit and to kill mice. It is destroyed by heat.

The enterotoxin may be detected best by means of the improved cat test described by Hammon in the American Journal of Public Health, 1941, 31, p 1191.

To perform the test, the cat, without anesthesia, should be placed on its

back on an animal board and the inner aspect of one thigh shaved to a point just below the knee. Slight pressure over the vein near the inguinal region enables one easily to see and enter the saphenous vein at about the level of the knee, or just above, with a sharp 26 gauge needle. After releasing the pressure above, the syringe and leg should be grasped together with one hand and the injection made slowly. Depending on the toxin, from 0.5 cc. to 5.0 cc. may be required. The toxin must, of course, be first treated to inactivate the lethal factor or factors. This is most conveniently done by heating a small amount for 30 minutes in a boiling water bath. The precipitate should be sedimented and the supernatant fluid used as inoculum. When heat is used, the supernatant fluid from well centrifuged cultures may be safely used without filtration.

A dosage of 2.0 cc. of toxin of average potency will produce a severe reaction on first inoculation. A somewhat less severe reaction will usually occur on repeating this dose, apparently regardless of the time interval following the previous inoculation. An increase of 50 to 100 per cent in dosage for the third inoculation gives a moderate or severe reaction. At the next injection a further increase in dosage is required. Because of the uncertainty of response and the increased volume of inoculum necessary, it is not advisable to use an animal a fourth time. One should not accept as final the result of a negative test unless repeated on at least 2 previously unused animals in a minimal dose of 3 cc.

The train of symptoms after an intravenous inoculation of enterotoxin is as follows: In from 15 minutes to 2 hours, most frequently in about 30 minutes, vomiting occurs, preceded by nausea. Coarse tremors are usually noted and the hair stands erect. The temperature rises to 104 degrees F. or 106 degrees F. attaining this maximum only after 2 to 4 hours. Diarrhea, although it occurs in most animals and is frequently very conspicuous, may occasionally be of a very mild nature. When present it usually persists for several hours. Vomiting may recur at intervals of from 5 minutes to 1 hour over a period of 2 or 3 hours. After 3 to 4 hours from the onset of symptoms the cat is frequently noticeably less ill and 24 to 48 hours later it appears normal if opportunity has been afforded for rehydration. Not infrequently animals develop diarrhea following inoculation of control materials, so vomiting only can be accepted as specific.

Since vomiting and occasionally diarrhea are conspicuous symptoms of a very common and highly infectious type of feline epizootic panleucopenia, it is quite important that the cats used be known to be in good health. Enders and Hammon (Proc.Soc.Exper.Biol & Med, 1940, 43,194) have described a satisfactory method of actively or passively immunizing experimental animals against this disease.

A moderately sized meal eaten shortly before the inoculation of enterotoxin has been found to increase the effectiveness of the vomiting stimulus, and noted refusal of the offered meal aids distinctly in the elimination of sick animals.

GAFFKYA

Gaffkya tetragena, a facultative saprophyte or parasite, is a gram-positive coccus occurring in tetrads. It may be found in sputum or saliva without having

any pathological significance. Occasionally the organism is found in the blood, in the pus of abscesses and in the spinal fluid of meningitis, indicating that some strains may be pathogenic for man.

GAFFKYA TETRAGENA

SARCINA

The genus Sarcina includes facultative saprophytes or parasites, which are gram-positive cocci dividing in three planes and producing regular packets.

(The term micrococcus is used to designate facultative saprophytes or parasites occurring in plates, groups or in irregular packets and masses while staphylococci are usually parasitic occurring singly, in pairs, in irregular groups and rarely in packets. When a few obviously contaminating colonies of staphylococcus-like colonies are obtained on culture, the term micrococcus may be employed rather than staphylococcus to indicate the probable non-pathogenic nature of the organism.)

STREPTOCOCCUS

There is a large and important group of spherically shaped organisms (cocci) which, because they multiply by division in one plane of space only and thus form chains of spheres, are grouped together. As a result of this solely morphological classification we have in one group a large number of organisms which differ markedly with respect to pathogenicity and biochemical and cultural characteristics. There is considerable overlapping of characteristics of members of this group and a considerable degree of variability exists. Sherman (Bact. Rev., 1937, 1, 3) discusses thoroughly the interrelationships of the members of this group.

For the classification of the streptococci, serological reactions, carbohydrate fermentation and action on erythrocytes have been used extensively. The sugar reactions are subject to considerable variation and do not always agree with serological groupings. Gunnison et al (J. Bact. 1940, 39, 689), however,

has by means of such studies been able to demonstrate some agreement between cultural reactions and the serological grouping procedures of Lancefield. Topley & Wilson's "Principles of Bacteriology and Immunity", (2nd edition), presents an excellent picture of the interrelationships of the three classification procedures.

Classification based on the changes produced on blood agar plates provides a simple practical working basis for the aerobic streptococci. Antigenic studies have revealed the fact that most of the streptococci pathogenic for man fall in one antigenic group (Group A of Lancefield) and that these organisms produce a soluble hemolysin. The use of a reliable and practical plating procedure for detection of these pathogens is of utmost importance. Since the hemolytic activity may be interfered with appreciably by small amounts of acid and by oxidative processes, the composition of the blood agar plate, the manner of inoculation of the plate (streak, pour or streak-pour) and the incubation procedure are of extreme importance. With these factors in mind the basic medium and candle-jar procedures described in this manual have been developed. The candle-jar procedure is a simple practical procedure permitting aerobic growth, supplying a small amount (about 3-4%) of growth stimulating carbon dioxide and reducing the oxygen tension to 17-16% so that inhibition of the hemolytic activity by this agent is reduced. The carbohydrate content of the basic medium is such as to permit maximum growth with minimum interference with hemolytic activity (Casman, J. Bact., 1942, vol. 43). (See Medium II in Appendix).

Using the above procedure, the surface streaking of blood agar plates may be employed for the isolation of the hemolytic streptococci(Beta), the viridans group of streptococci (Alpha) and those that produce no change on blood agar (Gamma). The following table (Table 17) presents these three groups of Streptococci.

TABLE 17.

Common Name	Brown's Classification	Changes on Blood Agar
Strep. viridans	Alpha (α)	Green zone with incomplete hemolysis
Strep. hemolyticus (pyogenes)	Beta (β)	Well defined clear zone of hemolysis
Strep. non-hemolyticus	Gamma (γ)	No change

Brown, recommending the use of poured rather than streaked plates differentiates the three groups by the changes produced in the deep blood agar as follows: Alpha colonies produce a somewhat greenish discoloration and partial hemolysis of the blood corpuscles immediately surrounding the colony, forming a rather indefinitely bounded zone 1-2 mm. in diameter, outside of which is a

second, narrow, clearer, not discolored zone. Under the microscope many corpuscles are seen in the inner zone, and these are obviously discolored, the discoloration varying in degree with different strains of streptococci. Very few corpuscles remain in the outer, clearer zone, and these are never discolored. These typical appearances may fail to appear after 24 hours, or even after 48 hours incubation, at the end of which time the narrow outer zone of hemolysis may not have developed. In such cases this zone makes its appearance during the subsequent 24 hours in the ice-chest.

The beta-hemolytic colonies are surrounded by sharply defined, clear, colorless zones of hemolysis. Under the microscope no corpuscles can be seen in this zone. These zones of hemolysis are often well developed after 18 hours incubation.

Alpha-prime (α') colonies are surrounded by a zone of hemolysis, which is slightly hazy, and less sharply limited than in the case of true beta-hemolysis. The colony itself is not sharply defined and examination with the microscope shows that the hemolysed zone contains, throughout, a moderate number of unaltered corpuscles which are most numerous in the immediate neighborhood of the colony. There is no discoloration.

Gamma (γ) streptococci develop in the blood agar without any change in the surrounding medium.

The above differences were noted using 5% horse blood in a meat infusion agar base. It has been observed that some strains giving alpha-prime colonies in horse blood agar may give typical alpha colonies in rabbit blood agar. Some streptococcal strains have been observed to give alpha type colonies on aerobically incubated blood agar plates and beta-hemolytic colonies on blood agar plates which were incubated anaerobically. Furthermore, all strains giving a beta-hemolytic type of colony on blood agar do not produce a soluble hemolysin. The production of such an hemolysin may be detected by growing the streptococcus in 20 per cent serum (use sheep or horse serum heated at 56 degrees C. for 45 minutes) broth for about 10 to 12 hours and then adding varying amounts (e.g. 0.1 to 1.0 cc.) of the culture to 0.5 cc. of a 5 per cent suspension of horse, rabbit or human red blood corpuscles. The mixtures are incubated at 37 degrees C. for two hours and then examined for hemolysis. Another convenient but crude procedure is to inoculate a blood agar slant and then add broth to almost the top of the slant. After 18 to 24 hours incubation, beta-hemolytic strains produce a zone of clearing in the agar which may be detected along the edges of the slant.

BETA-HEMOLYTIC STREPTOCOCCI.

From the clinical picture, the degree of purity of the culture and from the number of colonies of beta-hemolytic streptococci isolated from a specimen it is usually possible to estimate the etiological significance of these organisms. When only a few beta-hemolytic streptococci have been isolated from a specimen it may be desirable to learn more about the organism with regard especially to its pathogenicity. Determination of the Lancefield group into which the organism falls along with other studies may be of help in this respect as is indicated in Table 18.

TABLE 18.

Lancefield's Serological Group	Source	Relative pathogenicity for man	Tube hemolysis	Fibrinolysin	Hippurate	Trehalose	Sorbitol
A	Human	+ + +	+	+	-	+*	-
B	Human	+/-	+/-	-	+	+	-
	Bovine	?/-					
C	Human	+	+	+	-	+	-
	Animal	-		-	-	-	+
	Equine	-		-	-	-	-
D	Intestinal and genital	+/-	-	-	+/-	+*	+
E	Bovine	-	+	-	-	+	+
F	Human	+	-	-	-	+*	-
G	Human	+/?	+	+ or +/-	-	+*	-
H	Human	+/-	-	-	-	+*	-
K					-	-	-

(L)

(M)

* Some strains of A,D,F,G and H ferment neither trehalose nor sorbitol.

Such grouping may also be of help to the physician in therapeusis (Bliss, Long, Feinstone; South. Med. J., 1938, 31, 303-308). When other micro-organisms are also present in specimens or when hemolytic streptococci are isolated from milk or from nose and throat cultures, especially if they are examined in connection with outbreaks of streptococcal infections, additional data to indicate the probable pathogenic significance of the streptococci are essential. In such cases an attempt to determine the group antigenicity should be made.

It must be remembered, however, that mere demonstration of the presence of Group A streptococci is not always proof of the presence of pathogenic or highly infectious streptococci. Quarantine and release of convalescents from hemolytic streptococcus infections and of carriers cannot always be controlled by the demonstration of the presence or absence of Group A hemolytic streptococci. Such organisms have been demonstrated for long periods of time in the noses and throats of convalescents to whom no cases of infection could be traced.

While serological and cultural methods have been developed which permit a classification of the members of Lancefield's Group A into types, technical difficulties make their application for general use impractical. Griffith (J. Hyg.

1934, 34, 152) by means of a slide agglutination technique has been able to classify the Group A hemolytic streptococci into a large number of types. Keogh and Simmons (J. Path. and Bact., 1940, 50, 137-144) claim to be able to classify the members of Group A into types corresponding to Griffith's (although less completely) by their differential reactions on starch, mannitol and cellobiose, by colonial morphology in a special agar medium, and by the production of opalescence in serum broth.

For practical purposes the group precipitin test as described by Brown (See section on serological diagnostic procedures) may be used. Sera for Groups A, B and C, suitable for this test, are now available on the market.

If in an epidemiological study, sera for the demonstration of an antigenic relationship between strains isolated from food or another suspected vector on one hand and from the patient on the other, are not available,biochemical studies may be used to reveal a similarity or difference between these strains. While in some cases, as for example, in the study of strains isolated from milk in an epidemiological investigation of an outbreak of hemolytic streptococcus infection, it may be possible to differentiate Group B organisms commonly found in milk from Group A organisms by tests for hydrolysis of sodium hippurate, it is apparent from the above table that certain strains of Group C resemble Group A strains in this characteristic as well as in the fermentation of trehalose and sorbitol. Thus if biochemical characteristics are relied upon entirely, wrong conclusions may be made in regard to the group classification.

Surface colonies of beta streptococci are usually small, gray, rather opaque and show definite areas of hemolysis surrounding the colony. In the candle-jar incubation procedure the surface colonies frequently grow to a rather large size (5 - 7 mm. in diameter) and may be surrounded by only a narrow band of hemolysis (1 - 1.5 mm. or less).

A smear of the colony should be made and stained with Gram's. If there is any question as to the streptococcic nature of the organism, a tube of broth should be inoculated. In broth, chain formation is more easily detected. From this broth culture (after 18 to 24 hours of incubation) grouping by means of the precipitin test may be carried out. Chain formation in broth and production of hemolysin may be determined by inoculating a blood agar slant, covering it with broth and incubating for 18 to 24 hours.

If more information about the strain is desired the following media may be inoculated: phenol red broth base (See Appendix) containing 1 per cent trehalose, and the same base containing 1 per cent sorbitol, broth plus sodium hippurate, a Loeffler's serum slant and a tube of broth containing a few drops of serum or ascitic fluid, - the latter for a fibrinolysin test (Appendix) and, if desired, an hemolysin production test. If the Loeffler's serum slant shows evidence of liquefaction the organism is probably Streptococcus zymogenes. This is a proteolytic streptococcus belonging with Streptococcus liquefaciens (alpha or gamma hemolysis) to Group D of Lancefield, fermenting lactose, salicin, and mannite, liquefying Loeffler's medium and gelatin, hydrolysing sodium hippurate and peptonizing milk. Two weeks incubation may be required for the detection of the action on Loeffler's medium, gelatin, milk and sodium hippurate. For the detection of hydrolysis of sodium hippurate, the ferric chloride reagent of Ayers and Rupp is used (Appendix).

Reporting results.

The presence of hemolytic streptococci in a specimen should be reported as soon as possible to the physician who submitted the specimen. This should ordinarily be possible after overnight incubation of streaked blood agar plates.

The report of the results of group precipitation tests should follow promptly and include an explanatory note concerning the usual source of strains of the various groups. For example: "Hemolytic streptococci of Group A which is commonly associated with human infections." If the streptococci do not belong to Group A, the report should state that hemolytic streptococci were found which differ in their serologic properties from those usually encountered in human disease. Whenever, from the clinical history, the source of the specimen, or other data, further explanation of the findings seems desirable, the report should indicate that hemolytic streptococci of groups other than A occasionally incite disease in man and should suggest that a second specimen be submitted. The results of examinations of milk specimens are similarly reported, that is, hemolytic streptococci of serologic Group B which is commonly associated with bovine mastitis, hemolytic streptococci of serologic Group C which is commonly associated with infections in animals and occasionally with bovine mastitis.

ALPHA STREPTOCOCCI.

The term Strep. viridans (alpha streptococci of Brown and Smith) includes a large number of streptococci which on blood agar give a green coloration to the medium and produce a partial hemolysis. These organisms are saprophytic and parasitic and some may be moderately pathogenic.

The surface colonies are raised, convex, often quite hard and on smear may show round, elongated or pleomorphic gram-positive cocci in pairs and short or long chains.

Some enterococci (Strep. fecalis) require 48 hours of incubation before a green discoloration of the agar can be detected.

Occasionally strains of alpha streptococci are encountered which are so pleomorphic that their identity as streptococci may be missed. These strains when grown on solid media may show bacillary forms resembling diphtheroids and also swollen, round and elongated, coccoid, and less commonly, filamentous forms. When grown in broth, however, they are much less pleomorphic, usually taking on a typical streptococcic morphology.

Most strains of alpha streptococci grow in broth to give a coarse flocculent sediment. A uniform turbidity is not obtained. The Group D strains (Enterococci), however, produce a marked turbidity and a small amount of smooth sediment. A member of this group, Strep. liquefaciens, is capable of liquefying Loeffler's serum.

In the candle-jar incubation procedure some alpha streptococci may show colonies that are large and flat resembling the pneumococcus colony. The pneumococcus may be differentiated from the alpha streptococcus by smear, solubility in bile or in sodium desoxycholate, by the fermentation of inulin, by the flatness and greater transparency of the colony, by the demonstration of a capsule and by the type of growth in broth as indicated in the following table. (Table 19). (See section on Diplococcus pneumoniae - page 66).

TABLE 19.

DIFFERENTIATION OF PNEUMOCOCCI AND STREPTOCOCCI

Organism	Solubility, bile	Solubility in desoxycholate	Inulin*	Capsule‡	Growth in broth
Pneumococci	+	+	+	+	Uniform turbidity
Streptococci (Alpha)	-	-	-	-	Flocculent sediment (Usually)
Streptococci (Beta)	-	-	-	+/-	Flocculent sediment and uniform turbidity

* Some pneumococci fail to attack inulin and some alpha streptococci have been encountered which do attack this carbohydrate.

‡ Use beef or rabbit serum as diluent when the organisms are spread on the slide for staining.

GAMMA STREPTOCOCCI (Non-hemolytic, non-discoloring).

The non-hemolytic (gamma) streptococci produce no changes on blood agar. The surface colonies on blood agar are small, gray or colorless and translucent. Smears show gram-positive (frequently elongated) cocci in pairs, chains and small groups. As a rule the pathogenicity of this group is slight.

Strep. fecalis, an inhabitant of the intestinal tract, sometimes known as the enterococcus and almost identical with Strep. lactis of milk is sometimes classified as having alpha or gamma characteristics. It is characterized by its resistance to heat, by its fermentation of mannitol and lactose and by the fact that its growth is not inhibited by bile salts. It will resist exposure to a temperature of 60-62° C. for 30 minutes.

ANAEROBIC STREPTOCOCCI.

There are streptococci which while failing to grow on the surface of an aerobically incubated blood agar plate, will grow in anaerobic media or on a blood agar plate which is incubated anaerobically.

Such organisms may require anaerobic methods for primary isolation, but after cultivation, may grow aerobically.

Brewer's medium or better semi-solid shake tubes (0.1% glucose, 0.3% agar in the basic medium) may be employed for growing these organisms.

There is as yet no satisfactory classification of the anaerobic streptococci.

There are many micro-aerophilic types between the aerobic and anaerobic streptococci. The semi-solid shake tube may be used to demonstrate their presence.

DIPLOCOCCUS PNEUMONIAE

Rapid identification of the organism as a pneumococcus is obtained if on mixing with a homologous and suitable antiserum, swelling (Quellung) of the capsule is obtained. (See section on serological procedures for technique).

Type III pneumococci on blood agar give rise to large, moist, raised colonies which on further incubation become flat. In the candle-jar other types of pneumococci may grow in this manner. Type III pneumococcus may be differentiated from other pneumococci in that it forms chains although on solid media it may appear in the diplococcus form.

If pneumococci are growing on blood agar in what appears to be pure culture, they may be scraped off of the agar and suspended in saline for immediate typing by the Quellung test and for bile or desoxycholate solubility. The desoxycholate lysate may be cleared by centrifugation and a ring precipitin test performed with it against specific sera if desired (See section on serological procedures). When sodium desoxycholate is used to determine the solubility of organisms it is important that the medium used for the cultivation of the organism not contain dextrose. Acid formed as a result of the fermentation of this sugar tends to precipitate the sodium desoxycholate. Sodium citrate if present in appreciable quantity may precipitate the desoxycholate and so obscure the result of the test. The use of desoxycholate is advantageous in that the lytic action is obtained in a few minutes. It is done by adding 2 drops of 10% sodium desoxycholate to 1.0 cc. of the broth culture. Pneumococci are usually dissolved in less than one minute but the test should be held for 10 or 15 minutes before discarding as negative.

Since the basic broth (Medium I) contains added fermentable carbohydrate, desoxycholate solubility should not be used for the study of cultures in this broth unless the reaction of the culture is first carefully adjusted to one of about neutrality (pH 6.8-7.2). If bile is employed, such adjustment is not necessary.

Greey (J. Inf. Dis., 1939, 64, 206) (See Appendix) reported excellent results using dry Bacto-Oxgall sprinkled directly on blood agar plates for the detection of pneumococcus colonies. According to this procedure pneumococcus colonies are dissolved and disappear entirely, but leave evidence of their presence by means of the fixed blood cells in the clear medium. Colonies of Strep. viridans are not dissolved or otherwise altered. This procedure may be used to advantage in plates incubated in the candle-jar. Occasionally colonies of pneumococci encountered at primary isolation will resist lysis by this method. When transferred to broth or to a second blood agar plate they become susceptible to the lytic action of the bile. (See Appendix).

Since freshly isolated strains of pneumococci grow best in the presence of blood or serum, in order to obtain a good broth culture conveniently, a blood agar slant should be inoculated and then covered with broth. It may be advisable to wait two or three hours before adding the broth. Bile and desoxycholate solubility as well as typing may be done with such broth cultures.

Mouse inoculation is an excellent procedure for the isolation and typing of the pneumococcus from sputum. The mouse seems to be so selective a medium for the pneumococcus that when sputum is inoculated into the peritoneal cavity the peritoneal exudate contains a bacterial flora after about four hours in which the pneumococcus predominates. Typing may be done using this exudate. The mouse usually succumbs in 15 - 18 hours and the pneumococcus may be isolated in pure culture from the heart blood. The use of the mouse is recommended when the direct Neufeld typing of sputum is negative in cases of lobar pneumonia. If mice are not available the specimen may be inoculated into a modified Avery broth (Appendix) and the culture examined by the Neufeld technique after 3 to 6 hours incubation at 37 degrees C. If no pneumococci are found at this time the cultures should be reincubated and reexamined at intervals up to 16 - 18 hours after inoculation.

Inoculation of a Loeffler serum slant may be advisable if difficulty is experienced in attempting to demonstrate capsule formation. On this medium the pneumococcus appears to produce well defined capsules.

Because of the aid that may be provided the physician in his treatment of infections caused by the pneumococcus, typing of pneumococci should be executed as soon as possible whenever serum therapy may be indicated. Reports should be made promptly by telephone or telegraph.

THE GRAM-NEGATIVE COCCI

The strictly parasitic gram-negative cocci are, with the exception of N. gonorrheae, usually inhabitants of the nasopharynx. Certain members of the group are markedly pathogenic.

Growth requirements and carbohydrate fermentation are useful in the differentiation of these organisms as indicated in the following table. (Table 20)

TABLE 20.

Species	Growth in plain agar	Growth at 22 C	Carbohydrate fermentation			Pigment
			dextrose	maltose	sucrose	
N. gonorrheae	-	-	+	-	-	none
N. intracellularis	-	-	+	+	-	none
N. catarrhalis	+	+	-	-	-	none
N. sicca	+	+	+	+	+	none
N. perflava	+	-	+	+	+	yellow
N. flava	-	-	+	+	-	yellow
N. subflava	+ -	+ -	+	+	-	yellow
N. flavescens	+ -	?	-	-	-	yellow

The meningococcus (N. intracellularis) and gonococcus (N. gonorrheae) will not grow on most* agar media very well unless these are enriched by blood or serum. These organisms also differ from the other members of the group in that they do not grow below 30 degrees C. The gonococcus differs from the meningococcus with regard to growth requirements in that it is more fastidious. The meningococcus grows well on blood agar whereas the gonococcus yields moderate to sparse growth on this medium and may require chocolate blood agar for good growth. The growth of both organisms is favored by the presence of 5-10% carbon dioxide in the atmosphere.

The colonial morphology of these organisms may also be used for their differentiation. The colonies of N. intracellularis and N. gonorrheae are translucent, smooth and round and are easily emulsified. Those of the former are usually larger than those of N. gonorrheae being about 2-3 mm. in diameter and sometimes 1 cm. Colonies of N. catarrhalis are more opaque and white while those of N. sicca are so dry and adherent that they can be taken up entirely by means of a needle, or can be pushed about. Colonies of the pigmented members, i.e., N. flava, N. subflava, N. perflava and N. flavescens may resemble the meningococcus on the first day but they gradually become more opaque and develop a yellow color.

Fermentation of dextrose and maltose are useful for the differentiation of N. gonorrheae, N. intracellularis and N. catarrhalis (See Table 20). These studies may be done by inoculating tubes of semi-solid agar or shallow layers of broth containing the sugars and an indicator (See Appendix). Forty eight hours of incubation may be necessary before fermentation is detected.

The meningococcus is defined by Branham (Diagnostic Procedures and Reagents, 1941) as a "Gram-negative coccus, usually occurring in pairs with flattened adjacent sides, which ferments dextrose and maltose with the production of acid, forms characteristic colonies, does not produce pigment, usually grows only at incubator temperatures and is, as a rule, at some time agglutinable by polyvalent anti-meningococcus serum". These characteristics may be detected by inoculating blood agar with portions of colonies of gram-negative diplococci suspected of being N. intracellularis colonies and then from these pure cultures, inoculating, plain agar, two blood agar slants, a Loeffler slant (for the detection of pigment production) and flasks of shallow layer serum broth (or Proteose #3 peptone broth) containing 0.5% dextrose and 0.5% maltose. One of the blood agar slants should be kept at room temperature. An agglutination test may be carried out with polyvalent anti-meningococcic serum using the growth from one slant as a source of antigen. A one tube agglutination with diluted serum and controls is adequate. The antigen suspension should not be too heavy (#3 on the McFarland nephelometer scale). Three tubes may be set up as follows: (1) 0.5 cc. of polyvalent 1:20 meningococcus serum, plus 0.5 cc. bacterial suspension.

* See section on media for Mueller's starch agar medium for cultivating the meningococcus.

(2) 0.5 cc. normal 1:20 horse serum, plus 0.5 cc. bacterial suspension. (3) 0.5 cc. salt solution plus 0.5 cc. bacterial suspension. The tubes are shaken continuously and vigorously for 5 minutes and read. They are then incubated at 56 degrees C. for 30 to 60 minutes or longer and any difference in appearance noted. In view of the demonstration, by Miller and Boor, (J. Exp. Med., 1934, 59, 75) of common protein and polysaccharide antigens and haptenes among gonococci and meningococci, it is doubtful whether reliable differentiation can be made in all cases by serological methods.

Slide agglutination may be conveniently employed for the detection of the meningococcus with polyvalent antiserum or for typing with type-specific antisera. Antisera for types I, II, IIA and IV may be obtained from the Lederle Laboratories. In the performance of the slide agglutination test the antisera are diluted 1:10 with 0.85 per cent sodium chloride solution and a loopful of each placed on a slide. A small portion of the culture is rubbed up in these drops and the whole then examined either microscopically or macroscopically for agglutination. If there is any doubt as to the result obtained with the slide agglutination technic confirmation with the macroscopic tube agglutination procedure should be employed.

While many strains seem to be inagglutinable when first recovered from patients, practically all can be coaxed to agglutinate later.

The necessity for early administration of chemotherapeutic agents or therapeutic sera in meningococcic meningitis requires an early laboratory diagnosis usually from examination of the spinal fluid. In smears of spinal fluid the meningococci are usually found intracellularly. Since the organisms autolyze readily, it is not uncommon to experience difficulty in finding the organisms. One must be careful to avoid confusing the meningococcus with H. influenzae which, in spinal fluid, may take on a cocco-bacillus morphology.

The gonococcus is the most difficult organism in this group to cultivate. While ordinary blood agar will support its growth, the use of chocolate blood agar (Appendix) will give a higher percentage of isolations. The use of the "candle-jar" method of incubation furnishes the carbon dioxide which is an important adjunct for the support of growth of this organism. A fresh, moist medium is essential for successful cultivation of the gonococcus. The oxidase test may be used for detecting N. gonorrheae colonies. (See section on handling of specimens and also Appendix).

N. gonorrheae may be differentiated from the meningococcus by sugar fermentation (See above). The gonococcus colony on chocolate agar is convex, transparent, from 1 to 3 mm. in diameter and has undulated margins. It is grayish and slightly opaque by transmitted light. The organism fails to grow on plain agar and at room temperature (a few strains have been observed to grow at room temperature on unenriched media).

Routinely the laboratory diagnosis of gonorrhea may be made by (1) smear, (2) culture and (3) serological tests.

For diagnosis by smear, a Gram stained smear of the pus to be examined is made. The presence of intracellular gram-negative diplococci is usually considered diagnostic.

In medico-legal cases, when the findings by smear contradict the diagnosis, and in the absence of a history of exposure, confirmation by cultural procedures is necessary. The demonstration of culturally typical N. gonorrheae (by means of growth requirements, type of growth, oxidase test, sugar fermentation) is usually sufficient. The inoculation of chocolate and blood agar with the original specimen for the isolation of the gonococcus in pure culture, and then the inoculation of plain agar, two blood agar slants, chocolate agar, and shallow layer dextrose and maltose broths will provide the necessary information. (One blood agar slant should be incubated at room temperature).

The most satisfactory serological procedure for diagnosis of gonorrhea is the complement-fixation test. This test is described by Carpenter in Diagnostic Procedures and Reagents - 1941, pp 104 - 110.

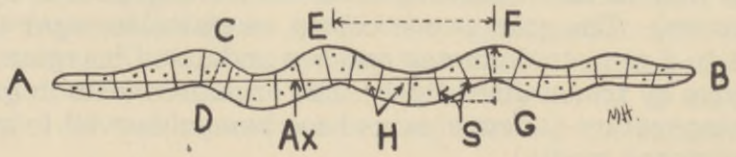
NOTE: Candle-jar (3-4% CO_2 and 16-17% O_2) incubation should be employed with all cultures except reduced tension slants when working with the gram-negative diplococci.

(B) SPIROCHAETES

Dark-field examination is the method of choice in examining material for spirochaetes, particularly for Treponema pallidum, the causative agent of syphilis. The spirochaetes have a poor affinity for dyes. Impregnation with silver nitrate, in smears by Fontana's method and in tissues by Levaditi's method requires considerable time and patience.

Species identification is based largely on morphology and movement of the spirochaete. The mean length and width, the number, length and depth of the spirals and the type and rapidity of movement are the chief distinguishing characteristics. There are six genera, three of which contain pathogenic species. The separation of these genera is based upon morphology and motility in the host and in cultures. The dimensions that are usually taken are given in the following figure:

DIAGRAM OF TYPICAL SPIROCHAETE



AB, length; CD, width; EF, spiral length; FG, spiral depth; AX, axial filaments; S, septa; H, metachromatic granules

It is difficult to determine the differential characteristics in smears, since the spiral morphology may be altered artificially. Motility and flexibility also cannot be relied on in smears.

The six genera are listed in Bergey's Manual as follows:

1. Spirochaeta - - Free-living forms in water, characterized by their large size.

2. Saprospira - - Free-living forms in foraminiferous sand, characterized by their large size, their shallow, irregular waves, and their chambered bodies.

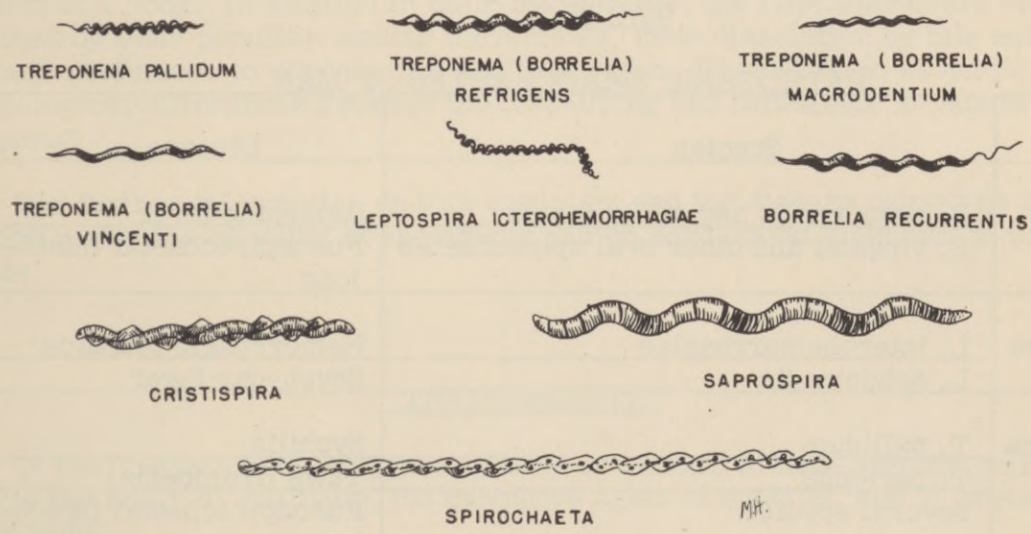
3. Cristispira - - Parasites in the alimentary canal of lamellibranchiate mollusks, characterized by their fairly large size, chambered bodies, and a band-like membrane or crista, which runs in a spiral manner along the parasite.

4. Borrelia - - This genus includes numerous nonpathogenic and pathogenic species. The usual habitat is the blood. The organism terminates in a fine terminal spiral filament. Growth in cultures is aerobic.

5. Treponema - - Pathogenic and nonpathogenic species are found in this genus. They are aerobic and anaerobic tissue parasites. The ends are pointed and the spirals are closely or loosely wound.

6. Leptospira - - The species of this genus are characterized by tapering ends bent in a semicircular hook and by numerous closely wound spirals. Growth in cultures is aerobic.

Typical species of each genus are sketched in the following figure:



The important differential characteristics of these genera are given in Table 21.

TABLE 21.

CLASSIFICATION OF SPIROCHAETES

	Spirochaeta	Saprospira	Cristispira	Borrelia	Leptospira	Treponema
Length in micra	100-500	100-120	45-90	8-16	7-14	6-14
Ends	Blunt	Blunt	Blunt	Pointed	Pointed Hooked	Pointed
Spiral filament at end	Absent	Absent	Absent	Present	Not recog-nized	Present
Chambered structure	Absent	Present	Present	Absent	Absent	Absent
Crista	Absent	Absent	Present	Absent	Absent	Absent
Relation to oxygen	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic	Aerobic Anaerobic
Habitat	Water	Foraminif-erous sand	Mollusks	Animals Blood Tissues	Animals Blood Tissues	Animals Tissues
Species parasitic to man	No	No	No	Yes	Yes	Yes

The following table presents some of the more important species which are causative agents of disease in man. (Table 22).

TABLE 22.

PATHOGENIC SPIROCHAETES OF MAN

Genus	Species	Disease
Borrelia	B. recurrentis and allied forms	Relapsing fever
	B. vincenti and other oral spirochaetes	Fusospirochaetal infect-ions
Leptospira	L. icterohemorrhagiae	Hemorrhagic jaundice
	L. hebdomadis	Seven-day fever
Treponema	T. pallidum	Syphilis
	T. pertenue	Yaws (frambesia)
	Several species	Fusospirochaetal infect-ions

BORRELIA

The genus Borrelia according to the classification of Bergey comprises two somewhat dissimilar groups or organisms: (1) the pathogenic blood spirochaetes and (2) certain commensal spirochaetes associated with the fuso-spirochaetal diseases. The latter are classed by many workers, perhaps more properly with the tissue parasites of the genus Treponema because of their general characteristics and habitat.

Borrelia recurrentis, the type species, in the blood, has a corkscrew-like rotation around its long axis and a lateral oscillation. It travels forward and backward for short distances. It is pathogenic for mice, rats, and monkeys. Rabbitts, guinea pigs and dogs are resistant. During the first febrile period in man the organisms are fairly numerous in the blood, but they may be difficult to find during the relapses. During the afebrile periods they are found mostly in the tissues, especially those of the brain and spleen.

Diagnosis may be made by examining cover-glass preparations of fresh blood for actively moving spirochaetes with or without dark-field technique. Blood films after wet fixation may be stained with Giemsa's stain. When organisms are scarce, as in relapses and during afebrile periods, injection of mice will produce in 24 hours, large numbers of organisms in the animal's blood. Any route of inoculation may be used but the cutaneous application of the infective material gives the highest percentage of experimental infections.

LEPTOSPIRA

The spirochaetes of the genus Leptospira are slender, highly flexible filaments with closely wound regular spirals and tapering extremities often bent in the form of a hook. In addition to their morphology, the Leptospirae are characterized by their peculiar lashing movements, their dissolution by bile salts and their resistance to saponin and distilled water. There are no essential morphological differences between the free-living and pathogenic members of this genus.

For further information on this organism and the disease caused by it see the sections on the handling of specimens and on serological diagnosis of the disease.

TREPONEMATA

Of the Treponemata, only two species are known to have specific pathogenicity for man: T. pallidum, the causative agent of syphilis, and T. pertenue, that of yaws.

T. pallidum is motile, showing corkscrew rotation about the long axis, a forward and backward gliding progression, and flexion of the entire body. It will grow when injected into rabbit's testes, and strains can be perpetuated by this means. However, the presence of T. cuniculi, the cause of rabbit spirochaetosis, complicates the experimental work with these animals. This organism is morphologically indistinguishable from T. pallidum and produces a localized infection which superficially resembles that caused by T. pallidum in the rabbit.

The laboratory diagnosis of syphilis is based upon the detection of spirochaetes in the lesions or upon serological procedures such as the Wassermann and floeculative tests. In cerebrospinal syphilis, special confirmatory tests may be made with the spinal fluid such as the colloidal gold and mastic tests. See the sections on the handling of genito-urinary specimens and special procedures (in the appendix) for further description of methods employed in the diagnosis of syphilis.

One should bear in mind that in specimens from the mouth T. pallidum may be confused with several oral spirochaetes which have a similar appearance. In genital lesions it must be differentiated from the more actively motile B. refringens.

T. pertenue, the causative agent of yaws or frambesia, is morphologically practically identical with T. pallidum. The Wassermann and floccula-tive tests give positive reactions in as high as 90 per cent of patients infected with yaws. The reaction depends upon the duration of the infection, the number and intensity of the lesions, and the number of superinfections. Serological and cutaneous tests are not of aid in differentiating yaws and syphilis.

Diagnosis in this disease is made as in syphilis by demonstrating T. pertenue in the lesions. The organisms are present in practically all intact lesions, in a high percentage of ulcerative lesions, and infrequently in old lesions. It is difficult to identify T. pertenue in ulcerative lesions since they may be few in number and masked by the presence of numerous extraneous spirochaetes. Smears stained with Giemsa's stain are examined or dark-field technique is employed. The Wassermann and flocculative tests furnish additional though incomplete evidence.

The mucocutaneous spirochaetes in association with fusiform bacilli, vibrios and cocci produce a wide range of acute and chronic lesions in various parts of the body. These diseases are probably produced by several species of spirochaetes in symbiotic relationship with the fusiform bacilli and other bacteria.

Table 23 gives the names of the more important fusospirochaetal diseases, their location in the body, and the common spirochaetes associated with the fusiform bacilli and other organisms.

TABLE 23.

FUSOSPIROCHAETAL DISEASES

Organ	Disease	Treponemata	Other Bacteria
Oral cavity and throat	Vincent's angina	B. vincenti T. macrodentium T. microdentium T. mucosum	Fusiform bacilli Cocci Vibrios Spirilla
	Trench mouth	Same B. buccalis	Same
	Gangrenous stomatitis (Noma)	Same	Same
Respiratory tract	Bronchial and pulmonary gangrene	Same	Same
Skin	Phagedenic ulcer	Same	Same
Genitalia	Gangrenous balanitis	Same T. balanitis and other genital spirochaetes	Fusiform bacilli Genital flora

Vincent's angina and necrotic gingivitis (trench mouth), the most common of these diseases are diagnosed in the laboratory by demonstrating the presence of the spirochaetes together with fusiform bacilli in large numbers. Smears stained by Gram's procedure, by several minutes of exposure to methylene blue or best by means of 10 to 15 seconds exposure to carbol fuchsin, are examined.

Smear from case of
VINCENTS ANGINA

(C) RICKETTSIAE

Rickettsiae, in recent years have come to be recognized as the etiological agents for a very important group of diseases: typhus, Rocky Mountain spotted fever, tsutsugamushi fever, Q fever and possibly trench fever. The rickettsiae are pleomorphic organisms of small size, usually less than half a micron in diameter, found intracellularly in arthropods and staining poorly with bacterial stains. The size of the organism depends upon its origin. In the human host it

is small, but in culture it becomes larger. Impression smears of the cut surfaces of organs made before fixation with formalin and stained with Machiavelli's stain give the best results. Rickettsiae in tissue section stain best with Giemsa's stain, but for tissue cultures the best method is the Machiavelli stain (see section on stains). They are bacteria-like in that they have a bacterial morphology and are easily visible. They resemble viruses, however, in that they can be cultivated only in the presence of living cells.

The experimental animal is best used for the isolation of these organisms. The organisms may be isolated from infected lice, ticks, fleas, or blood obtained from the patient in the early stages of the disease. After isolation through the use of the experimental animal, suitable culture media may be inoculated with the brain, spleen, blood, tunica vaginalis or scrotal tissue. Lice, tissue cultures and the yolk-sac of the developing chick embryo may be employed for culture.

The diagnostic test most commonly used in rickettsial disease is the Weil-Felix agglutination reaction (see section on serological procedures). This test, however, is positive in several diseases of this group. If facilities are available, more specific diagnostic information may be obtained by a study of the pathogenicity and immunological reactions produced by the rickettsiae in susceptible laboratory animals. These procedures, described below, require the services of specially trained personnel and facilities not available in most laboratories.

Previous to 1927 the problem of differentiation between the rickettsial diseases was not recognized since the various pathogenic members of this group appeared to be sharply separated on the basis of geographic distribution. Investigations carried out subsequently have completely broken down the geographic barriers so that it now seems probable that the rickettsiae of typhus and spotted fever may be present in practically all parts of the world.

The rickettsial diseases of man at present fall into four main groups: (1) the typhus group, (2) the Rocky Mountain spotted fever group, (3) the tsutsugamushi or Japanese river fever group and (4) Q fever. Trench fever is considered by some to be caused by a rickettsia and should perhaps be included among the rickettsial diseases. These diseases, the rickettsiae involved and some of the reservoirs and vectors are indicated in the etiological and clinical classification presented in Table 24.

It is possible that trachoma may be included in the rickettsial diseases since according to Cuenod and Natef (Brit. J. Ophth., 1937, vol. 21, p. 309), rickettsiae multiply in great numbers in the intestinal tracts of lice, following the injection of material from the conjunctiva of trachoma patients. With these cultures in lice they claim to have produced trachoma. This work has not been confirmed as yet.

TABLE 24.

ETIOLOGICAL AND CLINICAL CLASSIFICATION OF RICKETTSIAL DISEASES.

1		2	3
Typhus Fever		Rocky Mt. Spotted Fever Group	Japanese River Fever Group
Rickettsia prowazeki		Rickettsia rickettsi (Dermocentroxenus)	R. tsutsugamushi (R. orientalis)
var. prowazeki	var. mooseri		
Human strain cause of: "Classical" "European" or "Epidemic" - typhus	Murine strain cause of: "Mexican" "New World" or "Endemic" - typhus	cause of: "Tick" or "Tick-bite" - typhus	cause of: "Mite" "Scrub" "Tropical" "Rural" - typhus
Reservoir: Unknown	Reservoir: Rat	Reservoir: Rodents Dogs Rabbits	Reservoir: Field mice Rat
Vector: Louse	Vector: Rat flea	Vector: Ticks	Vector: Mites

with no primary sore		with primary sore	
In U.S.A.	In Brazil	In Mediter. area	In So. Africa
Rocky Mt. Spotted Fever	Sao Paulo or Brazilian typhus	Fievre boutonneuse	So. African tick-bite fever

4 "Q" Fever, a rickettsial (R. brunetti) tick-borne disease of Australia, immunologically distinct from the above and producing no rash. "Nine Mile" Fever (American "Q" Fever) a rickettsial (R. diaporica) tick-borne disease of Montana, identical with Australian "Q" Fever.

(5) Trench Fever.

A relatively new group of rickettsial diseases without a rash has been added to those hitherto recognized. "Nine Mile Fever" (American "Q" Fever) a rickettsial disease transmitted to man by the tick Dermacenter andersoni and not related to Rocky Mountain Spotted Fever has been observed in Montana. This disease appears to be identical with "Q" fever of Australia. The Weil-Felix reaction is negative in "Q" fever.

There is but one proved rickettsial disease that is limited to animals - - the tick-transmitted heartwater disease of sheep, goats and cattle.

The literature contains descriptions of a very large number of rickettsial diseases, often named after the regions where they occur, or after the individuals who first observed them. This degree of complexity appears to be neither necessary nor justifiable in the nomenclature of these various diseases. In so far as thorough studies of a number of these various diseases have been made, it has been found that they are variant strains of either typhus or of spotted fever.

Leaving trench fever out of consideration, therefore, the only other rickettsial diseases to be considered as separate from typhus and spotted fever are the mite-borne diseases of the Far East and the recently described "Q" fever and Nine Mile fever; so that in the United States, the diagnostic problem resolves itself into the differentiation between typhus, spotted fever, and Nine Mile fever. Typhus may be subdivided into endemic (murine) and epidemic (classical European) typhus.

TYPHUS GROUP

Two types of typhus are generally recognized; one, the classic European or epidemic type; the other, the murine or endemic type, so called because of its natural reservoir in rats. The European type is the epidemic type of world-wide occurrence. It exists in endemic form in many countries. Brill's disease in the United States is believed by some to be of the epidemic type.

The murine type is endemic in many countries, especially in warm climates. Murine typhus can be distinguished from classic epidemic typhus only by passage through the rat. There are also immunologic differences. In contrast to the European typhus it produces a febrile reaction in the rat and, after intraperitoneal inoculation, more exudate into the tunica vaginalis, accompanied by greater numbers of rickettsiae. The scrotal sac is considerably swollen when large (500 gram) guinea pigs are employed and Neil-Mooser bodies may be observed. The European type usually does not produce a scrotal swelling and Neil-Mooser bodies, although some exceptions may occur.

ROCKY MOUNTAIN SPOTTED FEVER GROUP

There appears to be no good reason for separating spotted fever disease as it occurs in the south and east of the United States from that of the northwestern states. This group includes, in addition, the Sao Paulo typhus of Brazil, Tobia fever of Colombia, fievre boutonneuse of the Mediterranean countries, and South African tick fever.

Fievre boutonneuse and South African tick-bite fever are characterized by a primary sore at the site of the tick bite, accompanied by adenitis of the regional lymph nodes. Cross immunity tests indicate that fievre boutonneuse (with primary sore) and South African tick-bite fever (with primary sore) may be distinct diseases. Clinically they are much alike.

Rocky Mountain spotted fever and the Sao Paulo typhus are not characterized by primary sores and buboes. They are without doubt the same disease.

JAPANESE RIVER VALLEY FEVER GROUP

The tsutsugamushi group - includes the classic tsutsugamushi disease of Japan, the rural typhus of Malaya and the mite fever of Sumatra. The tsutsugamushi disease of Japan and the mite fever of Sumatra develop primary sores at the site of the tick bite, accompanied by adenitis of the regional lymph nodes. The rural typhus of Malaya is not accompanied by a primary sore and bubo, although in all other respects the disease is identical with tsutsugamushi disease. In contrast to the Rocky Mountain spotted fever and typhus groups, the members of the tsutsugamushi group are difficult to establish in animals.

The intraperitoneal injection of white mice has been used for the isolation of the rickettsiae of scrub typhus.

DIAGNOSTIC PROCEDURES IN RICKETTSIAL DISEASES.

Clinically, the distribution of the rash is helpful. In spotted fever, the rash develops first on the extremities and then becomes generalized involving the palms and the soles, which are spared in typhus. This criterion is valuable only in cases which have a well developed rash. In many cases of the disease the rash may be mild in degree. In typhus the rash appears first on the trunk and then spreads to the extremities.

A definite history of tick bite is circumstantial evidence of spotted fever or "Q" fever infection, since typhus is never carried by ticks.

The Weil-Felix reaction is also of considerable diagnostic value. (See section on serological procedures). In typhus fever, and in spotted fever, agglutination with Proteus OX19 occurs in high titer, and with OXK in very low titer. In the Japanese River Valley fever group, however, agglutination is obtained with Proteus OXK but not with Proteus OX19. No agglutination of these strains is obtained in "Q" fever.

Probably the most important single process for establishing the diagnosis in these diseases is transmission to guinea pigs. This enables one to apply cross immunity tests with known strains of typhus and spotted fever and also to institute culture of the rickettsiae. The best method for establishing a strain is to inject comparatively large amounts (5 to 6 cc.) of blood into guinea pigs by the intraperitoneal route early in the disease. Immune guinea pigs do not occur in nature, and if the blood is obtained early in the disease, the more blood injected, the greater the chance of producing infection. Later in the disease smaller (2 to 3 cc.) amounts of blood should be injected because of the possible presence of protective antibodies in the patient's serum. It is advisable to permit the blood to clot and after discarding the serum to inject the ground clot.

In transferring epidemic typhus from one guinea pig to another, the brain is removed on the third febrile day. It is ground in a mortar and a 10 per cent suspension is made in saline. (The brain of a 500 gram guinea pig weighs about 3 grams). One cc. of the suspension is injected intraperitoneally.

To transfer murine typhus, the guinea pig is killed with ether on the second day of scrotal swelling. The testicle and tunica vaginalis are removed and dropped into an Erlenmeyer flask containing beads and 20 cc. of saline. After vigorous shaking, 2 cc. of the supernatant is injected intraperitoneally into another guinea pig.

For the transfer of Rocky Mountain spotted fever, 2 cc. of blood obtained by cardiac puncture on the third febrile day is injected intraperitoneally.

The period of incubation in guinea pigs will depend on the dose injected and the virulence of the strain. For Rocky Mountain spotted fever it will be from 5 to 7 days. For epidemic typhus it will be from 7 to 10 days, while for endemic typhus this period will vary from 4 to 7 days.

After injecting the guinea pig, there are two things to watch: first the temperature. A definite and sustained rise in temperature usually occurs between the fourth and the twelfth day in animals which become infected. The upper normal limit of a guinea pigs temperature is about 103.5° C. In very hot weather temperatures up to 105° C. in the middle of the day may not be abnormal. Early morning is the best time to take the temperature, in order to eliminate this hot weather reaction. Temperatures above 104° C. are considered significant.

In certain strains of both typhus and spotted fever, this rise in temperature may be only external evidence of infection. On the third and fourth days of the temperature rise, transfers of blood and of ground-up brain and spleen may be made into fresh guinea pigs as described above. Smears should also be made from the scrotal sac, for rickettsiae are often found here especially in murine typhus. In this disease the Neil-Mooser bodies should be looked for.

Secondly, attention should be paid to the scrotum of the inoculated animals. In spotted fever there is at first a light skin rash, then purpura, necrosis, sloughing and finally scarring. All of these are superficial skin reactions. In endemic typhus, the deeper tissues are affected. There is edema and injection of the vessels of the tunica. The tunica becomes so swollen that the testicle cannot be pushed through the abdominal ring. The skin may be erythematous and hot but appears normal. Epidemic typhus produces no obvious scrotal reaction. A typical scrotal reaction shows swelling, redness and edema, but occasionally a fibrinous exudate forms in the tunica vaginalis without any external evidence of its presence. This can often be detected by palpation. It has been found that the testes cannot be pushed up into the peritoneal cavity without breaking up loose fibrinous adhesions.

Guinea pigs which do not react at all to the blood injection should be tested for immunity to typhus and spotted fever after about three weeks. Complete protection of guinea pigs against a virulent strain of either disease is pretty sound evidence that they have received virus of that disease, even though they did not react, since guinea pigs are not naturally immune to rickettsial diseases.

Strains have often been established by killing a guinea pig without any evidence of infection on or about the twelfth day, and making transfers to fresh guinea pigs. Occasional strains of spotted fever have been of such low virulence

that they have produced evidence of infection in only about 10 or 20 per cent of the injected guinea pigs. Pinkerton and Bessey, (1939) have recently shown that rats suffering from riboflavin deficiency have a greatly increased susceptibility to typhus infection, and by utilizing this deficiency in rats and guinea pigs it may be possible to pick up a number of strains of rickettsial diseases which would otherwise be missed.

If the scrotal reaction occurs, the exudate can be used to carry on the strain, and by making smears one practically always finds rickettsiae in the serosal cells. The appearance of the organisms in smears has considerable diagnostic value. In endemic typhus we find cells distended with enormous numbers of rickettsize, somewhat like lepra cells, while in spotted fever we find 10 to 30 rickettsiae, somewhat larger and often having a characteristic diplococcoid appearance. Having established the strain, cross immunity tests in guinea pigs with known strains of typhus and spotted fever and injection of brain or spleen into the yolk sac of eggs may be carried out. This will enable one to establish the diagnosis beyond a reasonable doubt in the majority of cases.

The differentiation between epidemic or European and endemic or murine typhus is of some importance, since Zinsser (Zinsser and Castaneda, 1933; Zinsser, 1937) has recovered the European strain from a number of cases occurring near Boston and New York. This differentiation may be made on the basis of the lack of an obvious scrotal reaction in the European strain, and from the fact that murine typhus causes a febrile disease in the rat, with rickettsiae in the scrotal sac, while European typhus causes a completely inapparent infection in this animal.

Human blood is commonly infective for guinea pigs only during the first four to six days of fever. Even if the patient is convalescing, however, the diagnosis can often be established by cross protection tests. These tests are carried out by mixing the blood serum of the patient with infective doses of virus from both spotted fever and typhus and by injecting guinea pigs intraperitoneally with these mixtures, along with appropriate controls. If the patient's serum protects guinea pigs against typhus and not against spotted fever, for example, the diagnosis of typhus may be considered to be established. Definite diagnoses are often made by this method without transmitting the disease to guinea pigs.

In certain mild atypical strains of rickettsial diseases the various tests which have been described may give ambiguous results. In such cases the study of rickettsiae in tissue cultures, by the methods which Pinkerton and Hass (1932) elaborated several years ago, may be the only accurate method of differentiating typhus and spotted fever. (Except for the recently developed serological procedures). The important feature for differential diagnosis brought out by this method is the fact that typhus and spotted fever rickettsiae produce totally different patterns when they grow within mammalian cells in tissue culture. Typhus rickettsiae grow voluminously in the cytoplasm of the cells, but never invade the nuclei. Spotted fever rickettsiae, regardless of how atypical the strain from which they are derived may be, grow sparsely in the cytoplasm, but form compact spherical

colonies in the nuclei of infected cells. It is interesting to note that these distinctive patterns are also found in the cells of infected arthropod hosts (the tick and the louse) of these two viruses.

Nine Mile fever and Australian "Q" fever are easily recognized in tissue cultures from the pattern of cell infection and from the fact that the specific organism is found extracellularly as well as intracellularly.

Bengston. (1941) has described the use of the complement-fixation test in "Q" fever. Bengston and Topping, (1942) using a typhus antigen described the use of the complement-fixation test in the diagnosis of endemic typhus and found the test of value in the detection of active or past infection. It was found to be a better criterion of past infection with endemic typhus than the Weil-Felix test. They concluded that the complement-fixation test could probably be used to differentiate between endemic typhus and Rocky Mountain spotted fevers. Plotz (Science, 1942, 95, 441-442), using spotted fever rickettsial antigen has found the complement-fixation test valuable for the differentiation of spotted fever and typhus.*

Methods for the cultivation of rickettsiae in the embryonic tissue of developing chicks have been described by Cox (Public Health Reports, No. 51, vol. 53, 1938; Science, 1941, vol. 94, p. 399). In the latter reference this worker describes the preparation of rickettsial vaccines and methods for determining their potency.

The agar slant tissue culture method of Zinsser, Fitzpatrick and Wei (J. Exp. Med., 1939, vol. 69, p. 179) is an excellent method for growing most rickettsiae. This method, however, like other tissue culture methods, does not work very well for the cultivation of the rickettsiae of classical (epidemic) typhus. Plotz (personal communication), using the method has been able to grow the rickettsiae of "Q" fever in large quantities.

*Recent investigations reveal that epidemic and endemic (murine) typhus may be differentiated by means of the complement-fixation and mouse protection tests.

BIBLIOGRAPHY

Badger, L.F., Dyer, R.E., and Rumreich, A.: An infection of the Rocky Mountain spotted fever type. Identification in the eastern part of the United States. Pub. Health Rep., U.S.P.H.S.,1931,46[1],463.

Bengston - Proc. Soc. Exp. B. & Med., 1941, 46, 665-668.

Bengston & Topping - Am. J. Pub. Health, 1942, 32, p. 48.

Burnet, F.M., and Freeman, M.: Experimental studies on the virus of "Q" fever. Med. J. Australia, 1937, 2, 299.

da Rocha-Lima, H.: Zur Aetiologie des Fleckfiebers. Berl. klin. Woch., 1916, 53[1], 567.

Davis, G. E., and Parker, R.R.: Comparative experiments on spotted fever and boutonneuse fever (I). Pub. Health Rep., U.S.P.H.S., 1934, 49[1], 423.

Derrick, E.H.: "Q" fever, a new fever entity: Clinical features, diagnosis and laboratory investigation. Med. J. Australia, 1937, 2, 281.

Dyer, R.E.: A filter-passing infectious agent isolated from ticks, IV. Human infection. Pub. Health Rep., U.S.P.H.S., 1938, 53[2], 2277.

Maxcy, K.F.: An epidemiological study of endemic typhus (Brill's disease) in the southeastern United States with especial reference to its mode of transmission. Pub. Health Rep., U.S.P.H.S., 1926, 41[2], 2967.

Pinkerton, J., and Bessey, O.A.: The loss of resistance to murine typhus infection resulting from riboflavin deficiency in rats. Science, 1939, 89, 368.
-----and Hass, G.M.: Spotted fever. I. Intranuclear rickettsiae in spotted fever studied in tissue culture. J. Exp. Med., 1932, 56, 151.
-----and Maxcy, K.F.: Pathological study of a case of endemic typhus in Virginia with demonstration of rickettsia. Am. J. Path., 1931, 7, 95.
-----and da Rocha-Lima, H., see under D.

Plotz, H., and Wertman, K.: The use of the complement fixation test in Rocky Mountain Spotted Fever. Science, 1942, 95, 441-442.

Wolbach, S.B.: Studies on Rocky Mountain Spotted Fever. J. Med. Research, 1919-1920, 41, 1.
-----, Todd, J.L., and Palfrey, F.W.: The etiology and pathology of typhus. Harvard University Press, Cambridge, Mass., 1922.

Zinsser, H.: The rickettsia diseases. Varieties, epidemiology and geographical distribution. Am. J. Hyg., 1937, 25, 430.
-----and Castaneda, M.R.: On the isolation from a case of Brill's disease of a typhus strain resembling the European type. New England J. Med., 1933, 209,815.

BARTONELLA

Verruga peruana, or Enfermedad de Carrion, may include two very different clinical syndromes: (1) a severe, often fatal, febrile anaemia (Oroya fever), and (2) a cutaneous, verrucous eruption of haemangioma-like nodules (verruga peruana).

The etiology of this disease has been a controversial subject for many years. Recent investigation indicates the cause to be a round and rod-shaped organism occurring in the red cells. The rod-shaped forms measure approximately from 1 to 2u in length and from 0.2 to 0.5u in width. They are frequently slightly curved and occur singly and end to end in pairs, or in chains of 3, 4 and 5. Staining may be even or granular. The rounded forms measure roughly from 0.3 to 1u in diameter. The organism also occurs often in closely packed masses in the swollen endothelial cells, especially of the lymphatic glands, spleen, liver, and intestines. In 1913, the Harvard Commission described these organisms in detail and created the genus Bartonella for them. The organism was named Bartonella bacilliformis.

The bartonellae and the rickettsiae show certain resemblances from a morphological standpoint. Both are minute or pleomorphic in character and are gram-negative. In the human body they are more characteristically intracellular in nature. Bartonella, however, differs from the rickettsiae in that it is associated with the red blood corpuscles. Some of the species of Bartonella have been cultivated extracellularly while this has not been true of most of the pathogenic rickettsiae.

Noguchi was the first to succeed in cultivating the organism. He used a medium which he had developed for the cultivation of leptospirae. Pinkerton, H., & Weinman, D., (Proc. Soc. Exp. Biol. and Med., 1937, vol. 37,587) have cultivated Bartonella in tissue cultures, and Weinman and Pinkerton (Ann.Trop.Med. & Parasit., 1938, vol. 32, p. 215), recommend the agar slant tissue culture procedure of Zinsser, Fitzpatrick and Wei (See section on viruses) for the cultivation of Bartonella. Jiminez and Buddingh (Proc. Soc. Exp. Biol. & Med., 1940, vol. 45, p. 546) have obtained the most satisfactory growth and shown the behavior of B. bacilliformis in the developing chick embryo.

(D) VIRUSES

It is not within the scope of this manual to present in detail the methods used in the study and diagnosis of virus diseases. The average bacteriological laboratory is not provided with the equipment or the trained personnel necessary for the proper execution of these procedures. The nature of viruses and virus diseases, the handling of viral specimens and some diagnostic procedures will be briefly outlined. No attempt is made to review the considerable amount of investigation of recent years but some procedures are described in order that the laboratory worker may make himself familiar with some of the special technical procedures necessary for work in this field.

The etiology of about 150 diseases of animals and plants has been attributed to the ultramicroscopic viruses. Only a relatively small number of these diseases, about 25, affect man; although the etiology of many diseases of unknown cause, will undoubtedly be eventually proved to be caused by viruses. Rivers, in outlining the proof of viral etiology of infectious processes, states that the suspected virus must be intimately associated with host cells, and must always be found at the proper time in specific lesions of the disease. Filtrates of the infective material, known to be free from bacteria, when injected into a susceptible experimental host of the same or a different species, must lead to illness or death in a characteristic manner and should induce a typical pathological picture. In turn, similar filtrates of the blood or emulsified tissues of the experimental animals must be capable of transmitting the infection in series.

In demonstrating the viral etiology of a disease several sources of error should be avoided. It is necessary to be certain, if possible, that the animals used are free from natural viruses before they are used. (This may be difficult at times. Most adult mice for example are infected with the Theiler virus). The virus suspected of being responsible for a disease must be regularly, but not necessarily always, associated with the condition. The host (man) if possible should demonstrate antibodies to the isolated virus. Errors in interpretation in experimental animals have arisen from accidental cross infection with other viruses under study in the same laboratory. Contamination of tissue cultures or other materials with a second virus is also a possibility.

Because of their ultramicroscopic size and their failure to develop on ordinary culture media, the viruses are not recognized by the technical methods usually employed in bacteriology. With some exceptions most viruses are: (1) ultramicroscopic in size; (2) capable of passing through filters which retain bacteria; (3) incapable of growth on any but living cells; (4) able to survive at low temperatures for relatively long periods of time in 50 per cent neutral glycerol; (5) able to reproduce rapidly in the tissues of a susceptible host; (6) often able to produce characteristic "inclusion bodies" in the cells of the host; (7) in many instances, able to produce specific neutralizing and other antibodies in immunized animals or patients; (8) capable of producing disease which usually induces a permanent immunity.

In Table 25 a list of the diseases caused by filtrable viruses is presented.

TABLE 25.

DISEASES CAUSED BY VIRUSES

Virus Diseases of Man

Alastrim
Australian X disease
Common Cold
Dengue Fever
Epidemic Encephalitis
  Japanese Encephalitis Type A
  Japanese Encephalitis Type B
  St. Louis Encephalitis
Herpes simplex (febrilis)
Herpes zoster (shingles)
Inclusion blennorrhea
Influenza
Lymphocytic choriomeningitis
  (latent in mice)
Lymphogranuloma inguinale
Molluscum contagiosum
Pappataci fever
Parotitis (Mumps)
Poliomyelitis (Infantile Paralysis)
Psittacosis (Parrot Fever)
Rabies
Rubella (German Measles)
Rubeola (Measles)
Trachoma (according to Julianelle)
Vaccinia (Cow-pox)
Varicella (Chickenpox)
Variola (Smallpox)
Verruca (Common Warts)
Yellow fever

Virus Diseases of Horses or Cattle

African horse sickness
Borna disease
Equine encephalomyelitis
  (occasionally in man)
Equine influenza
Foot-and-mouth disease
  (occasionally in man)
Horse-pox
Malignant catarrh of cattle
Periodic ophthalmia of horses
Pernicious anemia of horses
Rinderpest (cattle plague)
Vesicular stomatitis

Virus Diseases of Sheep

Agalactia of sheep and goats
Catarrhal fever of sheep
Contagious pustular dermatitis of sheep
Louping-ill (occ.-in man-lab. infection)
Nairobi disease of sheep
Rift Valley fever (occasionally in man,
  also goats and cattle)
Sheep-pox

Virus Diseases of Hogs

Hog cholera (swine fever)
Swine influenza
Swine-pox

Virus Diseases of Fowl

Fowl plague
Fowl-pox (contagious epithelioma)
Infectious laryngo-tracheitis of fowl
Leukemia of chickens
Newcastle disease of fowl
Rous' sarcoma

Virus Diseases of Birds

Avian diphtheria
Canary-pox
Pigeon-pox
Psittacosis
Virus disease of owls
Virus of equine encephalitis

Virus Diseases of Dogs and Foxes

Distemper
Encephalitis of foxes
Pseudorabies (also cats and cattle)
Rabies

Virus Diseases of Rabbits

Infectious fibroma
Infectious myxomatosis
Infectious papilloma

DISEASES CAUSED BY VIRUSES (Continued)

Virus Diseases of Mice

Infectious ectromelia
Influenza-like disease in Swiss mice (reported by Dochez)
Lymphatic leukemia
Theiler's disease

Virus Diseases of Rats

Salivary gland disease of rats

Virus Diseases of Ferrets

Epizootic disease of ferrets

Virus Disease of Frogs

Carcinoma in frogs (recently reported by Lucke)

Virus Diseases of Fish

Carp-pox
Epithelioma of barbus
Lymphocystic disease

Virus Diseases of Bacteria

Bacteriophage--a transmissible lytic disease affecting many species of bacteria.

Virus Diseases of Rabbits (continued)

Rabbit-pox
Spontaneous encephalitis
Virus III

Virus Diseases of Guinea-pigs

Guinea-pig epizootic
Guinea-pig paralysis
Salivary gland disease of guinea pigs

Virus Diseases of Insects

Polyhedral ("Wilt") diseases
Sacbrood of honey bees
Silkworm jaundice

Virus Diseases of Plants

Mosaic disease--affecting tobacco, potato, tomato, cucumber, lettuce, cabbage, sugar cane and numerous other plants.
Rosette of wheat
Spotted wilt of tomato
Tulip break
Yellow of peach and a host of other diseases affecting many species of plants.

Table 26 lists some of these diseases, the specimens to be examined in each, the presence or absence of inclusion bodies, a brief description of the pathogenicity tests employed in each and the serological or immunological tests that may be employed for diagnosis.

One of the early steps in the study of a virus is to separate it from contaminating bacteria. This can be done by treatment with ether (Appendix) or by filtration. The passage of viruses through the materials usually employed in bacterial filtration is governed by the electrical charge on the filter, the charge of the material being filtered, the amount of foreign protein present, the pressure used, the size of the pores of the filter, the absorption of the virus by the foreign protein present and the duration and temperature of the filtration process.(A considerable amount of the virus may be lost in this procedure).

The demonstration of the presence of the virus may be accomplished by two methods. The first of these is the injection of experimental animals; the second, tissue culture. In some cases direct examination of tissue for the presence of in-

TABLE 26.

Some Representative Virus Diseases. (Guide to Laboratory Study.)

I. Insect-borne Diseases.

Name	Specimens to be examined for virus	Pathogenicity tests. Susceptible animals or insects	Route of infection	Inclusion bodies
Yellow fever*	Blood first three days of fever; tissues--liver and spleen	Man Monkey (Macacus) Mice(adapted virus) Aedes mosquitoes	Cutaneous Peritoneal Cerebral Ingestion	I.N. liver I.N. ganglion cells
Dengue fever	Blood first three days	Man Monkeys--from non-endemic regions.Aedes mosquitoes	Cutaneous and other routes Ingestion	
Pappataci fever	Blood first day of fever	Man Phlebotomus	Cutaneous Ingestion	
Rift Valley fever*	Blood plasma and cells during fever Internal organs	Man Monkeys (India, S.A.) Monkeys (African) Sheep Lambs Cattle Goats Insects suspected as vectors	Cutaneous Application to scarified skin, conjunctiva, or nasal mucosa; inoculation.	I.N. liver

II. Neurotropic Diseases.

Poliomyelitis*	Central nervous system Stool	Man Monkeys (M. rhesus) (Cotton rats, mice)	Nasal(?) G. I. (?) Cranial, spinal, ocular, nasal, periton., cutaneous or venous	

TABLE 26 (Continued).

Some Representative Virus Diseases. (Guide to Laboratory Study.)

II. Neurotropic Diseases (Continued).

Name	Specimens to be examined for virus	Pathogenicity tests. Susceptible animals or insects -	Route of infection	Inclusion bodies
Encephalitis Various etiological types:	Central nervous system	Man	Nasal (?)	
		Monkeys (Cebus)	Cranial	I. N.
(a) Herpes virus in some		Rabbits	Cranial	
(b) St. Louis epidemic (1933) of human encephalitis*	Central nervous system	Man	Nasal (?)	
		Monkeys	Cranial	Kidney
		White mice	Cranial; nasal	
(c) Australian X disease - - - - -		Horse	Cranial	
	Central nervous system	Man	(?)	
		Monkeys (M. rhesus)	Cranial	
		Cattle, sheep		
(d) Japan epidemic (1924)	Central nervous system	Man	(?)	
		Rabbit	Cranial	Resemble Negri bodies
Equine encephalomyelitis	Central nervous system	Horse	Cranial; nasal	Brain
		Guinea pig	Cranial; nasal;cut.	
		Pigeon	Cranial	
		Rabbit	Cranial	
		White mice	Cranial	
		Aedes mosquitoes	Ingestion	
Lymphocytic choriomeningitis	Central nervous system	Mice	Intracerebral	
		Guinea pigs	Intraperitoneal.	
Rabies	Saliva; central nervous system	Man	Cutaneous	Negri bodies (C) in ganglion cells of hippocampus
		Monkeys	Cranial; cutaneous	
		Rabbits,mice	Cranial	
		Dog, Cat	Cranial	
		Wolf, Horse	Cranial	
		Guinea pig	Cranial	

TABLE 26 (Continued).

Some Representative Virus Diseases. (Guide to Laboratory Study.)

II. Neurotropic Diseases (Continued).

Name	Specimens to be examined for virus	Pathogenicity tests. Susceptible animals or insects	Route of infection	Inclusion bodies
Louping ill*	Central nervous system	Man(lab. infect.)	Unknown	
		Sheep	Nasal; cerebral	In mice (C) nerve cells
		Hogs	Nasal; cerebral	
		Rats & Mice	Nasal; cerebral	
Distemper (Neurotropic ?)	Central nervous system Nasal secretion; blood	Dogs, wolf Ferrets, stoat	Nasal Cutaneous	
		Lynx, weasel Racoon, fox	Cerebral	
III. Dermo-neurotropic Diseases.				
Variola §‡	Skin lesions	Man Monkeys Rabbits Cattle	Dermal Corneal	I. N. and C. epithelium cells
Vaccinia	Skin lesions	Man Cattle Rabbits, horses, Rats,sheep	Dermal Corneal	(C) in epithelium cells
Varicella (?)	Skin lesions	Man Monkeys Rabbits	(?) Dermal	I. N. and C. (?)
Herpes zoster (?)	Skin lesions	Man Rabbits(?)	Cutaneous	I. N.
Herpes simplex*	Skin lesions	Man Rabbits,mice Guinea pigs Monkeys	Cutaneous Cut.;cerebral ; scarification of cornea(in rabbits)	I. N.

TABLE 26 (Continued).

Some Representative Virus Diseases. (Guide to Laboratory Study).

III. Dermo-neurotropic Diseases.(Continued).

Name	Specimens to be examined for virus	Pathogenicity tests. Susceptible animals or insects	Route of infection	Inclusion bodies
Animal pox diseases	Skin lesions	Lower Animals	Cutaneous	
Foot and mouth disease*	Skin lesions and blood early in the disease	Man Cattle Hogs, cats, sheep, Goats, rabbits and guinea pigs	Cutaneous Cutaneous Cutaneous	I. N.
Vesicular stomatitis	Skin lesions	Horses	Cutaneous	
	IV. Dermotropic Diseases.			
Verruca	Tissue lesions	Man	Cutaneous	I. N. and C.
Molluscum contagiosum	Tissue lesions	Man	Cutaneous	C.
Lymphogranuloma venereum	Gland tissue	Monkeys, mice, rabbits guinea pigs	Intracerebral	
	V. Respiratory Diseases.			
Rubeola (?)	Nasal secretion	Man Monkeys Rabbits (?)	Tracheal Tracheal Venous	
Rubella (?)		Man		
Parotitis (?)	Saliva Parotid gland	Man Monkeys (M. rhesus)	Parotid Parotid	

TABLE 26 (Continued).

Some Representative Virus Diseases. (Guide to Laboratory Study).

V. Respiratory Diseases(Continued).

Name	Specimens to be examined for virus	Pathogenicity tests. Susceptible animals or insects	Route of infection	Inclusion bodies
Psittacosis	Sputum Blood	Man Parrots Mice, rabbits, monkeys guinea pigs	(?) Nasal	C.
Influenza	Nasopharyngeal washings	Ferrets, mice	Nasal	
Common cold	Respiratory secretions	Man Chimpanzee	Nasal	

C - Cytoplasmic inclusion bodies.
I. N. - Intranuclear inclusion bodies.
* - Serological tests - Specific neutralization of virus.
§ - Serological tests - Precipitin, Complement -fixation, Neutralization of virus.
‡ - A- Test of McKinnon and Defries (1928, Am. Jour. Hyg., 8, 93, idem 107). Inoculate variolous material intracutaneously into normal and vaccinated rabbits. The normal rabbits show a red swelling from the second to the fourth day. This is firm and elevated, and 10 to 20 mm. in diameter. Later the center is straw-colored, desquamates, and by the twelfth day disappears, leaving no scar. Vaccinated rabbits show no reaction, or an allergic red nodule which appears and disappears rapidly. Material from varicella lesions produce no reaction in either animal.

B- Paul's test. This consists of the production of keratitis by the inoculation of variolous material on a rabbit's cornea. It may be positive in only 50 per cent of the cases.

clusion bodies may be carried out. (Negri bodies in brain tissue, inclusion bodies in eye scrapings.)

In animal work it is necessary to choose the animal which is most apt to respond to the suspected virus. For example, the ferret is the only animal which can be infected with the virus of epidemic influenza obtained directly from the nasal washings of human patients, while in poliomyelitis the monkey is the only animal which can be thus infected with any degree of regularity. The choice of the route of inoculation is also important, since infection of the animal will often be successful if one route is chosen and fail if another is used. For example, the virus of influenza will infect the ferret if instilled into the nose, but will produce no infection if given by any other route.

There are several tissue culture techniques which have proved successful in the cultivation of viruses, the most valuable being the use of the developing chick embryo. This procedure obviates many of the technical difficulties encountered in the maintenance of most tissue cultures. The shell of a twelve to fourteen day chick embryo is cut to make a window. The material to be cultured may be dropped through the window upon the chorio-allantoic membrane and the opening in the shell closed with Scotch tape or with a cover-glass held in place with valspar. (The Cox yolk-sac inoculation procedure may also be employed.) In order to demonstrate the presence of virus in the tissue-culture the following criteria are used: (1) demonstration of distinctive cellular changes with inclusion-bodies, if the latter are characteristic of the particular viral infection; (2) reproduction of these changes in subcultures; (3) proof of freedom from bacterial growth; and (4) production of the disease in susceptible animals inoculated with material from the tissue-culture.

The fact that viruses are antigenic may also be used to identify them. Specific antibodies in the serum of convalescent patients and animals, which will protect animals and give the reactions of precipitation, agglutination and fixation of complement with viruses, may be demonstrated and used in the diagnosis of viral disease.

Procedure used in the study of viruses.

I. Microscopic examination of tissues for the presence of inclusion bodies. The presence of inclusion bodies in the cytoplasm, in the nucleus or in both cytoplasm and nucleus may indicate that the tissue contains virus. The inclusion bodies may vary in their shape, may be either basophilic or acidophilic in their staining reactions and in some cases may contain lipoid substances which may be stained with osmic acid. In addition to the methods used routinely to prepare tissue for histological examination, many special stains have been employed in the study of inclusion bodies. For details of such staining methods the reader is referred to Gatenby and Cowdry, Microtomist's Vade Medum, 9th Ed., 1928, P. Blakiston's Son & Co., Philadelphia; also Ludford and Ledingham, System of Bacteriology, Medical Researsh Council, London, vol. 9, Chapter X, p. 130. Satisfactory results have been obtained with Giemsa's stain, eosin-methylene-blue, Mann's methyl-blue (China blue)-eosin, hematoxylin-eosin, Heidenhain's iron hematoxylin and Pappenheim's pyronin-methyl-green.

Films may be prepared with cells scraped with a scalpel from infected tissues such as smallpox lesions and spread on a clean glass slide; or impression films may be made from fresh specimens of brain as in the examination for Negri bodies. (See Appendix for technique of brain examination in rabies). Histological sections may be prepared by the usual technique or by a special procedure such as (1) Ludford and Ledingham's modification of Schridde's method or (2) Regaud's method. (See Appendix.) Giemsa's stain is useful for sections prepared from tissues fixed in Zenker's fluid.

II. Collection of specimens. (General principles). Specimens are collected for microscopic examination, for injection into experimental animals, for inoculation of tissue cultures and, in the case of serum, for the demonstration of specific antibodies. Specimens to be examined for the presence of living virus by culture or animal inoculation, should be collected aseptically, preferably in a cold dry receptacle, and should be examined as soon as possible in order to

avoid losing the virus. If the specimen is to be saved or shipped to another laboratory, several different preservation methods may be used depending on the nature of the material under study.

III. Preservation of viruses. (General directions). Fifty per cent glycerine in a buffered phosphate solution of pH 7.6 (See Appendix) is commonly used for the preservation of viruses in tissues. It is very important that cold temperatures be used in the preservation of viruses. Viruses present in tissue, unclotted blood, serum, pus or other materials may live for several months if stored at 2 to 5 degrees centigrade. Drying in vacuo is an effective method especially if the specimen is frozen at the time of drying (lyophilization).

Sawyer (Medicine, 1931, 10, B, 4, 509) has used the following freezing and drying procedure for the preservation of yellow fever virus: Blood containing the virus is drawn into a flask, defibrinated by shaking with beads, cleared of cells in the centrifuge and 0.5 or 1.0 cc. amounts are immediately put into loosely plugged test-tubes measuring about 13 x 100 mm. The bottom of each tube is immersed in a mixture made of alcohol and pieces of solid carbon dioxide, and is whirled until the serum freezes in the shape of a hollow thimble in the bottom of the tube. The tubes of serum are then put into an improved Hempel desiccator, previously chilled by packing in a salt and ice mixture, and are dried in vacuum over sulphuric acid. When the tubes are removed the next day the thimbles of pale yellow serum have shrunk and lie loose in the tubes. As quickly as practicable the tubes are sealed in the blast lamp and stored in the refrigerator. If tissues are to be preserved in this manner they may be ground to a paste and then frozen and dried as above.

IV. More detailed directions for collection and preservation of specimens. A simpler method of preservation of specimens to be examined for virus content is that described below for the preservation of specimens from suspected cases of neurotropic virus diseases. These diseases and the special tests which may be employed in their study are indicated in Table 27.

Specimens of blood which are to be examined by complement fixation and neutralization tests, should be collected aseptically in a Keidel tube or some other sterile container. The serum need not be separated from the blood clot. Each tube should be labelled with the patient's name, address, and the date, using adhesive plaster or a paper label covered by transparent tape. The tube should then be packed carefully and sent by first class mail to the virus laboratory. The package should be labelled "Specimen for Bacteriological Diagnosis-RUSH."

Blood specimens should be collected and mailed as soon as possible after onset of the illness, four weeks after onset of the illness and eight weeks after onset of the illness. The first blood specimen should be accompanied by a history of the case, including the clinical diagnosis and all pertinent clinical and laboratory data and the report of spinal fluid examination. The specimens sent after four and eight weeks should be accompanied by progress notes, including laboratory findings.

Specimens that are to be studied for the identification of the virus must be handled with special care. Virus studies are time-consuming and costly and unless the following directions are rigorously adhered to, all examinations are

TABLE 27.

Disease	Tests on serum			Tests on brain & spinal cord	
	Complement fixation	Neutralization	Virus studies	Virus studies	Histopath. exam.
Acute aseptic meningitis or Lymphocytic choriomeningitis	+	+	+	0	0
St Louis encephalitis	+	+	0	+	+
Lethargic encephalitis (von Economo)	0	0	0	0	+
Herpes simplex encephalitis	0	+	0	+	+
Rabies encephalitis	0	0	0	+	+
Equine encephalomyelitis	+	+	0	+	+
Poliomyelitis*	0	+	0	+	+
Postinfectious encephalitis**	0	0	0	0	+

* In poliomyelitis, serum specimens should be submitted for these special tests only when doubt exists as to the diagnosis. In all fatal cases of poliomyelitis, specimens of brain and spinal cord should be collected and forwarded for virus identification and histopathological examination.

** Mumps meningoencephalitis, postmeasles encephalitis, postinfluenzal encephalitis, postvaricella encephalitis, postvaccinal encephalitis, postrabic treatment encephalitis.

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pointless. The virus of lymphocytic choriomeningitis, for example, often can be recovered from the spinal fluid and blood during the acute stage of the disease. This virus, however, is thermolabile and dies rapidly unless it is kept in the frozen state.

At the time of diagnosis 10-15 cc. of blood should be taken in a dry sterile syringe and equal portions distributed in each of three sterile pyrex Wassermann tubes. The tubes should be stoppered with sterile corks held firmly in place with adhesive tape. The contents of the tubes should then be frozen in a mixture of alcohol and dry ice. The tubes are wrapped in cotton and packed carefully in a vacuum bottle. The dry ice may be broken up by wrapping it in a piece of cloth

and then crushing it with a hammer. (Caution: Do not touch dry ice with the fingers. Use a forceps or spoon to fill the bottle.) A small V-shaped slot should be cut longitudinally in the vacuum bottle cork or a large bore venepuncture needle placed through the center of the cork to allow the gaseous carbon dioxide to escape. A tiny hole should also be punched in the outer metal cap of the bottle.

The bottle should be stoppered and packed carefully in a suitable strong corrugated paper box. The package should be labelled: Specimen for Bacteriological Diagnosis-RUSH. The package accompanied by a history of the case should be sent by special delivery air mail to the virus laboratory. The laboratory should be informed by telegraph that the package is being sent so that the handling of the specimen will not be delayed. Specimens prepared and packed as directed above will remain frozen 24 to 36 hours.

In every case of acute nonsuppurative encephalitis a specimen of spinal fluid should be collected at the time of diagnosis and sent to the virus laboratory for special study. About 3 cc. of spinal fluid should be placed in each of three sterile pyrex Wassermann tubes. These should be stoppered, frozen, labelled and shipped to the laboratory as directed above for blood.

If the patient dies, as soon as possible after death, remove the brain with sterile precautions. This should be done before the thorax and abdomen are opened to prevent possible contamination from the viscera. Generous blocks should be taken from (1) the temporal lobe including the hippocampus, (2) the motor cortex, (3) the midbrain, (4) the thalmus, (5) the pons and medulla, (6) the cerebellum and (7) the cervical spinal cord. Place one block of tissue from each situation in a sterile container with at least 100 cc. of sterile buffered 50 per cent glycerol (See Appendix) and mail immediately to the virus laboratory. Duplicate blocks from each of the above, fixed in 10 per cent formalin, should be sent with other autopsy material for histologic study.

V. Preparation of specimens for study. Fluid material such as blood or serum may be used for inoculation of animals either undiluted or diluted with various substances including water, physiological saline, Ringer's solution, Locke's solution, Simm's solution, broth, etc. Since it has been shown that sodium chloride has a deleterious effect on certain viruses, suspensions made with salt solution should be used with as little delay as possible. This inhibiting action of sodium chloride may be decreased by the addition of 10 per cent normal serum.

Suspensions of solid or semisolid tissues may be prepared as follows: If the specimen has been preserved in glycerol this material should be removed by washing several times with the sterile diluting fluid. It is then cut into small pieces which are weighed, placed in a sterile mortar with a small amount of sterile sand or particles of Pyrex glass and ground to a homogeneous paste. Sufficient diluting fluid is then added to make a 10 or 20 per cent concentration of tissue. The gross debris may be removed from such a suspension by centrifuging at 2000 r.p.m. for twenty minutes. The supernatant material may be used at once or, if preferred, may be filtered prior to the inoculation in order to insure the absence of bacteria.

VI. Inoculation of animals. The animals commonly employed in the laboratory are white mice, white rats, guinea pigs, rabbits, cats, Syrian hampsters, dogs, ferrets and monkeys. Other domestic or wild animals may also be used such as

birds, fowl, reptiles or insects.

It is often desirable with specimens from cases of unknown etiology to inoculate different amounts of the material into various species of laboratory animals by several different routes. Closely related strains of animals may be markedly different in their susceptibility or immunity to a specific virus. In some instances a virus may develop in an animal without the production of recognizable symptoms and by serial passage the pathogenicity of such a virus may be so increased that relatively small amounts will produce severe typical infections.

The affinity possessed by some viruses for special tissues may be used as a guide to the best route of experimental infection. Dermotropic viruses should be injected into the skin, while neurotropic viruses should be injected into the nervous system.

VII. Cultivation. Methods for the cultivation of viruses on living tissue cells are described in detail in the following references:

Tissue culture:

General Reference: Parker, R. C., "Methods of Tissue Culture", Paul B. Hoeber, Inc., New York, 1938.

Special References:

Preparation of reagents ---	Sanders, M., Arch. Path., 1939, vol.28, p.541.
Suspended cell cultures ---	Maitland, H.P. & Maitland, M.C., Lancet, 1928, vol. 2, p. 596. Li, C.P., Rivers, T.M., J. Exp. Med., 1930, vol. 52, p. 465. Plotz, H., "Culture des virus" in Levaditi, C. and Lepine, P., "Les Ultravirus des maladies humaines". Paris, 1938.
Agar slant tissue cultures -	Zinsser, H., Fitzpatrick, F., and Wei, H., J. Exp. Med., 1939, vol. 69, p. 179.
The chorio-allantoic membrane of the fertile hen's egg. - - - - -	Burnet, F.M., "The use of the developing egg in virus research." Medical Research Council: Special Report Series #220, 1936.
Cox method for inoculation of fertile eggs (for cultivation of various rickettsiae and the viruses of lymphogranuloma inguinale and influenza) - - -	Cox, H., Pub. Health Rep., vol. 53, 1938, p. 2241. Nigg. C., Crowley, J. H., Wilson, D.E., Science, 1940, vol. 91, p. 603.

VIII. Titration of virus. Viruses may be titrated by diluting them in a suitable fluid such as Locke's solution or Simm's solution (See Appendix) using a fresh pipette for each dilution and then testing each dilution by inoculating suitable ani-

mals or media. Tissues must be thoroughly ground in order to liberate the intracellular viruses before titration is attempted.

IX. Serological tests. Precipitins and complement-fixing antibodies may be detected in the serum of man or animals infected with certain viruses. Tests for specific neutralizing substances in serum, the so-called "neutralization tests", have been used for the differentiation of viruses and for the diagnosis of viral diseases. They are of special value in determining immunity to these diseases. These tests are based on the fact that if the virus and serum are mixed in suitable proportions and for an adequate period of time, the virus will be non-infective when injected into a susceptible animal. Descriptions of the techniques employed may be found in the following references:

Sawyer, W. A., & Lloyd, W., Jour. Exp. Med., 1931, 54, 533.
Francis, T., Magill, T.P., Rickard, E.R. & Beck, M.D.; Am. J. Pub. Health, 1937, vol. 27, p. 1141.

The technique of the complement-fixation test with influenza virus is also described in the above reference by Francis, Magill, Rickard and Beck.

Craigie, J. and Tulloch, W.J., Medical Research Council, special report series, No. 156, 1931, London, describe a variola-vaccinia flocculation reaction which is useful for the diagnosis of doubtful cases of small-pox or generalized vaccinia but will not differentiate between these two diseases. It consists of a precipitative reaction obtained by mixing immune serum from a rabbit injected with vaccinia virus and extracts of crusts removed from the patient which represent the antigen.

(E) FUNGI

I. CLASSIFICATION AND GENERAL DESCRIPTION (Reference: "Medical Bacteriology," Belding & Marston, 1938).

The position of the fungi in the plant kingdom is indicated in the following scheme:

Spermatophyta - - - - - - - - - - - - - - Seed plants.
Pteridophyta - - - - - - - - - - - - - - Fern plants.
Bryophyta - - - - - - - - - - - - - - - Mosses.
Thallophyta - - - - - - - - - - - - - - - Irregular thallus bodies not differentiated into roots, stems and leaves.

(1) Algae (containing chlorophyl)
(2) Fungi (non-chlorophyl bearing)
(3) Lichens (peculiar plant forms composed of algae and fungi living in symbiosis).

The fungi include the Schizomycetes (bacteria), the myxomycetes (slime molds) and the Eumycetes (true fungi). The Eumycetes (or true fungi) comprise the mushrooms, leaf spot fungi, molds and yeasts. The true yeasts are unicellular, while other true fungi, under certain conditions, maintain both unicellular and multicellular forms.

Mycology is the study of that branch of botany dealing with fungi, the morphological description of which necessitates the introduction of botanical terms with which the bacteriologist may be unfamiliar. These terms are essential for an understanding of the structure and classification of the fungi. A few of the more important terms are presented below:

Hyphae - elongated thread-like filaments; with or without septations.
Mycelium - a loose or compact network or collection of intertwined or branched hyphae.
Septa - partitions, dividing the hyphae into cells or sections.
Oidia - cylindrical or round cells which separate from the mycelium by splitting, (arthrospores) or by budding (thallospores) (blastospores). Unlike the spores, they cannot survive long periods of dormancy.
Chlamydospores - thick-walled cells or asexual spores stored with food material. These resting cells are formed, when conditions for growth are unfavorable, by the contraction of the protoplasm and the secretion of a thick wall. They maintain their viability through long periods of dormancy.
Spores - cells specialized for reproductive purposes. They separate from the parent stock and germinate to form new individuals. They may survive long periods of dormancy, but are not so highly specialized in this respect as bacteria. There are sexual spores, formed when there is fusion of the nuclei of two cells; and asexual spores, formed when no nuclear fusion takes place. Spores are useful for the classification and identification of fungi owing to their characteristic color, form and position.
Asexual spores - may arise directly from the walls of the ordinary hypha or

they may arise on specialized branches (sporophores). When these spores are formed in a sac or case at the end of a sporophore, the spores are known as sporangiospores and the sac or case inclosing them, the sporangium. The sporophore bearing the sporangium is called a sporangiophore. When the spores are not enclosed by a case or sac and are formed at the tips of the sporophores they are called conidia and the sporophore is called a conidiophore. When the conidia are not liberated at maturation but are set free by the disintegration of the mycelium they are called aleuriospores.

Fuseaux - fusiform (spindle-shaped) septate spores. They are formed in the aerial mycelium and are composed of several cells. They range in appearance from boat-shaped to club-shaped forms.

Zoospore - endospores that are free and provided with locomotive flagellae.

Endospore - a general term applied to any spore formed within the membrane of the parent cell.

SEXUAL SPORES:

Ascospores - a special class of endospores which are formed in a membrane called the ascus, the number of spores in the sac usually being limited to two, four, or eight and constant for the particular species producing them. The parent cell from which ascospores are produced has originally two nuclei which fuse into one before again dividing to form the ascospores. This fusion is regarded as a rudimentary sexual process.

Basidiospores - when the sporogenous cells instead of forming spores internally (e.g. asci), cut off the spores externally, they are known as basidia and the spores as basidiospores.

Zygospores - sexual spores of the higher forms of the Phycomycetes are formed by the fusion of two similar hyphae. The cell develops a thick wall, usually with irregular projections from its surface. Zygospores form only in those forms having non-septate mycelia.

Oospore - sexual type of spore produced by the fertilization of a female cell by a differentiated male cell.

Columella - the swollen tip of the sporangiophore forming the supporting center of the sporangium.

Sterigma - a short stalk bearing conidia. In Aspergillus the sterigmata arise from the inflated end of an unbranched conidiophore. In Penicillium, conidia are borne upon sterigmata which are given off from short branches, metulae, at the tip of the conidiophore.

Stolon - a runner-like branch of the mycelium which extends outward from the growing mold and gives rise to a new complete fungus where the tip comes in contact with the medium. This point of contact is called the node and the portion of the stolon between these points of contact is called the internode.

Rhizoids - small, filamentous, root-like branches which serve to attach the mycelium to the substrate.

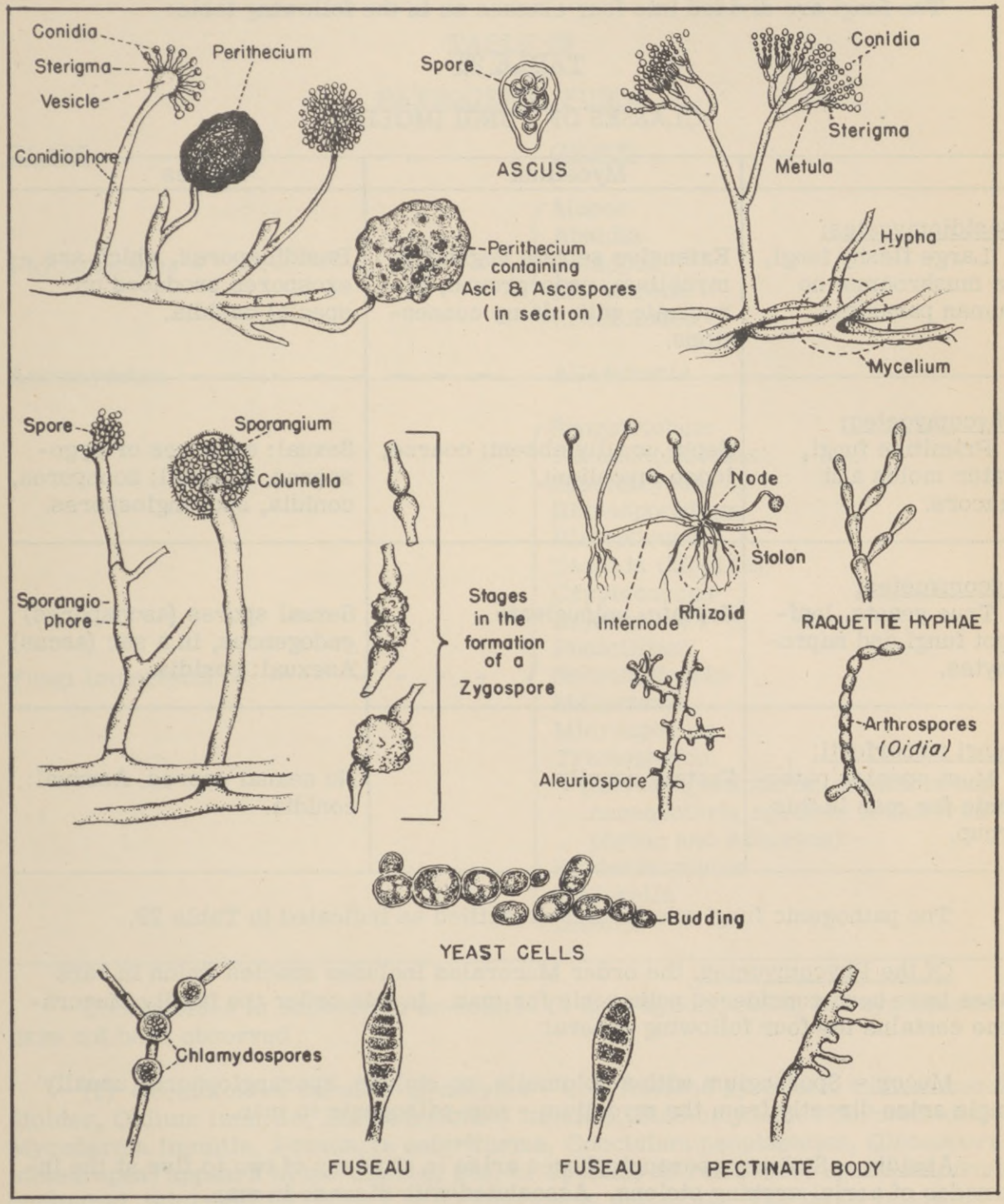
Vesicle - the swollen end of a hypha from which sterigmata, bearing conidia, arise.

Raquet mycelium - hyphal cells swollen at one end; arranged in series in such fashion that the small end of one is attached to the swollen end of the adjacent one.

Pectinate bodies - hyphae, often curved at the tip, so branched as to make a comb-like structure in which the teeth are widely and irregularly spaced.

Phialides - flask-shaped sterigmata.
Scutulum - disc-like crust.
Perithecium - the wall and cavity in which the asci are borne.

SOME TYPES OF STRUCTURES FOUND AMONG THE FUNGI



Because of our incomplete knowledge of the fungi and a lack of agreement between botanical classification and clinical-pathological groupings their classification is rather difficult. The gross appearance, the microscopic structure and especially the type of spore produced are relied on for the classification and identification of the fungi.

The fungi are divided into four classes as in the following table:

TABLE 28

CLASSES OF FUNGI (MOLDS)

	Mycelium	Spores
Basidiomycetes: Large fleshy fungi, as mushrooms; no human parasites.	Extensive septate vegetative mycelium, underground, binucleate with clamp connections.	Basidiospores, which are exospores produced on special basidia.
Phycomycetes: Primitive fungi, water molds and mucors.	Septa usually absent; coarse, loose mycelium.	Sexual: oospores or zygospores. Asexual: zoospores, conidia, sporangiospores.
Ascomycetes: True yeasts, leaf-spot fungi and saprophytes.	Septate, uninucleate	Sexual spores (ascospores) endogenous, in a sac (ascus). Asexual: conidia.
Fungi imperfecti: Most species pathogenic for man in this group.	Septate usually.	No sexual spores. Asexual: conidia.

The pathogenic fungi are further classified as indicated in Table 29.

Of the Phycomycetes, the order Mucorales includes species which in rare cases have been considered pathogenic for man. In this order the family Mucoraceae contains the four following genera:

Mucor - Sporangium with a columella, no stolons, sporangiophores usually single arise directly from the mycelium - non-pathogenic to man.

Absidia - Stolons, sporangiophores arise in clusters of two to five at the internodes of aerial arching stolons. Associated with disease in man.

Rhizopus - Sporangiophores arise singly or in clusters at the nodes of aerial arching stolons. Rarely associated with disease in man.

Mortierella - the mycelium forms a closely fitting mat of hyphae over the medium and is not typically aerial. The sporangia are without columellae. Rarely associated with disease in man.

TABLE 29

PATHOGENIC FUNGI

CLASS	GENUS
Phycomycetes	Mucor Absidia Rhizopus Mortierella Coccidiodes
Ascomycetes	Allescheria
Fungi imperfecti	Sporotrichum Paracoccidioides Blastomyces Rhinosporidium Histoplasma Candida (Monilia) Cryptococcus Aspergillus Penicillium Scopulariopsis Malassezia Microsporum Trichophyton (including ectothrix, endothrix and neoendothrix species, Endodermophyton and Achorion) Epidermophyton Madurella Indiella

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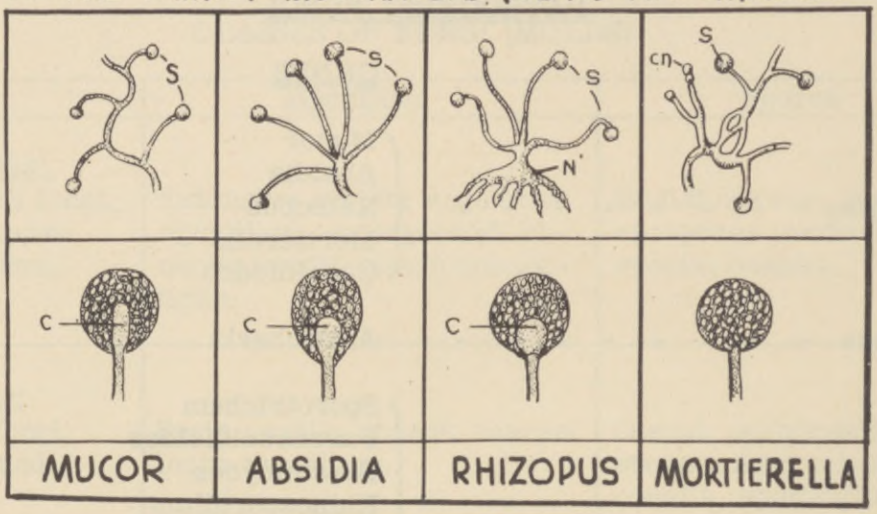
Coccidioides is believed to be related to the Phycomycetes, but zygospores have not been observed.

(1) Coccidioides immitis (Synonyms - Coccidioides pyogenes, Oidium coccidioides, Oidium immitis, Blastomycoides immitis, Blastomycoides dermatitidis, Mycoderma immitis, Posadasia esferiforme, Coccidium neoplasicum, Glenospora meteuropea) appears in the tissues, pus, or sputum, as refractile, spherical, doubly contoured, thick-walled cells, 5 to 80 microns in diameter. The large yellowish

cells contain many small spores 3 to 6 microns in diameter, which are liberated when the cells rupture. Multiplication in the tissues takes place by the formation of endospores. In cultures the cells develop as a coarse septate and branched mycelium without conidia. Chlamydospores are abundant. It is the cause of coccidioidal granuloma which is diagnosed by finding the round unicellular organisms, the larger containing spores, in the pus or sputum.

DIAGRAMMATIC REPRESENTATION OF FOUR GENERA OF THE FAMILY *MUCORACEAE* (CLASS PHYCOMYCETES



C = Columella.
S = Sporangia.
N = Node.
C N = Conidia

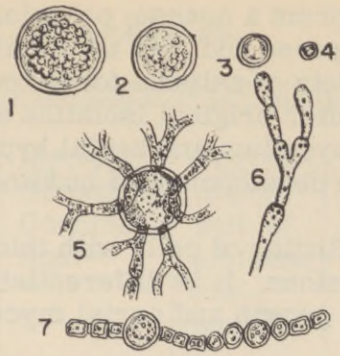
The Ascomycetes, characterized by the formation of sporangia, asci, endogenous ascospores and septate mycelium, are divided into twenty orders. Fungi of this group are of little importance as agents of disease, although Saccharomyces cerevisiae rarely causes a thrush-like infection and rarely invades deeper tissues.

The Ascomycetes include a few forms which are of medical interest: (a) Onygenaceae, (b) Gymnoascacae, (c) Allescheria, and (d) some of the ascospore-producing species of Aspergillus.

(a) The species of the family Onygenaceae lead a saprophytic existence on animal substrates such as hoofs, horns, and feathers. Strictly speaking they are not true parasites.

(b) In the family Gymnoascacae the asci are imbedded in a loose hyphal mass and the ascogonium grows in coiled branches. Many authorities believe that the dermatophytes may be imperfect forms of this family.

(c) Allescheria boydii is an infrequent cause of mycetoma. Its imperfect form, Monosporium apiospermum is more commonly seen.



COCCIDIOIDES IMMITIS

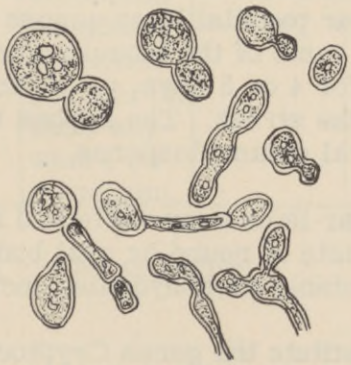
1 to 4, Cells containing Spores in tissues; 5, Development of Mycelium from large round cell; 6, Mycelium in culture; 7, Old Mycelium with Chlamydospore.

(2) Histoplasm capsulatum produces fatal chronic infections characterized by anemia, emaciation, leukopenia, splenomegaly, cirrhosis of the liver and pseudotubercles in the lungs and intestines. In the tissues the cells appear as small budding yeast cells with thick hyaline capsules.

(3) Rhinosporidiosis is characterized by polypoid growths principally in the nose, but also in the throat, eye and ear. R. seeberi also known as R. kinealys and Coccidium seeberi is found in the connective tissues of the lesions as round cells from 6 to 300 microns in diameter, the smaller homogeneous, the larger filled with spores. The parasite has not been cultivated.

(4) Blastomyces dermatitidis is the cause of American blastomycosis, a generalized infection of the skin and subcutaneous tissues. Synonyms for the organism are Cryptococcus gilchristi, Cryptococcus dermatitidis, Oidium dermatitidis, Mycoderma dermatitidis.

In the tissues this fungus appears as large ovoid or spherical, thick-walled, highly refractile, yeast-like cells, 8 to 20 microns in size, which reproduce by budding. The large cells often contain one or more refractile vacuoles in the granular protoplasm.



BLASTOMYCES DERMATITIDIS

In cultures the organism forms a coarse, occasionally septate, raquet, aerial mycelium with chlamydospores and both terminal and lateral pyriform conidia on short sterigmata. A variety of cultural forms occur, depending upon the medium, temperature, interval after original isolation and frequency of subculture. At 22° C. the production of the mycelium and aerial hyphae is favored, whereas a temperature of 37° C favors the development of budding yeast-like forms.

Diagnosis is based on the finding of cells with thick refractile walls and a single bud in the pus from the lesions. It is differentiated from the yeasts and Moniliae in cultures by its tough growth and aerial mycelium.

(5) Paracoccidioides brasilensis resembles Blastomyces both in the tissues and in culture, but differs in producing multiple buds in tissue, and lesser pathogenicity for animals. It gains access to man through the digestive tract and skin. The organisms are abundant in the lymphatics and the disease resembles coccidioidal granuloma.

(6) Species of Candida (Monilia) are simple yeast-like fungi which reproduce by budding, form a septate mycelium under suitable conditions, and do not show the production of ascospores. They differ from the yeasts by the formation of a mycelium, and cannot be classed as Eremascaceae since ascospore formation has not as yet been observed. The best known pathogenic species have been incorrectly designated as Monilia and the diseases - the moniliases.

The infections caused by the Moniliae in contrast to the blastomycosis caused by Blastomyces dermatitidis are characterized by their generally superficial nature and by the rather feeble invasive powers of the organisms. It is difficult to differentiate between the Moniliae of the gastro-intestinal tract in health and in disease, since Candida (Monilia) albicans is present in over 50 per cent of gastric samples and 10 per cent of feces in normal individuals.

The most common of the Monilias producing lesions in man is Candida albicans (Syn. Oidium albicans, Monilia albicans). It is the cause of thrush and may appear in lesions in both the myceloid and budding stages, the latter predominating. The mycelium has numerous oval budding cells and large terminal chlamydospores.

The common pathogenic species, Monilia (Candida) albicans can usually be identified solely on the basis of examination of a cornmeal agar plate culture. The inoculum is placed in three or four parallel lines across the agar plate, the needle cutting through the agar to place some of the inoculum below the surface. After incubation at room temperature for 4 or 5 days, most strains of C. albicans form hyphae extending laterally from the streak. The hyphae bear characteristic clusters of buds and thick-walled, spherical chlamydospores.

The culture on dextrose agar is creamy white and has a pasty consistency. It has a yeast-like odor and consists of round or oval budding cells. Anaerobic conditions and the absence of fermentable carbohydrates increase mycelium formation.

(7) The species which constitute the genus Cryptococcus are yeast-like but do not form ascospores. Partial differentiation is obtained by colonial growth, and

TABLE 30.

DIFFERENTIATION OF MONILIAS* (CANDIDA)

Species	Colony on Blood Agar	Mycelial Growth on Cornmeal Agar	Agglutination with M. albicans antiserum	Pathogenicity for Rabbits	Sugar Fermentation			
					Dextrose	Sucrose	Lactose	Maltose
M. albicans	Dull gray, size 1.5 mm. smooth circular	Tree-like. Chlamydospores on tips of branches. Spherical spore clusters. Buds usually at ends of mycelial segments.	+	+	⊕	+	-	⊕
M. parapsilosis (C. parakrusei)	Pearly white, size 0.7 mm. smooth and circular	Produced with difficulty. No chlamydospores. Irregular spore clusters. Buds usually at end of mycelial segments.	+ -	-	⊕	-	-	-
M. candida (C. tropicalis)	Grayish white, size 2.0 mm. mycelial fringe	Mycelium abundant. No chlamydospores. Buds anywhere on mycelium.	+	+ in large doses	⊕	⊕	-	⊕

(continued on next page)

+ = Acid; ⊕ = Acid and Gas; - = No reaction.

TABLE 30 (continued)

DIFFERENTIATION OF MONILIAS* (CANDIDA)

Species	Colony on Blood Agar	Mycelial Growth on Cornmeal Agar	Agglutination with M. albicans anti-serum	Pathogenicity for Rabbits	Sugar Fermentation			
					Dextrose	Sucrose	Lactose	Maltose
M. krusei	Dull grayish white, size 0.2 to 1.0 mm. variable in shape	Naked threads with branching at wide intervals. No chlamydospores. Buds often in whorl at tips of mycelium	-	-	⊕	-	-	-
M. mortifera (C. pseudotropicalis)	Size 0.5 mm. variable in shape	Similar to M. parapsilosis	-	-	⊕	⊕	⊕	-
M. stellatoides	Size--large, elevated central zone with radiating "arms"	Similar to M. albicans	-	-	⊕	-	-	⊕

* Adapted from a Practical Classification of the Monilia by Martin, Jones, Yao and Lee (Journal of Bacteriology, 1937, 34:99).

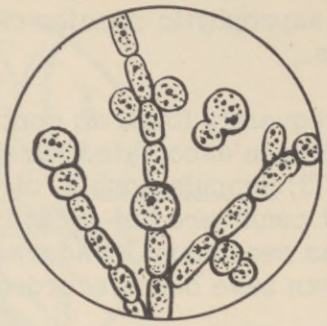
* * * * *

serological characteristics. The pathogenic species, Cryptococcus neoformans (Torula histolytica, Cryptococcus hominis) is typical of the species. It produces pulmonary, neurological, and general lesions.

In tissues, the spherical or slightly subspherical cells vary from 1 to 50 microns in diameter. A gelatinous capsule twice the diameter of the cell is formed in the tissues and in cultures. The cells reproduce by budding, form no mycelium, and do not produce ascospores. In old cultures large thick-walled cells filled with granules are observed.

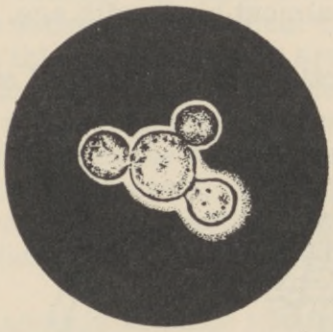
In culture the organism has a moist cream-colored mucoid growth which later becomes yellow or brown with age. No gas is formed in carbohydrate media,

but acid is formed in dextrose and levulose regularly, and gelatin is liquefied slowly. In liquid media there is a thick pellicle and a slimy sediment.



CANDIDA (MONILIA) ALBICANS
(THRUSH FUNGUS)

Two general types of infection have been associated with the Cryptococci: (1) superficial or deep-seated cutaneous infections at times becoming generalized and (2) fatal infections of the nervous system. The former, called European blastomycoses are differentiated from American blastomycoses by the formation of gelatinous or myxomatous abscesses. The latter are referred to as Torula infections. Although pulmonary symptoms are not prominent, the primary infection is usually in the lungs. Other possible portals of entry are the intestinal tract and the skin.



CRYPTOCOCCUS NEOFORMANS
(Torula Histolytica) (C. hominis)

When the nervous system is involved, there is a diffuse meningitis or cerebral abscess giving symptoms similar to those of tuberculous meningitis or of brain tumor. In meningeal involvement the spinal fluid is turbid and slimy or gelatinous. As a rule laboratory animals are fairly resistant to infection, rats and

mice being most susceptible. Miliary nodules may be found in the viscera of inoculated animals.

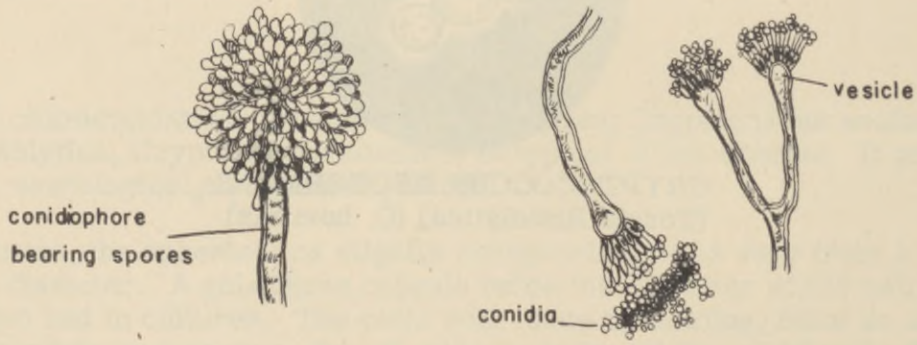
Positive complement-fixation reactions have been reported with the serum of infected individuals, and positive cutaneous reactions have been obtained with extracts of the organism. Some saprophytic species can be differentiated from C. neoformans by agglutination tests.

(8) The Aspergillae are frequently found as contaminants in bacteriological laboratories. Three genera have been associated rarely with disease in man: (1) Aspergillus, (2) Pencillium, and (3) Scopulariopsis, of which the first two are best known. These organisms are not considered of great importance as primary human pathogens. More often they act as secondary invaders. Nevertheless, instances of definite pulmonary infection in man have been recorded.

The genus Aspergillus is identified by the characteristic one-celled conidia formed by sterigmata which arise from the inflated end of an unbranched conidiophore. In some species the stalk-like sterigmata are branched. In the species forming ascospores, the perithecia develop from peculiarly coiled hyphae and contain asci with eight spores. The various species are identified largely by the color and microscopic appearance of the spores and by the number and arrangement of the sterigmata.

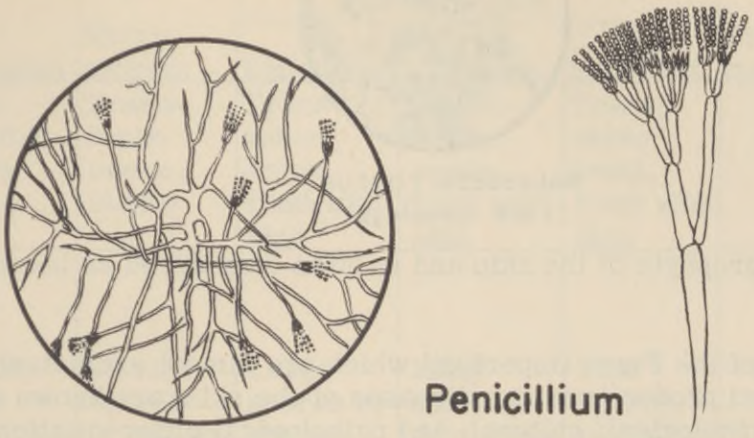
Four species, A. fumigatus, A. ruger, A. nidulans and A. flavus have been associated with human disease. A.nidulans which is chiefly associated with ear infections, has a bright green color, possesses branched sterigmata with parallel rows of conidia and has pink to purplish perithecia. A. fumigatus is the most prevalent pathogenic species and the cause of wide-spread aspergillosis in birds. In man it produces three forms of disease: (1) otomycosis, (2) dermatomycosis, and (3) pulmonary mycosis. Cultures grow well at 37° C. and their color varies from green to dark green, becoming almost black with age.

A. niger also causes infections of the external ear.



ASPERGILLUS

The genus Penicillium in the subfamily Aspergillae is characterized by a brush-like spore head. Conidia are borne upon sterigmata which are given off from short branches, metulae, at the tip of the conidiophore.



Penicillium

A few species have been reported as pathogenic for man, but a true etiological relationship is very doubtful. The classification of the genus is based on symmetrical and asymmetrical branching of the spore heads.

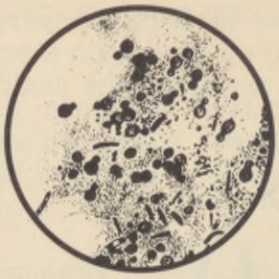
Most species of Penicillium are obligate aerobes which grow best below 30° C and not at all at 37° C. As a rule the colonies are green but occasionally are colorless, yellow, or even red.

The presence of the characteristic brush-like penicillium identifies the genus. The determination of the species requires the services of an expert mycologist.

(9) The pathogenic fungi causing superficial skin diseases may be classified according to their pathological action. The clinical classification is at present perhaps the most satisfactory. In this the organisms are divided into saprophytes and parasites. The former which produce no true infection but by their existence on the epidermis cause a superficial disease, include several unrelated species among which are (1) Malassezia furfur, the cause of pityriasis versicolor, and (2) Actinomyces minutissimus, the cause of erythrasma. The latter, a group of closely related species referred to as the Dermatophytes, actively invade the skin and other tissues. The same disease may be produced by any of several different species.

Species of the genus Malassezia produce the cutaneous infection known as pityriasis versicolor or tinea versicolor. The principal species found in this infection is Malassezia furfur which is found in abundance in the epidermis. It appears in the form of septate hyphae about 3 microns in diameter and refractile spores from 3 to 8 microns in size. It is difficult to isolate by culture and grows slowly if at all. The organism may invade the hair follicle but does not involve the shaft of the hair.

Erythrasma, another superficial epidermal infection is caused by Actinomyces minutissimus. The lesions, consisting of round scaling patches with a characteristic erythema, are usually located in the axilla or the groin. The causative organism



MALASSEZIA FURFUR
(skin scrapings)

is essentially a saprophyte of the skin and is often considered as belonging to the actinomycetes

The species of the Fungi imperfecti which are almost exclusively parasites of the epidermis and produce various diseases of the skin, are known as dermatophytes. The morphological, cultural, and pathological differentiation of the various dermatophytes is given in Table 31.

TABLE 31.

FUNGI IMPERFECTI PRODUCING DERMATOMYCOSES

			Trichophyton				
	Achorion	Microsporum	Endothrix	Neo-endothrix	Ectothrix	Epidermophyton	Endodermophyton
Disease	Favus	Ringworm	Ringworm	Ringworm	Sycosis, Ringworm	Ringworm	Ringworm
Host	Animal Man	Animal Man	Man	Animal Man	Animal Man	Man	Man
Pathology Location	Scalp, occasionally skin	Scalp, skin	scalp, occasionally skin	Scalp, skin	Scalp, skin	Moist folded skin	Skin
Skin lesion Suppuration	Yes	No	No	No	Yes	No	No
Scutula	Yes	No	No	No	No	No	No
Ring	No	Yes	Yes	Yes	Yes	Yes	Yes
Elevated	Yes	No	No	No	Yes	No	No
Skin lesion Scaling (marked)	No	Yes	No	No	Yes	No	Yes
Deep	No	No	No	No	Yes	No	No
Inflammation	Marked	Slight	Slight	Moderate	Marked	Slight	Slight
Heals in center	Yes	Yes	Yes	Yes	Yes	No	Yes

(continued on next page)

TABLE 31. (Continued).

FUNGI IMPERFECTI PRODUCING DERMATOMYCOSES (Continued).

			Trichophyton				
	Achorion	Microsporum	Endothrix	Neo-endothrix	Ectothrix	Epidermophyton	Endodermophyton
Hairs	Split longitudinally	Transverse break, stump	Transverse break, flush with skin	Transverse break, flush with skin	Transverse break, flush with skin	----	----
Ident. of org. in lesion: Inside hair	Articulated mycelium	Small spores in chains, mycelium	Large spores in chains, mycelium	Many spores, mycelium	Few spores, mycelium	None	None
Outside hair	Articulated mycelium	Clustered spores in mosaic	None	Few spores and mycelium	Spores in row	Articulated mycelium	Articulated mycelium
Ident. of org. in culture: Colonies Texture	Waxy	Velvety	Downy	Downy	Powdery Downy	Downy	-----
Color	Yellow to white	White to brown	White-yellow-violet	White to brownish	White yellow pink	Greenish yellow	-----
Appearance	Smooth to wrinkled	Cottony with radial furrows	Radial furrows concentric zonation	Wrinkled	Stellate or entire	Radial and concentric folds	Wrinkled
Mycelium	Irregular articulate	Twisted, lateral branches	Fine septate	Fine septate	Fine septate	Irregular, articulate	Irregular articulate
Conidia	Usually none or few	Clavate	Clavate to spherical	Clavate to spherical	Clavate to spherical	None	Few
Chlamydospore	+	+	+	+	+	+	None
Spindle-spores	+	+		+	+	+	None

The following classification based upon the clinical and pathological background appears best suited for the clinician:

I. Lesions of the scalp.
   A. Yellow scutula, hairs split longitudinally or containing air bubbles.
      Trichophyton (Achorion) schoenleini.
   B. No scutula.
      1. Non-suppurative
         (a) Hairs broken off above skin
            Microsporum
         (b) Hairs broken off flush with skin
            (1) Growth within hairs
               Endothrix trichophyton
            (2) Growth mostly within but also on hairs
               Neo-endothrix trichophyton
      2. Suppurative
         (a) Growth in and on hairs
            Ectothrix trichophyton

II. Lesions of the smooth skin
   A. Lesions confined to moist surfaces. Parasite not in hairs.
      Epidermophyton or Trichophyton
   B. Lesions on open skin
      1. Intricate concentric patterns, parasite not in hairs.
         Endodermophyton
      2. Red concentric patterns, not raised
         Microsporum, occasionally Endothrix Trichophyton
      3. Elevated red scaly pustular lesions
         Ectothrix trichophyton

Favus, is produced by species of Trichophyton (Achorion) or Microsporum but usually by one species, T. schoenleini. The lesions of the scalp and skin are characterized by the formation of a yellow cup-shaped, crusty mass, the scutulum. The mycelium is irregular and the cells vary in shape and form. A characteristic feature is the appearance of a series of air bubbles in the hairs. On culture at 30° C. yellow waxy colonies develop which become wrinkled with age and which produce in some strains a white aerial mycelium. The fungus invades the hairs forming parallel bundles of mycelium through their centers. The hairs become dull, split longitudinally and finally drop out. Diagnosis is made by microscopic examination of the scutulum or of the hairs, and by cultures. (See figure below).

Ringworm is a cutaneous infection due to several species of Microsporum and Trichophyton. In ringworm of the scalp caused by Microsporum, irregular clusters of polyhedral cells are formed outside the hair, and in the interior of the hair are short articulated filaments of mycelium which tend to break into chains of arthrospores. In cultures there is a fine septate mycelium with swollen cells which develop into chlamydospores. The aerial mycelium is peculiarly twisted with numerous short lateral branches, and pluri-septate spindle-spores with fine hair-like processes are formed at the ends of the long branches of aerial mycelium. The presence of mycelium within the hairs and of irregular clusters of polyhedral cells in mosaic arrangement outside is sufficient for microscopic diagnosis, which may be confirmed by culture. (See figure below).

Endothrix trichophyton ringworm is commonly caused in man by T. acuminatum, T. crateriforme, and T. violaceum. Chains of round, oval or cylindrical spores are found in the interior of the hairs. These spores are produced by fragmentation of the mycelium.

The creay white colonies of T. acuminatum have a central projecting conical peak, a folded peripheral portion, and are covered with a fine powdery coat. Pear-shaped spores with or without stalks are borne laterally at the tips of the filaments. The colonies of T. crateriforme are sunken in the middle. The colonies of T. violaceum are violet colored and smooth.

Clinical diagnosis depends upon the presence of a scalp lesion in which the hairs break off flush with the surface. Chains of round or ladder-like spores are present within the hairs. (See figure below).

Ectothrix Trichophyton ringworm is caused by two groups of Trichophyton (1) those with small spores, 3 microns in size and (2) those with large spores, 5 to 7 microns in size. The spores are found both within and on the hairs (see figure below) but occur in chains rather than in a mosaic as in Microsporum. The large-spored species more often infect the smooth skin, scalp and beard of adults, while the small-spored forms produce lesions of the scalp, face and hands of children.

Neo-endothrix Trichophyton ringworm is found chiefly in Germany and Austria. The lesions which are found on the scalp, beard and smooth skin are of a type transitional between the Endothrix and Ectothrix trichophyton (See figure below).

Epidermophyton ringworm, or tinea cruris, or eczema marginatum is an infection of the moist skin or folded surfaces of the body. In India it is known as dhobie itch. The lesions differ from those of the other ringworms by not healing in the center. The species associated with this disease, E. floccosum, is characterized by spindle-spores, lemon-green colonies and an articulated mycelium which breaks up into chains of oval or round cells. (See figure below).

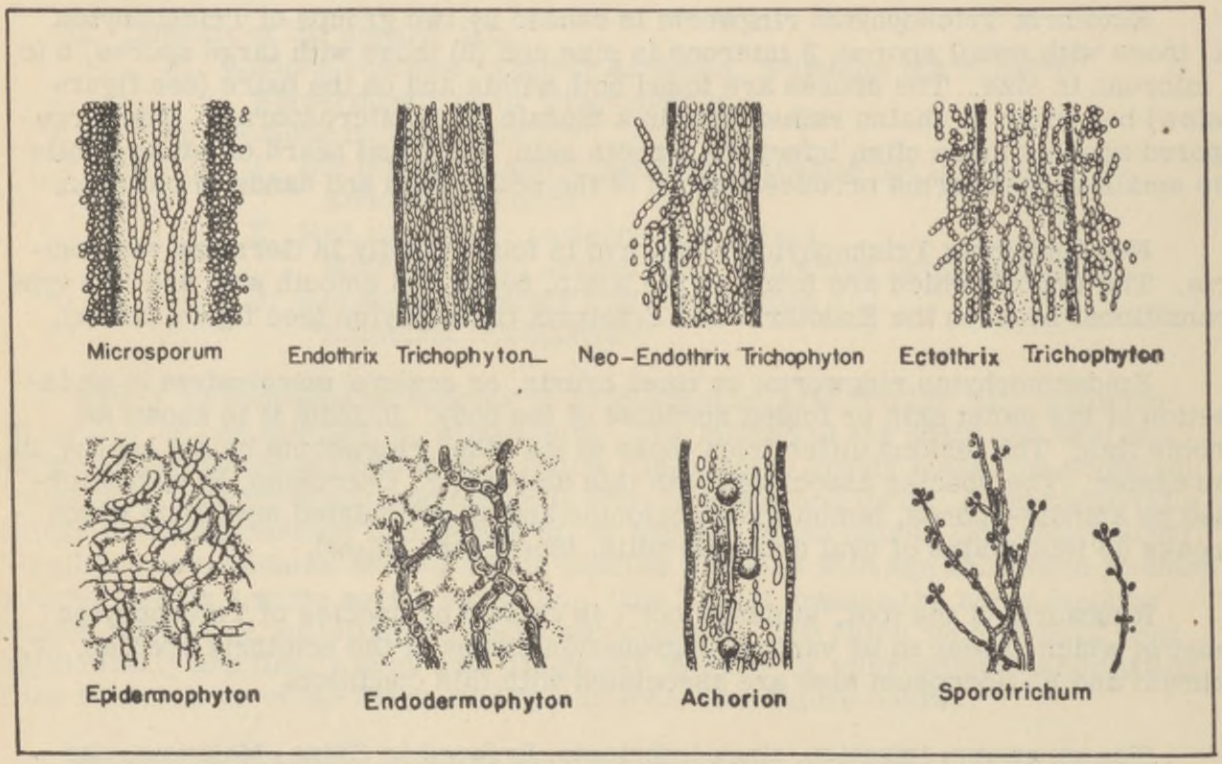
Ringworm of the foot,"athletes foot", is caused by species of Trichophyton most of which appear to be various degradation stages of the ectothrix species. T. rubrum and E. floccosum also are associated with this condition.

The ringworm infection, tinea imbricata, is found in China, Malaysia, and the Islands of the Pacific. At least five species have been identified. The organism may be cultivated with difficulty in liquid media. It forms no spores. The lesions show marked scaling and a complicated pattern of concentric white rays. The organism grows between the superficial and the deep layers of the skin and may be found in the scales. (See figure below.)

Sporotrichosis is a chronic infection of the skin, subcutaneous tissues, and lymphatics caused by a species of the genus Sporotrichum. A number of pathogenic species which differ in pigment production, spore-formation, and sugar fermentation have been described.

In the tissues, the organism appears as an oval or spindle-shaped cell 2 to 10 microns in length. In pus the gram-positive organism may be found either within or without the polymorphonuclear leukocytes. In cultures there is a tangled branched septate mycelium and free pyriform conidia. The latter arise from all parts of the mycelium, both laterally and terminally, singly and in clusters. Frequently these spores have short stalks. Chlamydospores are also present in the mycelium. (See figure below). Cultures are at first white and soft but become a shiny, brownish to black, leathery, wrinkled mass of closely woven mycelium which spreads peripherally. At times aerial hyphae produce raised areas of hairy appearance.

Diagnosis from a discharging lesion is difficult because few organisms are ordinarily present. Cultures of aspirated material may yield the organisms. Intra-abdominal injection of rats produces an inflammation of the peritoneum and testicles which is diagnostic.



THE DERMATOPHYTES AND SPOROTRICHUM

Although Madura foot is often thought of as an actinomycotic infection, almost half the cases are caused by fungi usually of the genera Madurella, Indiella, Glenospora, and Monosporium. The majority of non-actinomycotic mycetomata are caused by members of the genus Madurella. This genus is characterized by a branching, septate mycelium the hyphae of which are 1 to 10 microns in diameter and may divide to form chlamydospores. Young cultures are white but produce dark brown pigment with age.

The genus is associated with the black grained mycetomata and is less apt to involve bony structure than are the Actinomycetes.

II. EXAMINATION OF SPECIMENS AND DIAGNOSTIC PROCEDURES (Reference: Diag. Procedures and Reagents - 1941; Pathogenic Micro-organisms, Park & Williams - 1939; Dodge, Medical Mycology - 1935.)

REAGENTS, STAINS, and CULTURE MEDIA:

1. Approximately 20 per cent aqueous sodium hydroxide.
2. Gram stain for smears. (See Appendix)
3. Beauverie stain for ascospores. (Appendix)
4. Sabouraud's agar.
5. Malt agar. (Difco-dehydrated)
6. Potato-carrot agar. (For development of pigment)
7. Plain agar. (Basic agar medium)
8. Broth with carbohydrate and indicator for fermentation studies.
9. Calcium lactate milk.

COLLECTION OF SPECIMENS:

Surface lesions are cleaned with 70 per cent alcohol. Diseased hairs and scales, or a thin surface section taken with a razor blade from the outermost portion of the lesion (where the process is most active) are placed in sterile containers.

From moist surfaces material may be collected by scraping with a sterile scalpel or by collecting the material on a sterile swab.

Material from pustules or abscess-like lesions may be aspirated with a sterile syringe. In the case of vesicles on the soles or other surfaces, the vesicle may be transfixed with a needle, curved scissors inserted under the needle and the roof of the vesicle snipped off. For direct examination, the roof of the vesicle should be examined upside down.

Sputum is best collected before breakfast after the patient has thoroughly rinsed his mouth.

HISTORY OF INFECTION:

Since laboratory identification can often be aided by a knowledge of the clinical history and picture, the following information should be obtained:

1. Approximate duration of the lesions.
2. Clinical description of the lesions, their character, location and extent.
3. Tentative clinical diagnosis.

In primary cutaneous mycosis with (usually) no definite or important systemic involvement, the following groups of fungi may be expected in the indicated clinical conditions:

1. Tinea
   (a) Microsporum
   (b) Trichophyton
   (c) Epidermophyton

2. Favus
   (a) Trichophyton (Achorion) schoenleini.

In primary cutaneous and/or mucous membrane infections with frequent systemic involvement; or primary visceral infection without or with occasional cutaneous involvement:

1. Monilia and other yeast-like forms.
2. Blastomyces.
3. Coccidioides.
4. Sporotrichum.
5. Actinomyces.
6. Aspergillus.
7. Cryptococcus.

Weidman, in Park & Williams' "Pathogenic Micro-organisms", 11th Ed., 1939, suggests the following procedures for the approximate identification of fungi, as determined by their characteristics in tissue and pus:

1. If the organism is chained or filamentous, has the dimensions of bacteria and is branching, the following are to be considered: actinomycosis, nocardiosis, and erythrasma. If not branching, the leptothrices should be considered.

2. If the organism measures 2 micra or more in diameter, is perhaps segmented, and branching, and reproduction occurs essentially by means of arthrospores, the following are to be considered: aspergillosis, mucormycosis, penicilliosis, dermatophytosis, favus, endodermophyton infections, oidium infections and the more or less rare tropical dermatoses.

3. If in addition to the mycelium, budding cells are present, the following are to be considered: thrush, moniliasis, and tinea versicolor.

4. If only budding cells are present, the following are to be considered: blastomycosis, torulosis, cryptococcus, and saccharomyces infections (intertrigo saccharomycetica). When of the dimensions of cocci, consider seborrheic dermatitis, histoplasmosis, epizootic lymphangitis and sporotrichosis. The comparatively large, dark brown, hyaloid bodies of chromoblastomycosis are not budders in the strict sense; they are chlamydospores of a special type.

5. If a spherical, double-contoured cell is observed, which does not bud but reproduces by endosporulation, coccidioidal granuloma and rhinosporidiosis should be considered.

6. If an organism like that described in 5 but differing in that buds extruding through pores in the capsule are seen, paracoccidioidal granuloma infection should be considered.

7. Extremely minute bodies suggesting Leishmania, occurring abundantly within endothelia (spleen, liver, etc.) should suggest the possible presence of histoplasmosis or epizootic lymphangitis.

"It is emphasized that the foregoing arrangement pertains only to forms

occurring in tissue, including pus. In culture they are almost invariably different in form, unless special methods are devised to reproduce tissue forms. When exceptions are met they concern the simpler, more minute forms such as Pityrosporum and perhaps some of the threads."

METHODS OF EXAMINATION AND IDENTIFICATION:(From Stovall and Almon in Diag. Proc. & Reagents, A.P.H.A., 1941.)

Divide all specimens into three portions, if possible; one for direct examination, one for culture and one for animal inoculation.

A. Direct Examination:

1. Preparation of specimens:

a. Scales from skin and nails; hairs--Mount in a drop of 20 per cent sodium hydroxide solution. Apply cover glass. Seal edges of cover glass with vaseline. Let stand for 1/2 hour or longer; then examine with 4 mm. and 1.8 mm. objectives. Gentle warming will hasten clearing. Thick scrapings may have to stand overnight.

b. Sputum--Select representative flakes, granules, or dense portions of the specimen. Mount on slide; apply cover glass with pressure to crush the material beneath. Examine immediately with the higher powers of the microscope.

c. Biopsy or autopsy material--Make frozen or paraffin sections of biopsy material. Stain with hematoxylin and eosin, or Gram stain. Imbed autopsy material; stain by Gram's method.

d. Purulent material--Mount some of the material in 20 per cent sodium hydroxide and examine without staining. Clearing with hydroxide may be superfluous in some instances. Make smears and stain by Gram's method.

2. Key to the identification of fungi found by direct examination of tissues or exudates:

For the most part only genera can be determined by these methods.
For subdivision to species, cultural methods are necessary.

a. Both spores and hyphae found--

(1) Spores are of the arthrospore type without buds; found in or around hairs and cutaneous scales:

(a) Spores round, 2 to 4 microns in diameter; irregularly arranged, chiefly on the outside of hairs:

Microsporum

(b) Spores spherical, ovoid, or cylindric; large type about 8 microns, small type about 3 microns in diameter; arranged in chains either within (Endothrix) or on the outside (Ectothrix) of hairs:

Trichophyton

(c) Spores irregular in shape; large, and irregularly arranged; both hyphae and spores relatively sparse, so that the structure of the hair is always clearly visible between them. Air bubbles may be present:

Achorion

(2) Spores are yeast-like in type; often found detached from the hyphae:

Monilia, Geotrichum, Endomyces

(a) Cultural studies are necessary for further identification within this group.

b. Spores or yeast-like cells present; hyphae absent.

(1) Budding forms:

(a) Small (3 to 5 microns); with gelatinous capsules:

Torula (Cryptococcus)

(b) Larger (5 to 20 microns); without gelatinous capsules; budding infrequent:

Blastomyces (Zymonema)

(2) Non-budding forms:

(a) Round with refractile capsules; 5 to 80 microns; sporangia, containing many spores, may be found:

Coccidioides immitis

(b) Cigar-shaped, non-septate bodies; 3 to 10 microns long, 1 to 3 microns wide; very difficult to demonstrate; sometimes found in macrophages:

Sporotrichum

c. Hyphae or mycelial masses found without spores--

(1) Hyphae made up of oblong, double-contoured cells; invade epidermis, but not hair:

Epidermophyton (Trichophyton and Microsporum in lesions of globuous skin)

(2) Mycelium has few septations; oval cells (not true spores) are sometimes present in addition:

Aspergillus

(3) Entire fungus mass situated in characteristic "sulphur granule" which is yellow or dark brown in color. When crushed or sectioned the mass reveals a central area of interlacing fine thread and a peripheral zone of "clubs" which are the swollen ends of some of these threads. The central zone stains Gram-positive; the clubs stain Gram-negative:

Actinomyces

B. Examination by Culture:

1. Preparation of specimens:

a. Hairs, cutaneous scales, nail scrapings--

(1) Soak the specimen for 2-8 minutes in 70 per cent alcohol which is free from molds (determined by culture control) to free it from contaminating bacteria.

(2) Transfer specimens to agar slants.

b. Sputum--

(1) Select suitable flakes or nodules.

(2) Grind them up with 2 cc. of sterile saline in a sterile mortar.

(3) Use one drop of the resulting suspension for each inoculation.

c. Spinal fluid, or pus aspirated from unopened lesions need not be treated.

d. Biopsy tissue--

(1) Grind up a small piece with twice its volume of sterile saline.

(2) Use several drops of the resulting suspension for each inoculation.

2. Inoculation:

a. Use slants or prepare plates by pouring 20 cc. of the appropriate melted medium into each Petri dish and letting it harden. For each specimen prepare two plates with plain agar (Basic Agar Medium) and two with Sabouraud's agar.

If Actinomyces is anticipated, inoculate three plain agar plates.

b. Place scales, hairs, etc., upon the medium with sterile forceps or needle. Streak saline suspensions or drops of pus over the entire surface with a sterile cotton swab or inoculating loop.

c. Inoculate two tubes of 1 per cent glucose broth with each specimen.

3. Incubation:

a. Incubate one plain agar plate and one Sabouraud's agar plate from each specimen at room temperature and the other two at 37 degrees C.

b. If Actinomyces is anticipated, incubate the additional plain agar plate under anaerobic conditions at 37 degrees C.

c. Keep plates for at least 2 weeks before discarding as negative. If mixed growth develops in broth tubes, keep until bacteria have settled to the bottom and fungus is growing on top. Then inoculate a plate with the surface growth.

d. Use some device, such as jars or Petri dish cans, for keeping the plates moist during the entire incubation period.

4. Observation of colonies:

a. Macroscopic morphology--Observe at the first signs of growth and at 3 day intervals thereafter until the colonies attain close to their maximal size, taking special note of the presence of surface down, color changes, and differences in color between top and bottom of the colony and between center and border.

b. Microscopic morphology--Mount portions of the colonies in saline under cover glasses. In order to assure full representation of structures, select material from the edge of the colony and from the center.

Typical structures of most species require at least 1 week to develop.

5. Study of subcultures:

a. Tests for ascospores--

(1) Make a heavy suspension of the growth in saline solution and let it stand at room temperature for 1 week. Mount some of the sedimented cells and stain them by Beauverie's method, or make a wet mount and examine without staining.

(2) Inoculate a malt agar plate by the pour method. Let the plate dry in the incubator until the medium is completely dehydrated. Make a saline suspension of some of the dried culture and inoculate another malt agar plate by streaking. Mount some of the cells from the top of 48 hour colonies and stain by Beauverie's method, or make a wet mount and observe directly without staining.

b. Observation of Monilia colonies for hyphal fringes--

(1) Inoculate malt agar plates by streaking so as to obtain isolated colonies.

(2) Observe at 48 hours, with the low power of the microscope.

c. Observation of fermentation reactions and reaction in calcium lactate milk.

(1) Inoculate glucose, maltose, and sucrose broth and calcium lactate milk with 1 drop of a saline suspension made of the growth from a young malt agar slant culture in 5 cc. of saline.

(2) Observe for fermentation at 48 hours and 1 week; observe milk for coagulation at 3 days.

d. Key to the genera as determined in culture--

The descriptions given below are for recently isolated cultures. Among some groups, principally the dermatophytes, pleomorphism is very common. Cultures kept on artificial media for varying lengths of time cannot be expected to conform to these descriptions.

(1) Growth on solid media shows powdery, velvety, downy or cottony surface early. Colonies consist of hyphae, and, in most cases, spores of one or more types.

(a) Spores not borne aloft on special fruiting structures, but interspersed irregularly throughout the growth.

(a1) Hyphae are fairly regular in outline. Spores are chiefly conidia, variable in size and shape; usually produced abundantly. Growth seldom if ever brightly colored. Spirals and rudimentary, blunt fuseaux formed by some species:

Trichophyton

(a2) Hyphae more irregular than in the previous genus; pectinate bodies and raquet mycelium may be found. Spores may be conidia, chlamydospores, and fuseaux. The latter, when formed, are spindled-shaped, multiseptate, and sometimes bear spiny outgrowths on their walls. Yellow or red pigment formed by some species:

Microsporum

(a3) Growth usually pigmented; yellow to yellowish-green. Chlamydospores or club-shaped fuseaux present; conidia lacking:

Epidermophyton

(a4) Growth white to yellow color. Rather large, variable conidia, borne laterally, may be found.

Achorion

(a5) Growth may or may not possess surface down in young cultures (see (2), (a2e), below). Mycelium is characteristically of the raquet type. Chlamydospores and arthrospores are common.

Coccidioides immitis

(b) Fruiting structures specialized and borne aloft. Hyphae long; septations, if present, widely spaced.

This group contains most of the air contaminants. Very few species are known to be pathogenic.

(b1) Conidia borne in radiating chains on sterigmata which arise from a vesicle:

Aspergillus

(b2) Conidia borne in chains on sterigmata which arise directly from the end of the conidiophore without vesicle:

Penicillium

(b3) Mycelium without septations. Spores borne enclosed in a spherical or ovoid sac (sporangium):

the family, Mucoraceae

(2) Growth on solid media, though rugose in some genera, devoid of powdery, velvety, downy, or cottony surface in early stages.

(a) Slow-growing colonies; frequently somewhat rugose, at least when old.

(a1) Composed of irregular, coarse, short hyphae with large budding outgrowths. All elements surrounded by relatively thick walls. Carbohydrates not fermented:

Blastomyces

(a2) Composed of relatively fine, long hyphae. Colonies often colored. Spores usually of the conidial type.

(a2a) Colonies violet:

Trichophyton violaceum

(a2b) Colonies with ochre center:

Trichophyton ochraceum

(a2c) Colonies tan; spongy when old. Ends of some hyphae swollen, forming "favic chandelier":

Achorion Schoenleini

(a2d) Colonies white to dark brown or black; composed of long, fine hyphae, bearing pear-shaped conidia laterally and terminally, singly and in small clusters:

Sporotrichum

(a2e) Colonies begin as paraffin-like plaques, later covered by fluffy gray aerial hyphae (or (a5), page 122). Raquet mycelium, arthrospores, and chlamydospores formed:

Coccidioides immitis

(b) Rapidly growing colonies; always moist and pasty and relatively smooth when young. Composed chiefly of yeast-like cells.

(b1) Some hyphae produced on most media.

(b1a) Ascospores produced:

Endomyces

(b1b) Ascospores not produced:

(b1b1) Definite pellicle produced in liquid media. Glucose, fructose, and mannose fermented with acid and gas:

Mycoderma

(b1b2) Pellicle, if produced, is very thin.

Monilia(See key to species, below)

(b2) No hyphae produced on any of the usual media during ordinary periods of incubation.

(b2a) Ascospores produced:

Saccharomyces and other genera of true yeasts.

(b2b) Ascospores not produced:

Torula (Cryptococcus)

(c) Growth only on plain agar. Colonies small and bacterial in character, but adherent to the medium. Sometimes yellow to orange. Composed of branching, gram-positive filaments; sometimes of gram-positive rods:

Actinomyces

e. Key to the species of the genus Monilia--

(1) No gas formed in maltose or sucrose. Average size of blastospores (48 hour growth on malt agar) about 4.5 microns. Calcium lactate milk not coagulated in 3 days:

Monilia parapsilosis

(2) Gas formed in maltose, but not in sucrose. Forty-eight hour surface colonies on malt agar lack hyphal fringe. Calcium lactate milk coagulated in 3 days:

Monilia albicans

(3) Gas formed in maltose and sucrose. Forty-eight hour surface colonies on malt agar have definite hyphal fringe. Calcium lactate milk not coagulated in three days:

Monilia candida (Candida tropicalis)

(4) In addition, Fisher and Arnold describe four unidentified types of Monilia.

STUDIES ON PATHOGENICITY FOR ANIMALS.

A. Dermatomycoses:

1. Impregnate a piece of sterile sandpaper with a suspension derived from the specimen submitted.
2. Scarify the shaved skin of a rabbit, guinea pig, or mouse with the paper thus prepared.
3. Observe daily for lesions.
4. Obtain specimen from lesions as directed in section on "Collection of specimens." (above).
5. Observe as directed in section on methods of examination and identification.

B. Coccidioides:

1. Inoculate a guinea pig or rabbit subcutaneously in the groin or intraperitoneally with 0.2 cc. of pus from suspected coccidioidal granuloma.
2. Proceed with observation as directed above. Kill the animal after 3 to 4 weeks.

C. Sporotrichum:

1. Inject some of the exudate from suspected sporotrichosis subcutaneously or intraperitoneally into a young male rat.
2. Examine testes, joints, and tail at three day intervals for lesions. Tail lesions are cutaneous papules.
3. When lesions are well developed, proceed with observation as directed above.
4. In any event, autopsy in 3 to 4 weeks.

D. Monilia:

1. Grow culture in glucose broth for 48 hours.
2. Centrifuge and take up organisms in saline. Count suspension.
3. Inoculate 2 rabbits intravenously with doses amounting to 6.0 millions per 100 gm. of body weight.
4. Kill one animal after 24 hours and observe the lungs for petechial hemorrhages:

Numerous petechiae	Monilia albicans
Few petechiae	Monilia candida
No petechiae	Monilia parapsilosis

5. If the other rabbit does not die within 7 days, kill it on the 7th day. Examine dead animal for abscesses, particularly in the kidneys. Many lesions with enlargement of the kidney, and numerous lesions elsewhere in the body (peritoneum, diaphragm, skeletal muscle):

Monilia albicans

Moderate number of lesions in kidney with little enlargement; few, if any, lesions elsewhere in the body:

Monilia candida

No lesions:

Monilia parapsilosis

E. Blastomyces:

1. Grow the organism for 2 weeks on malt agar.
2. Suspend the growth from one slant in 5 cc. of saline.
3. Inject 0.5 cc. of this suspension into the ear vein of a rabbit, or intraperitoneally or intratesticularly in mice or rats.
4. If death does not supervene, kill the animal in 2 weeks.
5. Observe for widespread lesions (liver, spleen, lungs, brain) having the gross appearance of tuberculosis and containing the characteristic budding organisms.

SEROLOGICAL TEST FOR IDENTIFICATION OF SPOROTRICHOSIS ANTIBODIES IN PATIENT'S SERA.

1. Obtain serum from coagulated blood of patient.
2. Make suspension of sporotrichum by grinding growth from week-old cultures with saline in sterile mortar, and filtering through cotton.
3. Adjust turbidity of suspension to approximate that of No. 2 barium sulphate standard (McFarland), using saline as diluent.
4. Prepare a series of serum dilutions starting with 1:20 and extending through 1:5,120, using 1/2 cc. amounts in agglutination tubes. Use 0.85 per cent saline as diluent.
5. Add 1/2 cc. of the organism suspension to each serum dilution and also to a tube containing 1/2 cc. of saline, as control.
6. Shake rack vigorously for 1 minute.
7. Incubate at 37 degrees C. for 2 hours and at room temperature overnight.
8. Read in comparison with the saline control.

IV SEROLOGICAL AND IMMUNOLOGICAL METHODS OF DIAGNOSIS

Disease may be diagnosed by taking advantage of the specificity of reactions that are obtained when diagnostic serum is mixed with bacteria or certain bacterial products. Such diagnostic serum may be prepared by the injection of small, gradually increasing doses of bacteria or their products, into animals, usually rabbits or guinea pigs. Therapeutic sera are prepared by injecting horses, goats, calves, or rabbits.

Serological procedure is employed in the diagnosis of the disease by one or both of two methods: (1) The patient's serum may be mixed with known organisms; (2) Organisms isolated from the patient may be mixed with known diagnostic sera.

AGGLUTINATION TEST

The identification of organisms by the agglutination test using known antiserum of high titre is of considerable value in the confirmation of identification by means of carbohydrate fermentations and other biological characteristics. It is especially useful in the identification of members of the Eberthella, Salmonella, Shigella, Pasteurella, and Brucella groups.

The culture of the organisms to be identified (the antigen) is grown in broth for 18 to 24 hours. If the culture is too turbid it may be diluted with physiological saline to a density comparable to tube No. 3 of the McFarland nephelometer (Appendix). The antigen may also be prepared by washing an 18 to 24 hour growth from an agar slant with sterile saline or broth and diluting to the proper density. It is often desirable, at this point, to kill the organisms either by heat (56 degrees C. for 1 hour) or by the addition of 1 or 2 drops of formalin, as a precautionary measure. Ten small, clean, clear test tubes are set up in a wire rack. To the first tube 0.9 cc. of saline is added. In each of the remaining tubes, 0.5 cc. of saline is added. To the first tube, 0.1 cc. of known antiserum is added and mixed thoroughly with the saline. 0.5 cc. of this serum-saline mixture is transferred to the second tube and after thorough mixture, 0.5 cc. of this mixture transferred to the third tube. This procedure is repeated until the ninth tube is reached. 0.5 cc. is discarded from the ninth tube instead of being added to the tenth tube which is used as an antigen control. The serum dilutions obtained by this procedure are 1:10 in the first tube, 1:20 in the second tube, 1:40 in the third tube, etc. until a dilution of 1:2560 is reached in the ninth tube. To each tube 0.5 cc. of the antigen is now added. The final dilutions are now 1:20 in the first tube, 1:40 in the second tube and so on until a dilution of 1:5120 is obtained in the ninth tube. The rack containing the test tubes is now shaken and the mouth of each tube flamed. This last point of technique is a precautionary measure since occasionally, when a pipette is placed into or taken out of a small test tube, a drop of culture from the tip of the pipette is accidentally placed on the rim of the tube.

The tubes are now subjected to body or higher temperature for a period of time varying with the type of antigen (See Appendix) and examined for agglutination. The tubes may be refrigerated overnight and then again examined.

When reading the test, the tubes are first examined for turbidity. They are then gently shaken and examined for the amount and character of agglutina-

tion. Complete agglutination is indicated by absolute clearing of the supernatant fluid and settling of the organisms in large white particles in the bottom of the tube. Partial agglutination is indicated by incomplete clearing of the supernatant fluid and diminution in size of the bacterial clumps. The final agglutinating titer will, of course, depend on that of the antiserum used and may vary due to differences in antigens.

Occasionally an organism on primary isolation will fail to agglutinate with its specific antiserum. For example an organism typically typhoid as determined by biochemical and other characteristics and isolated from a known case of typhoid fever, may fail to agglutinate with the typhoid antiserum. This may be due to an antigenic structure of the newly isolated strain (which usually contains Vi antigen) which is not identical with that of the strain used in preparing the antiserum. Rapid transfer of the culture may bring about a change in the antigenic structure of the organism so that agglutination by the antiserum occurs. One loopful of a broth culture is transferred to a fresh tube of broth daily for 4 or 5 days. The agglutination test is then repeated.

Instead of the agglutination technique described above, a microscopic technique and a macroscopic slide technique may be employed.

The microscopic technique is employed when only a small quantity of antigen is available. The mixture of serum and bacterial suspension is made on a clean cover-glass and inverted over a vaseline-rimmed depression in a hollow-ground slide. Tilting the slide at intervals prevents undue sedimentation of the organisms and insures thorough mixing of the ingredients. Reading may be made with the microscope after incubation for one hour at 37 degrees C. Clumping of the bacteria and loss of motility are looked for.

The macroscopic slide agglutinative test is a convenient time saver and may be applied to isolated colonies from a plate. A portion of the colony may be smeared, stained and examined as a check upon the type of the organisms and the purity of the colony. Another portion is emulsified in two drops of salt solution on a clean slide. A loopful of the mixture is placed on the opposite end of the slide to serve as a control. To the original drop, a drop of known immune serum is added and mixed well with a clean loop. The reaction should be observed against a black back-ground. The appearance of fine to coarse granules in the mixture is indicative of agglutination. No reaction should occur in the control drop.

This method can also be used with known bacteria against unknown sera to determine the presence or absence of specific antibodies. A good example of this is the rapid slide agglutination test for Brucellosis. This test will detect agglutinins against Brucella in the serum of infected individuals in three or four minutes. A glass plate ruled with one inch squares and a specially prepared and accurately standardized antigen must be used. The preparation of this antigen is tedious and time consuming and not practical for the average hospital laboratory. However, satisfactory antigens may be secured commercially.

In performing this test the patient's serum is distributed in 0.08, 0.04, 0.01 and 0.004 cc. amounts in squares on the glass plate. A drop of antigen is placed in each drop of serum. Serum and antigen are mixed with a toothpick,

care being taken to progress from the smallest to the largest amount of serum. Agglutination occurs immediately and is very marked and easy to read. Results obtained with this method should agree closely with those obtained in the test tube agglutination test.

By proper selection, or by special treatment of the antigen it is possible to detect the presence of one or more types of antibody reacting specifically with a given organism. Thus the presence of flagellar (H) and somatic (O) agglutinins may be detected. Such differential analysis of the antibody content of serum of possible typhoid fever patients may aid in differentiating between antibody production due to infection and antibody due to past infection, recent vaccination or an anamnestic reaction. The simplest procedure for gaining this information is one in which the test is repeated after a few days. A rise in titer usually permits diagnosis of the disease.

Macroscopic slide agglutination is also used in the Pampana test to detect roughness in the antigenic composition of organisms, (Pampana, E.J., J. Hyg., 1933, 33, 402-403). The reagent consists of a 1:500 solution of trypaflavine in normal saline. A drop of the solution is put on a slide. Close to the drop, but not in the drop, a minute fraction of a loopful of the bacterial colony to be examined is placed. The flamed and cooled loop is moistened with a small drop of physiological saline and the material on the slide is gradually emulsified. Finally it is mixed with the drop of trypaflavine solution. If the colony is rough agglutination takes place immediately or within a few seconds. The reaction is more easily read if the surface of the slide is illuminated by oblique light against a dark background.

Care must be taken in the gradual mixing of the bacteria with the test solution because mixing of the bacterial suspension with the whole droplet of trypaflavine at once may cause a pseudo-agglutination.

H & O AGGLUTINATION

For the preparation of H antigens which are to be used in detecting agglutinins against Eberthella typhosa, Salmonella schottmülleri (paratyphosa B), Salmonella paratyphi (paratyphosa A), and Salmonella suipestifer, smooth motile strains are used. Agar slants are inoculated with actively motile organisms. After eighteen to twenty-four hours of incubation, the growth is washed off of the agar slants with physiological saline containing 0.2% formaldehyde, viz., 0.05 cc. of 40 per cent formaldehyde per 10 cc. of sterile saline. The bacterial suspension is then stored in sterile bottles in the refrigerator for 7 to 10 days so that all bacteria will be killed. The suspension if found to be sterile may then be stored in the refrigerator as the stock antigen. When needed it is diluted with saline to a turbidity of #3 on the McFarland nephelometer scale although this density may be varied according to individual preference.

A culture of live organisms may be used for macroscopic agglutination tests, but formalized suspensions are recommended because their use avoids the risk of laboratory infection and because they can be stored in the refrigerator for a long period of time.

O antigen should be prepared for Proteus X19. If H and O agglutinations are to be done in the "Widal" test, an "O" antigen should be prepared from Bact. typhosum.

There are two accepted methods of preparing bacterial suspensions for use as O antigens. The easier and perhaps the more satisfactory of the two methods consists of preparing a phenolized (0.5% phenol) saline suspension of a non-motile strain. The main obstacle to this procedure is the difficulty in obtaining satisfactory non-motile strains of organisms. However, a satisfactory non-motile strain of E. typhosa "0901" can be obtained either at the Standards Laboratories, Oxford or at the Lister Institute. The procedure to be followed with such a strain is that described above for the preparation of H antigen. If, however, non-motile strains are not available, the method of White (Med. Res. Council Spec. Rpt. Ser. 103, 1926) may be used. The growth from an agar slant culture is suspended in 1.0 cc. of absolute alcohol and heated at 60° C. for 1 hour. The organisms are sedimented by centrifugation, the alcohol decanted and the bacilli resuspended in 0.5 cc. of normal saline.

Suspensions of Shigella paradysenteriae (Flexner) and Shigella dysenteriae (Shiga) for use as antigens are prepared as H antigens. Five strains of Flexner (V,W,X,Y and Z) or a polyvalent Y strain are employed. If spontaneous agglutination is obtained, reduction of the salt concentration from 0.85 to 0.5% usually results in the removal of this difficulty.

For the preparation of Brucella antigen, Brucella abortus is usually used. Antigenically it is almost identical with Brucella suis and Brucella melitensis. Forty-eight hour agar slant cultures are washed off with a small amount of normal saline. For the Brucella antigen the saline should be phenolized, not formalized, using 0.5% phenol in saline solution. The suspension should be refrigerated, tested for sterility and diluted to the proper turbidity for use.

A non-virulent strain of Pasteurella tularensis should be used for the preparation of P. tularensis antigen. Such a non-virulent strain, "B-38", may be obtained from the National Institute of Health.

Forty-eight hour growth on cystine-blood agar is washed off with normal saline containing 0.5% formaldehyde and the suspension stored in the refrigerator for 7 to 10 days or until needed. After testing for sterility it is diluted for use to the desired turbidity.

The agglutination test is performed by diluting the serum as described above, adding the antigen and after exposure to a standard temperature for a fixed period of time examined for clumping of the bacteria. All agglutination tests should be incubated at 50-55 degrees C. for five hours and refrigerated overnight except those for the typing of D. pneumoniae or H. influenzae. These are incubated at 37 degrees C. for two hours and then refrigerated overnight. (See Appendix).

Reading of the agglutination test is carried out by shaking the tube gently and observing the character and amount of the agglutinated particles. H. agglu-

tinins produce large flakes of the floccular type, which are easily broken up, while O agglutinins produce granular small-flaking agglutination.

For O agglutinins: the significant titres are those above 1 to 50 since a few normal individuals have titers as high as 1 to 50 and rarely 1:200 or above. For H agglutinins: titers of 1:80 or above appear to have some diagnostic significance.

In interpreting the results of an agglutination test such variable factors as past history of infection, vaccination, the time at which the specimen was taken, naturally occurring agglutinins, etc. must be taken into consideration. Because Eberthella typhosa and Salmonella enteritidis share a common O antigen, when in the Widal test only O and no H agglutination is obtained, S. enteritidis should be used as an H antigen also. If it is agglutinated the infection is due to S. enteritidis and not to Eb. typhosa.

Negative results may be due to taking the sample of blood before the appearance of the agglutinins in the serum, so that the later appearance of agglutinins after a negative result is usually significant.

Positive results may be interpreted as follows:

In typhoid fever:

(a) Definite infection may be indicated by a titer of 1:160 with O antigen and 1:80 with H antigen. (Here the titer obtained with the O antigen is higher than that obtained with the H.)

(b) An anamnestic reaction, past infection, or recent vaccination may be indicated by a 1:160 titer or under with H antigen only.

Examples of some possible Widal findings and their interpretation are presented in Table 32.

In tularemia and in brucellosis positive reactions in dilutions of 1:160 or over usually suggest definite infection. As in other infections no arbitrary statement can be made as to what titre means infection. Early in the disease titers of 1:10 or 1:20 may be found and in these cases second specimens should be requested. Agglutinins do not often appear in the blood during the first week of illness.

Francis and Evans (1926) showed that there is frequent cross-agglutination between Pasteurella tularensis and either Brucella abortus or Brucella melitensis and advise that sera from suspected cases of tularemia and undulant fever be set up against Pasteurella tularensis and either Brucella abortus or Brucella melitensis, unless the clinical history definitely points to one or the other of the diseases.

They concluded that a serum which shows a marked difference in titer for tularensis on the one hand, and for abortus or melitensis on the other, can usually be classed by the higher titer as due either to tularemia or to one of the varieties of Brucella melitensis. In routine work if a positive agglutination

TABLE 32.

POSSIBLE WIDAL FINDINGS

(1) Twelfth day of illness	Patient uninoculated	
	"H"	"O"
T	1/1500	1/600
A	0	0
B	0	0
(2) Tenth day of illness	Patient uninoculated	
T	1/25	1/50
A	1/400	1/50
B	1/25	0
(3) Tenth day of illness		
T	1/150	1/50
A	1/50	0
B	1/100	0
(4) Fifth day	Uninoculated	
T	0	1/100
A	0	0
B	0	0
(4a) Tenth day	Same Case	
T	1/1000	1/500
A	0	0
B	0	0
(4b) Tenth day	Same Case	
T	0	1/300
A	0	0
B	0	0
S. enteritidis	1/500	-

Antigenic structure: Eb. typhosa-IX, d; S. enteritidis-IX, gom.

Interpretation of Table 32:

(1) Active typhoid infection.

(2) "H" and "O" agglutinins are within normal limits except those of S. paratyphi, which are suspiciously high. Report as almost certainly paratyphoid A. Repeat in four or five days.

(3) All findings are suggestive of previous T.A.B. inoculation, nothing pointing to active infection. Repeat in four or five days. Obtain history as to inoculation. A later rise will indicate enteric infection or might possibly be due to anamnestic reaction.

(4) Typhoid "O" suspicious but just within possible normal range. Repeat.

(4a) Widal now diagnostic of typhoid infection.

(4b) Alternate finding. Significant rise in typhoid "O" agglutinins means that the patient may have had typhoid, but no "H" agglutinins have appeared. The bacteriologist, therefore, has included a suspension of S. enteritidis (Gaertner) "H", this organism having the same "O" antigen as Eb. typhosa. This suspension is agglutinated, showing that infection is due to Gaertner's bacillus.

test is obtained with one of these antigenically related antigens, the test should be repeated using both antigens. They suggest that a serum which agglutinates all three organisms to the same, or nearly the same titer, should be subjected to agglutinin absorption tests. A description of the technique of agglutinin absorption may be found in Zinsser and Bayne-Jones, 8th Edition, pp. 931-932.

THE WEIL-FELIX TEST

Various Proteus X strains have been used as an aid in the diagnosis of rickettsial diseases although they are in no way connected with the rickettsiae and have no etiological significance in the diseases. The common useful ones are Proteus OX 19, and Proteus OXK. Their usage is indicated in Table 33.

TABLE 33.

	Typhus Group	Tsutsugamushi Group	Spotted fever Group
Name of disease	European or epidemic typhus (Louse borne)	Tsutsugamushi fever	Rocky Mt. spotted fever
	Mexican typhus-Tabardillo (Louse borne)	Rural typhus of Malaya	Sao Paulo typhus
	Murine or endemic typhus (Flea borne)	Mite fever of Sumatra	Fievre boutonneuse
	Brill's disease (vector unknown)		
	Shop typhus of Malaya		Tick-borne typhus of South and North Africa, India, Kenya, etc.
Vector	Lice and rat fleas	Mites	Ticks
Reservoir of virus	Rats and mice	Field mice & rats	Ticks, rodents, dogs
Agglutination	OX 19 + + + OXK -	OX 19 - OXK + + +	OX 19 + + + OXK - or ±

When one leaves trench fever out of consideration, the only other rickettsial diseases to be considered as separate from typhus and spotted fever are certain mite-borne diseases of the Far East (the tsutsugamushi group) and Australian Q fever and Nine Mile fever (American Q fever).

In the United States we need be concerned only with the differentiation between typhus, spotted fever and Q fever, the first of which is divided into endemic and epidemic typhus. (See section on Rickettsiae.)

In typhus and in spotted fever there is agglutination in high titer with Proteus OX 19 and in very low titer with OXK. No agglutination is obtained with these strains in Q fever. The use of strain OXK is therefore not necessary in this country. Welch (Diagnostic Procedures and Reagents, 1941) suggests taking a series of three blood samples to establish diagnosis. The first taken as soon as the nature of infection is suspected or when the rash appears; the second taken between the 12th and 15th days; and the third taken during early convalescence. A marked increase in titer is helpful in reaching a diagnosis. He describes certain precautions which should be taken in the preparation of antigens as well as the technic of the macroscopic tube and slide agglutination tests.

Since flagellar (H) Proteus agglutinins are occasionally present in the sera of healthy individuals it is necessary to use O antigens in performing the agglutination test. This antigen contains the fraction which reacts specifically with sera from typhus and spotted fever cases.

Due to the production of variants from the X 19 strain, the diagnostic antigen may become practically inagglutinable in sera of clinical cases of Rocky Mountain spotted fever. Certain of these variants may agglutinate directly in heterologous sera. The Proteus strain used therefore should be maintained under conditions which will preserve it as a pure O variant. In this respect lyophilization of the culture is helpful.

The cultures should be carried on dry slants of nutrient agar and all strains except HXK should be kept in the non-motile state.*

Changes in morphological, biochemical and serological characteristics are evidence of instability and should be looked for.

In order to exclude spontaneously occurring variants, agglutination tests should be made at least once every three months with 5 specific antisera, Eberthella typhosa, Salmonella schottmulleri, Salmonella paratyphi and Shigella paradysenteriae Army and Flexner.

The antigen to be used in the macroscopic tube agglutination test is prepared by suspending 18-24 hour cultures in 0.85 per cent saline. The turbidity is adjusted to that of tube #3 of the McFarland nephelometer scale.

* If the O antigen is prepared from a motile strain by heating in absolute alcohol at 60° C. for one hour, this direction may be ignored.

The agglutination test is set up in agglutination tubes (75 x 10 mm.) by mixing thoroughly 0.5 cc. of serum dilutions and 0.5 cc. of antigen suspension. Serum dilutions of 1:10 through 1:640 (final dilutions 1:20 through 1:1280) are usually sufficient. The usual control tube containing 0.5cc. of antigen and 0.5 cc. of saline should be included as well as a control titration using positive serum. Tests and controls are incubated for 5 hours at 50 to 55 degrees C. followed by overnight refrigeration at 8-10 degrees C.

For a description of the rapid slide test (Antigen preparation and standardization, technique of test) see pages 223-226 of Diagnostic Procedures and Reagents - American Public Health Association - 1941.

For the agglutination test used in the diagnosis of Weil's disease (infectious jaundice) a special dark-field technique is employed. Serum-antigen mixtures are incubated at 32 degrees C. for three to four hours and observed under dark-ground illumination, using a low power objective and a compensating ocular. If living organisms are used, agglutination is seen to occur in the lower dilutions and lysis in the higher. With formalized or phenolized suspensions, agglutination alone is seen. Two or three different strains should be used, including one of Lepto. canicola.

Agglutination Technique for Leptospira in Detail

Antigen: Living four to six days old cultures of Leptospira icterohemorrhagiae and Leptospira canicola grown in Verwoort-Schuffner medium (Appendix) are used.

The test: Using sterile tubes, the serum of the patient is diluted with Verwoort-Schuffner buffer solution (See Appendix) in the following manner:

Tube	1	2	3	4
Buffer	1.2 cc.	0.9 cc.	0.9 cc.	0.9 cc.
Serum*	0.3 cc.			
Dilution	1:5	1:50	1:500	1:5000

* Transfer 0.1 cc. of the serum buffer mixture from #1 to #2, from #2 to #3 and from #3 to #4.

Two sterile porcelain plates are used for each specimen to be examined. The tests are set up as in the following model, taking care to avoid contamination:

L. icterohemorrhagiae			L. canicola		
Row 1	Row 2	Row 3	Row 4	Row 5	Row 6
.15 cc. 1:5 .15 cc. antigen (1:10)	.05 cc. 1:5 .10 cc. buffer .15 cc. antigen (1:30)		The same procedure as that used for L. icterohemorrhagiae except for the use of an L. canicola antigen.		
.15 cc. 1:50 .15 cc. antigen (1:100)	.05 cc. 1:50 .10 cc. buffer .15 cc. antigen (1:300)				
.15 cc. 1:500 .15 cc. antigen (1:1000)	.05 cc. 1:500 .10 cc. buffer .15 cc. antigen (1:3000)				
.15 cc. 1:5000 .15 cc. antigen (1:10,000)	.05 cc. 1:5000 .10 cc. buffer .15 cc. antigen (1:30,000)	.15 cc. buffer .15 cc. antigen (control)			

The porcelain plates are covered to prevent drying and incubated for 3 to 4 hours at 32 degrees C. or six hours at room temperature. Each dilution is then examined by darkfield for agglutination or lysis. Agglutination often occurs in the lower dilutions and lysis in the higher dilutions. Both of these reactions are specific. No reaction in the 1:10 dilution with positive results in higher dilutions may be observed. The darkfield examination is done on a glass slide without a coverslip using the low power objective and a compensating ocular.

The detection of Vi agglutinins by using a special antigen rich in Vi and poor in O antigen (Blatnagar's Vi I strain) has been used for the detection of typhoid carriers (Felix et al. J. Hyg., 1935, 35, p. 421; Pijper and Crocker, J. Hyg. Camb., 1937, 37, 332; Bhatnagar, Brit. Med. Jour., 1938, 2, 1195; Horgan and Drysdale, Lancet, 1940, 1084-85; Eliott, Am. J. Hyg., Sect. B, 1940, 31, 8-15.)

The finding of bacteriophages that specifically lyse typhoid organisms possessing Vi antigen (Craigie, J. Bact., 1936, 31, 56; Sertic and Boulgalkov, Compt. rend. Soc. de Biol., 1936, 122, 35) has led to a classification of Bact. typhosum organisms containing Vi antigen based on their susceptibility to serologically distinct Vi-phages. (Craigie and Yen, Canad. Pub. Health J. 1938, 29, 448.) The ability to type strains of Eberthella typhosum by this method has provided us with a valuable epidemiological tool (Yen, Chinese Med. Jour., 1940, 57, 330.)

Precipitation.

When the sera of animals immunized with bacteria or bacterial products are mixed with these soluble bacterial products in correct proportions, macro-

scopically visible precipitates are produced. Like the agglutination test this reaction is a specific one and may be employed in the identification of bacteria and in the diagnosis of disease. The test is not as practical as the agglutination test because,while a reasonably potent serum can be diluted several thousand times before the agglutinating power is exhausted, in precipitation reactions the serum will lose precipitating power if diluted more than, at most, twenty times. In titrating precipitating sera the antigen solution is diluted and can be detected with a potent precipitating serum, in dilutions of from one to many thousand times.

In the general diagnostic laboratory the precipitative reaction has a limited range of usefulness but has wide application in the field of immunity. It has been used in medico-legal work for the identification of stains caused by animal fluids and in detecting adulteration of various food products. In these applications, specific precipitative sera are produced against the protein material to be tested for. Thus in the case of suspected adulteration of meat-products a positive precipitative reaction obtained with a mixture of an extract of the meat-product and an antiserum prepared against the suspected adulterant, will establish the presence of the suspected protein in that product.

The "ring" technique in which dilutions of the antigen are layered over the antisera is usually used. A positive reaction is indicated by the formation of a white precipitate at the junction of the two liquids. The contents of the tube may then be shaken and allowed to stand overnight at room temperature. Final readings of the precipitate are then made. Lancefield, (Proc. Soc. Exp. Biol. and Med., 1938, 38, 473) describes such a ring test using very small quantities of the reagents.

A convenient precipitation test is the hanging drop technique described by Brown (J.A.M.A., 1938, 111, p. 310) for the grouping of beta hemolytic streptococci and that for the typing of pneumococci (Schaub and Reid, J.A.M.A., 1938, 111, p. 1285.) In this test loopfuls of antiserum and antigen are mixed and examined for the presence of precipitate with the 16 mm. objective of the microscope.

By the use of the following simplified technique described by Brown, beta hemolytic streptococci may be grouped according to the Lancefield classification. Group antisera are available commercially. In grouping streptococci from human infections, Groups A, B, C and D sera should always be used. If possible, it is also desirable to use antisera against Groups E, F, G, H and K, as occasionally minute beta hemolytic streptococci of Group F and organisms of the Groups G, H and K are isolated from clinical and autopsy material.

1. Preparation of the Antigen - The culture is grown in 5 cc. of infusion broth or basic broth containing 1 per cent of dextrose for from 18 to 24 hours at 37 degrees C. Many strains grow in the form of a sediment at the bottom of the test tube; others need to be centrifuged. All but about 1 cc. of the supernatant broth is pipetted off and discarded. Two drops of metacresol purple indicator (0.04 gram dissolved in 60 cc. of 95 per cent alcohol and then diluted to 100 cc. with distilled water) are added to the remaining sediment suspension. From a drop bottle, 2 per cent hydrochloric acid (which may be prepared by diluting about 6 parts of concentrated HCl to 100) is added until the indicator turns slightly

pink (about pH 3.0). Congo red paper which turns blue at this pH may be employed. The tube of sediment is heated in a boiling water bath with occasional shaking for 15 minutes and then colled in running cold tap water for 10 minutes. From a drop bottle 2 per cent sodium hydroxide is added until the color of the indicator passes through yellow and begins to darken (about pH 7.5) but is not noticeably purple. The tube is then centrifuged for about 15 minutes, and the clear supernatant fluid used for the precipitin test. It is not necessary to dilute the antigen.

2. Technique of the Test - On the bottom surface of a nearly optically perfect Petri dish rule 12 mm. squares by means of a wax or diamond pencil. On the abscissa indicate the sera to be used; e. g., A, B, C, and D. On the ordinate indicate the antigens. Both inside and outside surfaces of the bottom of the Petri dish must be very clean and free from lint, dust, and finger prints, but need not be sterile. Within the appropriate squares on the inside surface of the bottom of the dish place one loopful of antigen from a small (2mm.) platinum loop and one loopful of serum, mixing the serum with the antigen as added so as to make rather flat, hanging drops when the dish is inverted. A platinum loop is specified because some of the cheaper substitutes give off alkali. To avoid carbon particles in the drop it is essential to burn off the loop thoroughly. This can best be done by dipping it into water to remove most of the serum each time before flaming. It may be necessary to centrifuge the sera occasionally to free them from particles of native precipitate. One should be careful not to form a precipitate by introducing a hot loop into the serum or antigen. Into the lid of the Petri dish is placed a disk of moist, but not too wet, white filter paper, and the dish is closed by inverting the bottom part and placing it, bottom uppermost, in the lid. If the Petri dish is not optically perfect, a piece of clear plate glass will do.

3. Reading of Results - The assembled Petri dish is placed, bottom up, on the stage of a microscope and the drops are observed through a 16 mm. objective. Positive reactions usually develop in thirty minutes and are indicated by the appearance of large clumps of precipitate. Sometimes, as the precipitate is forming, it assumes a diffuse, ground-glass appearance, and clumps may be caused to form by carefully rocking the dish. Negative results are indicated by the absence of such precipitate. (Brown, J.H., J.A.M.A., 1938, 111, 310.)

For the inexperienced worker, the ring precipitin test described by Lancefield (Proc. Soc. Exp. B. & Med., 1938, 38, 473) may be found to give more clear-cut results.

A small conical tube is prepared by slightly drawing 7 mm. glass tubing in a flame. The drawn tubing is cut in the center to make 2 tubes. The narrow end is sealed into a knob to prevent the tube from slipping through the rack. The 7 mm. diameter is retained at the open end to facilitate pipetting, and the lower end has a bore of about 3 mm., holding 0.1 cc. in a column 8 to 10 mm. high. Good results have been obtained by putting 0.05 cc. of the extract into the tube first, and then 0.05 cc. of serum. Since the serum is heavier, it sinks below the saline extract and forms a layer with about the right amount of mixing. With the usual technic employed in ring-tests of placing the serum in the tube first, too narrow a ring is formed due to insufficient mixing in a tube of such small caliber. If, due to an air

bubble, the fluid fails to lodge at the bottom of the tube, no attempt is made to shake it down. The result can easily be read at any level, and shaking is apt to interfere with the formation of a good plane of junction. Positive reactions are usually obvious at once, and can be safely recorded after ½ hour at room temperature. In order to save serum, exceptionally potent antisera may be diluted, but not beyond the point where an immediate heavy ring-reaction is obtained with extracts of the homologous group.

In case of doubt, the dilution-method with large volumes is used, or a comparable dilution-method can be employed with the microtechnic in the following manner: 0.05 cc. each of undiluted extract and of 1:4 and 1:16 dilutions of extract are pipetted into 3 small tubes, and 0.05 cc. of serum is added to each. The small tubes are observed for ring formation, as usual, at the end of a half hour. The tubes are then shaken, incubated at 37° C. for 2 hours and placed in the icebox overnight before final readings are made.

Gordon (Med. Research Council, Special Rep. Series, No. 98, H. M. Stationery Office, London, 1925) described a precipitin reaction with an extract of vaccinal lesions against vaccinal antiserum produced in rabbits. A similar test may be performed for the diagnosis of variola (Craigie et al, Tulloch.) Ledingham (Bull. John Hopkins Hosp., 1935 a, 56, 247; 1935 b, 57, 32) has claimed that there is some specificity separating vaccinia and variola flocculation (precipitation) tests, but in the routine application of this test this differentiation is not possible. The test may be employed, however, for the differentiation of these two diseases from chicken-pox and from generalized impetigo.

Ascoli's precipitin reaction has been found useful in making a rapid diagnosis of infection by B. anthracis (See sect. on gram-positive rods.)

The flocculation of diphtheria toxin by its antitoxin is a precipitative phenomenon which is not used in the diagnostic laboratory but is a convenient method for titrating these substances. The Ramon method of flocculation is one based on the fact that precipitation occurs first in that tube of a series containing various amounts of the two reagents in which these substances are present in equivalent proportions. For a description of the procedure followed see page 933 of Zinsser and Bayne Jones' Textbook of Bacteriology, Eighth Edition, 1939.

COMPLEMENT (Alexin) FIXATION

An important diagnostic serological procedure is that depending upon the binding or fixation of a normal constituent of serum (complement) when an antigen and its specific antibody are brought together. Since this phenomenon is an invisible one it is detected by determining whether a measured amount of complement has been utilized by an antigen-antibody system through the later addition to the same system of a second (indicator) antigen-antibody system. In the second antigen-antibody system the antigen is usually sheep cells and the antibody sheep hemolysin. For lysis of the sheep cells (an obviously visible phenomenon) by the hemolysin, the presence of complement is necessary. If the complement is fixed by the first antigen-antibody combination, there is no complement left for hemolysis of the sheep cells and the test is positive. When the sheep

cells are hemolysed, on the other hand, no complement fixation by the first system was obtained and the test is reported as negative.

The outstanding example of a complement-fixation diagnostic procedure is the Wassermann test in which the antigen is an alcoholic solution of lipoidal substances extracted from beef heart muscle.

Complement fixation tests for bacterial infections are applicable to only a few diseases, principally gonorrhea, glanders and rarely tuberculosis. The technique of the test is the same as that of the Wassermann, but the antigen consists of preparations of the specific bacteria. The test appears to be valuable, however, in the diagnosis of rickettsial diseases. (See section on rickettsiae.)

No attempt will be made here to describe in detail the technique of the complement fixation test. The Wassermann test is described in detail in Diagnostic Procedures and Reagents, Am. Pub. Health Assn., 1941. For a detailed description of the technique for the bacterial complement fixation test the reader is referred to the Textbook of Bacteriology by Zinsser and Bayne-Jones, Eighth Edition - 1939.

Neufeld in 1902 described a reaction between the pneumococcus and its specific antiserum which he pictured as a swelling (Quellung) of the capsule. This reaction is now used extensively for the typing of pneumococci. The test may be applied directly to the sputum and is therefore of considerable value because of the ease and speed with which a diagnosis may be made.

In typing directly from the sputum, one small loopful of sputum (if thick and tenacious wash and emulsify in saline) is mixed with two or three loopfuls of undiluted antiserum. The mixtures are stained lightly by adding a small drop of Loeffler's alkaline methylene blue and are covered at once with cover slips to prevent drying. Examination is made with the oil immersion lens, with the light dimmed. When a positive reaction occurs, which is usually within a few minutes, there is a decided swelling of the capsule of the pneumococcus. The swollen capsule is of a light greenish-gray color, is much less translucent than one that is not swollen, having a ground glass appearance, and has a definite outline. The definite outline is one of the most characteristic features of a positive reaction. In the preparations in which no reaction is evident, the capsule of the pneumococcus appears as a halo of refracted light. In all preparations the body of the pneumococcus stains a definite blue.

Combinations of monovalent antiserum (rabbit) are used instead of making separate preparations of sputum with each of the monovalent serums. After determining in which combination (pool) of anti-sera the organism falls, the sputum is mixed with the individual monovalent serums of the (pool) in order to determine the type of the organism. In some instances the capsular swelling may take as long as 60 minutes to appear. When the sputum contains a large number of pneumococci (especially Type III) it is necessary occasionally to dilute the sputum with salt solution before any swelling of the pneumococcus becomes evident.

It is also advisable to smear and Gram stain the sputum prior to doing the Quellung test in order to determine whether other organisms such as Friedlander's may be the predominating organism and whether there are enough pneumococci present to justify the execution of the Quellung test.

Organisms falling in certain of the numerically higher types may give typical but less extensive swelling of the capsule. Such positive reactions may be missed if a hasty examination is made.

At times because of a poor sample of sputum it may be convenient to inject a mouse with the sputum and to type the pneumococcus from the peritoneal exudate. The mouse appears to act as a selective medium for the virulent pneumococcus which grows readily in the peritoneal cavity of this animal.

About 0.5 cc. of sputum is injected intraperitoneally and as soon as the mouse dies, which is usually in 18 to 24 hours, it is autopsied. If the autopsy cannot be done immediately the dead mouse should be kept in the refrigerator until the examination can be made. The peritoneal cavity is washed out with one to two cc. of sterile saline and Quellung preparations set up with these peritoneal washings following the same procedure described for the typing of pneumococci from sputum. It is sometimes possible to obtain suitable peritoneal washings about four hours after injection of the mouse. This may be accomplished without killing the animal by using a syringe and needle to inject two or three cc.

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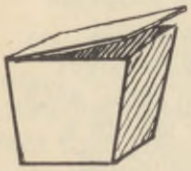
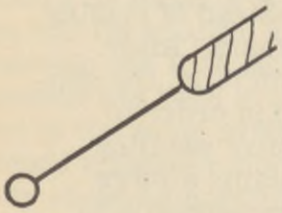
PNEUMOCOCCUS TYPING



1.
Sputum from patient suspected of, or known to have, Pneumococcus infection.

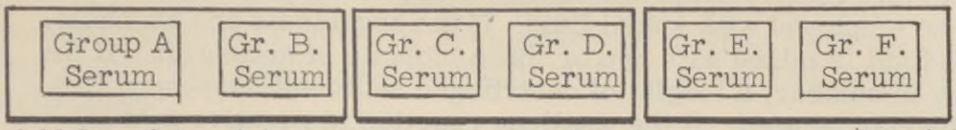
2.
Gram's stain of representative sample. If no gram-positive diplococci having typical lancet-shaped morphology are present repeat the preparation. If 2-3% or more of the organisms appear to be pneumococci perform the routine typing.

3.
Mix fleck of sputum thoroughly with methylene blue (Loeffler's alkaline solution) and typing serum on a slide. Use one loopful of sputum, a drop of methylene blue and 2 or 3 loopfuls of typing serum. Two preparations may be used per slide. Flame the loop after using so as not to mix or contaminate the sera.



Preliminary Grouping

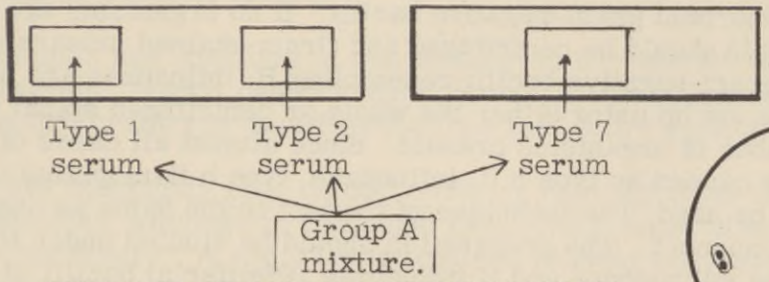
A. One loopful of sputum and drop of methylene blue.

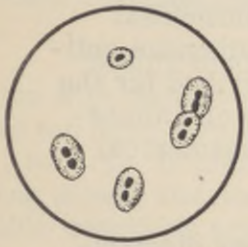


B. Add 2 or 3 loopfuls or one capillary tube of group serum (Gr. A to F)
C. Mix well with toothpick (one for each mixture) and cover with coverslip.
D. Examine under oil immersion lens with light partially dimmed.

Final Typing of Pneumococci

If any one or more Group serum-sputum mixtures show capsular swelling around the pneumococci, repeat the preparations outlined above using the Type sera included in the Group serum mixture which showed capsular swelling. For example: if typical capsular swelling was observed in the Group A mixture (Group A contains Types 1, 2 and 7 sera), a loopful of sputum and a drop of methylene blue is mixed on a slide with the separate types 1, 2 and 7 sera. The mixtures are covered with a coverslip and examined after 5 to 30 minutes.





Positive Reaction

THE ONE OR MORE MIXTURES THAT SHOW POSITIVE CAPSULAR SWELLING ARE REPORTED AS THE TYPE OR TYPES OF PNEUMOCOCCI PRESENT IN THE SPECIMEN SUBMITTED.

Negative Reaction

* * * * * * * * *

of saline intraperitoneally and then using the same syringe and needle to withdraw the washings. A sharply pointed pipette may also be used.

In the Neufeld typing of pneumococci in mouse peritoneal washings, agglutination of the organisms may be detected on microscopic examination and conveniently used in identifying the type of pneumococcus.

In cases of pneumococcus meningitis, the Quellung reaction may be used for the typing of pneumococci directly from the spinal fluid. When a sample of spinal fluid is sent to the laboratory for bacteriological study, and a Gram-stained smear of the material shows organisms morphologically similar to pneumococci, a Quellung test should be set up immediately. Such typing of the organisms definitely establishes its identity as a pneumococcus. If the diagnosis is based on morphology alone there is always a possibility of error. The organism

may be identified by cultural methods, but this procedure is slow and the immediate diagnosis of the causative organism in meningitis is usually of considerable help in that it permits rapid institution of specific serum therapy and the selection of the most suitable chemotherapeutic agent. The general procedure used with sputum may be employed with spinal fluid. If the organisms are abundant, a loopful of spinal fluid is mixed with two or three loopfuls of antiserum. If the organisms are few in number, a loopful of centrifuged sediment suspended in a few drops of saline may be used. If the Quellung reaction is negative with all of the group sera, the preparations should be set aside and re-examined at the end of one hour.

The Neufeld Quellung test may be applied to material obtained from lung puncture or from empyema pus. The approximate number of organisms should be determined and if large numbers are present (more than fifteen or twenty per oil immersion field) it is necessary to dilute the original material. To make a dilution suspend a loopful of the original material in a few drops of sterile saline.

Rapid diagnosis of meningitis caused by Hemophilus influenzae may be made by performing a Quellung test with the spinal fluid. Gram-stained smears should first be made of the material and observed carefully for the presence of small and often pleomorphic gram-negative bacilli. If no organisms are seen in the smears, the fluid should be centrifuged and Gram-stained smears made from the sediment. If gram-negative bacilli resembling H. influenzae are seen, Quellung tests should be set up using either the whole or centrifuged spinal fluid according to the number of organisms present. Since almost all cases of influenzal meningitis are caused by type b H. influenzae, type b Hemophilus influenzae antiserum should be used. The technique of the test is the same as described for the typing of pneumococci. The preparation should be studied under the oil immersion lens of the microscope and if influenzae (Pfeiffer's) bacilli of serological type b are present, a typical Quellung reaction will be observed.

Injection of organisms or their products into the skin is another diagnostic procedure of considerable value.

The intracutaneous (Mantoux) test is the most delicate method of determining past or present infection with tubercle bacilli. An injection of 0.1 cc. of a 1:1000 dilution of Koch's Old Tuberculin (OT) or of the first dilution (0.0002 mg. per cc.) of purified protein derivative of tuberculin (Tuberculin P.P.D.) is made intracutaneously into the flexor surface of the forearm for routine exclusion purposes. A control of 0.1 cc. of concentrated broth as a test for protein sensitivity must be included when O.T. is used. Tests are read at 24 and 48 hours. A positive reaction with Old Tuberculin consists of an area of redness, swelling and induration at least 5 mm. greater in diameter than the control reaction. With P.P.D. a positive reaction consists of an area of swelling which is 5 mm. or more in diameter.

Von Pirquet's method of performing the tuberculin test is convenient but not as accurate as the Mantoux. Two small drops of Old Tuberculin are placed on the skin of the front of the forearm about two inches apart and the skin is slightly scarified, first at a point midway between them and then through each of the

drops. A wooden tooth-pick with chisel-shaped end is a convenient scarifier. It is held at right angles to the skin and rotated 6 to 12 times with just sufficient pressure to remove the epidermis without drawing blood. In about 10 minutes the excess of tuberculin is wiped away gently with cotton. No bandage is necessary. A positive reaction is shown by the appearance in 24 to 48 hours of a papule with a red areola, which contrasts markedly with the small red spot left by the control scarification.

The Schick test is an intracutaneous test used to determine susceptibility to diphtheria. An individual having at least 1/30 of a unit of diphtheric antitoxin per cc. of blood will show no reaction.

An intradermal injection of 0.1 cc. of standard diphtheric toxin containing 1/50 M.L.D. is made into the flexor surface of the forearm. The same amount of diphtheric toxin which has been heated to 75 degrees C. for 10 minutes to destroy the toxin is injected as a control into the other arm. Another form of control (The Moloney test) may be used. In this control a formalin toxoid, diluted 1:20, is used. Since in such a control there is a greater amount of foreign material injected than in the test injection Moloney reactors may often be Schick negative. It has the advantage that those who react to it are also apt to react unfavorably to immunizing doses of toxoid.

Certain individuals are sensitive to the proteins in the mixture and show reactions to this fraction. Such a pseudo-reaction may, at times, mask the true toxic reaction, although ordinarily pseudo-reactions fade more rapidly.

Schick tests are commonly read on the fourth or fifth day to avoid confusion with pseudo-reactions, although in combined reactions a lapse of seven days to ten days may be necessary. A red, circumscribed, slightly infiltrated area 1 to 2 cm. in diameter is considered positive. It persists for 7 to 15 days, fading slowly and showing superficial scaling and brown pigmentation. Four possible reactions are shown in Table 34.

The Dick test is used to determine susceptibility to scarlet fever. One skin test dose (STD) of the toxin in 0.1 cc. is injected intradermally into the flexor surface of the forearm. The other arm is injected with 0.1 cc. of the toxin heated to 100 degrees C. for 2 hours. Reactions are read at 18 to 24 hours. An area of redness and slight infiltration at least 1 cm. in diameter with a negative control is indicative of a positive reaction. The possible reactions to this test are shown in Table 34.

Brucellergin (a standardized nucleo-protein suspension extracted from Brucella abortus, Brucella suis or Brucella melitensis), is used to detect brucella infection.

0.1 cc. of brucellergin is injected into the flexor surface of the forearm and the reaction read 24 and 48 hours later. The diameter of the reaction varies from 2 to 10 cm. It is characterized by erythema and slight edema and may persist for 48 to 96 hours and occasionally even longer. In infected individuals the local reactions may be accompanied by more marked manifestations of symptoms;

focal reactions, especially, may be noted; hypersensitive persons will show severe systemic reactions. Those persons who have not been sensitized to Brucella and who are probably susceptible to infection show no local or systemic reaction. In normal individuals an erythema without edema may appear about the point of the injection. These nonspecific reactions subside between the 24 and 48 hour readings, while specific reactions may increase. A positive reaction indicates a sensitivity to the nucleoprotein and should be interpreted as meaning past or present exposure to the brucella organism.

TABLE 34.

Possible results of intradermal tests with toxic filtrates

Test arm (toxin)	Control arm (protein)	Reading	Interpretation
+	-	Positive	Not immune Not sensitive
-	-	Negative	Immune Not sensitive
+ larger	+	Combined	Not immune Sensitive
+ equal	+ equal	Pseudo	Immune Sensitive

Another allergic skin test discovered by Frei and named after him is used in the diagnosis of lympho-granuloma venereum. The original Frei test was performed by injecting intradermally 0.1 cc. of a 1 to 10 dilution, in saline solution, of pus aspirated from a suppurating inguinal node. In persons having the disease, a papule surrounded by a red areola is developed in 48 to 72 hours at the site of the injection. Due to the difficulty of obtaining sufficient material from human subjects a substitute for the Frei antigen has been prepared in the form of a saline emulsion (1 to 5 or 1 to 10) of the brains of infected mice. When used with a suitable mouse brain control this antigen seems to give satisfactory results although some workers feel that because of false reactions that may be obtained with this antigen, it is not suitable for the routine diagnosis of lymphogranuloma venereum.

More recently a Lymphogranuloma venereum antigen of chick embryo origin has been made available. This antigen contains little non-specific extraneous matter, and false reactions due to such matter are therefore at a minimum.

Schultz and Charlton in 1918 described a skin test bearing their name which is sometimes used as an aid in the diagnosis of scarlet fever. By injecting scarlet-fever convalescent serum into the reddened skin of a scarlet fever patient a local blanching was obtained. One tenth cc. of potent scarlatinal antitoxin produced in horses (or 0.5 cc. of convalescent serum) is injected intradermally into an area of red skin rash. In a positive test blanching usually begins to appear in 6 to 8 and up to 14 hours. When no blanching at the site of injection is obtained the test is negative. A positive test is one in which blanching occurs for several cm. around the site of injection. The swelling of the follicles in the area may disappear, with the skin assuming a normal color and appearance. The blanched appearance usually lasts throughout the duration of the rash.

The absence of a positive Schultz Charlton test does not make the diagnosis negative and the value of the test progressively diminishes with the age of the rash.

The Use of Animals

Animals used routinely in the laboratory are: the guinea pig, rabbit, white mouse and white rat. Occasionally the monkey, ferret, cat, horse, fowl, canary and Syrian hampster are used. They are useful for the determination of virulence of the organism under study, for the isolation of organisms that are not readily grown on culture media, for immunity studies and for the maintenance of such infectious agents as cannot be kept on culture media.

Before mixing with regular stock all animals received from an outside source should be isolated for from 10 days to three weeks and found to be free from disease.

Animal quarters should be kept clean, dry, and completely free from vermin. The optimum temperature for most animals is 65 degrees F. to 70 degrees F. Large (10-1/2 inch) and small (8 inch) animal jars are suitable for mice and rats; the large jar can be used for a small guinea pig. The bottom of the jar should contain an absorbent bed material, such as wood shavings; hay or straw may be used in large cages. The animal quarters should be cleaned and the bedding changed twice per week.

The diet recommended for rabbits consists of commercial "Rabbit Pellets" supplemented once or twice per week with feeding of green-stuff, such as carrots, lettuce, or celery tops. A diet consisting of equal parts of oats, wheat, and barley, plus 10 per cent of legume, soybean, or linseed meal is suitable. Alfalfa or timothy hay will serve both for food and bedding. Plenty of water and a small piece of rock salt should always be kept in the cage.

Rabbits may be afflicted with one or more of several diseases:

(1) "Coccidiosis", an intense and fatal enteritis, is the most serious disease. New rabbits should be observed for this disease several days before adding to stock.

(2) "Ear mange", which is caused by a mite, can be cured by local application of a parasiticide.

(3) "Snuffles", is a cold-like disease caused by a filterable virus. Infected rabbits should be isolated until three weeks after recovery.

Guinea pigs receive the diet recommended for rabbits except that they must have supplementary feeding of green stuffs at least twice per week to supply vitamin C. A vitamin C deficiency is characterized by coarse hair and mangy appearance. It is transmissable to the young through the mother.

Guinea pigs may be infected with a Balantidium coli type of enteritis. In case of Salmonella infections, all potentially infected animals should be destroyed, the room and cages sterilized and new stock obtained.

Commercial dog or fox-chow checkers furnish an ample, balanced diet for the growth and breeding of mice. Occasionally a piece of carrot or some other green stuff should be added. There should be a supply of fresh clean water in the cage at all times. Mice will also do well on simpler diets such as mixed grain diet listed above for rabbits, or dry bread with water or skimmed milk, with addition of cod liver oil once per week. Salmonella infections are common and should be handled as described above for guinea pigs.

Rats receive the diet described for mice. They are usually very resistant to disease.

Monkeys do well on dog-chow checkers plus canned tomatoes, with occasional feeding of fruits and nuts (oranges, apples, bananas, peanuts, sunflower seeds, etc.) Pneumonia (usually fatal) and tuberculosis may be found in these animals.

For the identification of a few animals a description of the color, sex and peculiarities of each animal may be sufficient. Numbered ear tags and leg bands may also be used. It is also possible to number animals by systematically arranging holes and notches in their ears.

Precautions:

Animals which are to be injected with material in which the infectious agent may be carried by the animal's ecto-parasites should be dipped in an antiseptic solution prior to use. They should then be placed in glass jars covered with fine mesh gauze to prevent access or escape of any parasites.

When handling infected animals, living or dead, the hands and arms should be protected by wearing rubber gloves and a long-sleeved gown.

The use of animals in the diagnosis of some diseases is indicated in Table 35.

TABLE 35.

DIAGNOSIS OF INFECTIOUS DISEASES BY ANIMAL INOCULATION

Etiological Agent	Disease	Animal of Choice	Usual Method of Inoculation	Sources of Material	Diagnostic Characteristics
Bacteria					
Diplococcus pneumoniae	Pneumonia	Mouse	Intraabdominal	Sputum	Type determination from peritoneal exudate and bacteria by Neufeld, precipitative, or agglutinative tests. Blood culture.
Corynebacterium diphtheriae	Diphtheria	Guinea pig	1. Endermal 2. Subcutaneous	Culture	1. Skin lesion. 2. Local edema, hemorrhagic effusions, and hemorrhagic suprarenals. Control guinea pig injected with 500 units of antitoxin - negative.
Bacillus anthracis	Anthrax	Mouse, Guinea pig	Subcutaneous	Pustule(man) Blood(animals)	Numerous bacilli in spleen and blood.
Clostridium botulinum	Botulism	Mouse, Guinea pig	Subcutaneous	Suspected food	Death from cardiac or respiratory failure, at times with paralysis. Control animals, protected by antitoxin - negative.

TABLE 35 (Continued).

DIAGNOSIS OF INFECTIOUS DISEASES BY ANIMAL INOCULATION

Etiological Agent	Disease	Animal of Choice	Usual Method of Inoculation	Sources of Material	Diagnostic Characteristics
Bacteria					
Clostridium tetani	Tetanus	Guinea pig, Mouse	Subcutaneous	Wound or culture*	Spastic paralysis. Control animal protected by antitoxin-negative.
Clostridium welchii	Gaseous gangrene	Guinea pig, Pigeon	Intramuscular	Wound or culture	Local dissolution of muscles with crepitation and general gaseous infection. Control animal protected by antitoxin - negative.
Brucella melitensis, B. abortus, and B. suis	Brucellosis	Guinea pig (male)	Subcutaneous	Blood Urine Lesions	In 4 to 12 weeks caseous nodules in spleen, liver, lymph glands, and at times epididymitis. Agglutinins may be demonstrated.
Pasteurella pestis	Plague	Rat, Guinea pig, Mouse	Subcutaneous, Cutaneous	Bubo Blood Sputum	Hemorrhagic lymphadenitis, caseous lesions in spleen, liver, and lymph glands.
Pasteurella tularensis	Tularemia	Guinea pig	Cutaneous, Subcutaneous	Lesion Lymph gland Blood	Within one week lymphadenitis and caseous areas in spleen and liver

*Mix specimen with sterile emory dust.

TABLE 35 (Continued).

DIAGNOSIS OF INFECTIOUS DISEASES BY ANIMAL INOCULATION

Etiological Agent	Disease	Animal of Choice	Usual Method of Inoculation	Sources of Material	Diagnostic Characteristics
Bacteria					
Mycobacterium tuberculosis var. hominis	Tuberculosis	Guinea pig	Subcutaneous	Sputum Urine Pleural fluid Spinal fluid	In six weeks tuberculous nodules in regional lymph glands, spleen, and liver.
Mycobacterium tuberculosis var. bovis	Tuberculosis	Rabbit	Subcutaneous	Glands Bone Lesions	In ten weeks miliary tuberculosis with nodules in lungs, kidneys, lymph glands, and at times in spleen and liver.
Vibrio comma	Cholera	Guinea pig	Intraabdominal	Culture	Peritonitis with vibrios present. Control animal with anticholeric serum shows lysis of vibrios. (Pfeiffer's phenomenon.)
Spirillum minus	Rat-bite fever	Mouse, Rat, Guinea pig	Intraabdominal	Blood Local lesions Lymph glands	Organisms in blood in 5 to 14 days.
Malleomyces mallei	Glanders	Guinea pig (male)	Intraabdominal	Lesion	General granulomata. Orchitis with plastic exudate. (Straus reaction.)

TABLE 35 (Continued).

DIAGNOSIS OF INFECTIOUS DISEASES BY ANIMAL INOCULATION

Etiological Agent	Disease	Animal of Choice	Usual Method of Inoculation	Sources of Material	Diagnostic Characteristics
Spirochaetes					
Borrelia recurrentis	Relapsing fever	Mouse	Intraabdominal	Blood	Organisms in blood in 1 to 4 days.
Leptospira ictero-hemorrhagiae	Infectious jaundice. (Weil's disease.)	Guinea pig	Intraabdominal	Blood Urine	Leptospirae in blood and liver in 7 to 12 days. Hemorrhage, jaundice and fever.
Fungi					
Coccidioides immitis	Coccidioidal granuloma	Guinea pig	Subcutaneous	Pus from lesions	In four weeks tissue lesions with organisms showing endogenous sporulation.
Rickettsia					
Rickettsia prowazeki	Old World typhus. Epidemic typhus.	Guinea pig (young male)	Intraabdominal	Blood	Slight mortality. Rare slight scrotal reaction? (See section on Rickettsiae.)
Rickettsia prowazeki (murine)	New World typhus. Endemic typhus.	Guinea pig (young male) Rat	Intraabdominal	Blood	Slight mortality. Scrotal swelling? (See section on Rickettsiae.)

TABLE 35 (Continued).

DIAGNOSIS OF INFECTIOUS DISEASES BY ANIMAL INOCULATION

Etiological Agent	Disease	Animal of Choice	Usual Method of Inoculation	Sources of Material	Diagnostic Characteristics
Rickettsia					
Rickettsia rickettsi	Rocky Mt. spotted fever	Guinea pig (young male)	Intraabdominal	Blood	High mortality. Western type - Marked scrotal lesions? Eastern type - Rare scrotal swelling? (See section on Rickettsiae.)
Ultramicroscopical viruses*					
Epidemic Encephalitis	Epidemic encephalitis	Rabbits Monkey Mice(St. Louis)	Intracerebral	Brain	Encephalitis with histopathological lesions of brain.
Herpes febrilis	Herpes febrilis	Rabbit	Corneal	Vesicular fluid	Keratitis with intranuclear inclusion colonies in epithelial cells of cornea.
Influenza	Influenza	Ferret, then to mouse.	Intranasal	Nasal secretions	Atypical pneumonia in mice.
Lymphogranuloma inguinale	Lymphogranuloma inguinale	Mouse	Intracerebral	Pus from bubo	Encephalitis

* See section on viruses - Table 26.

TABLE 35 (Continued).

DIAGNOSIS OF INFECTIOUS DISEASES BY ANIMAL INOCULATION

Etiological Agent	Disease	Animal of Choice	Usual Method of Inoculation	Sources of Material	Diagnostic Characteristics
Ultramicroscopical viruses*					
Poliomyelitis	Poliomyelitis	Monkey	Intracerebral	Saliva Brain Cord	Paralysis with degeneration of motor cells in cord and brain.
Psittacosis	Psittacosis	Mouse	Intraabdominal	Sputum Blood	Focal necrosis in liver and spleen with inclusion colonies.
Rabies	Rabies	Rabbit Mouse	Subdural	Brain Cord	Negri bodies and lesions in central nervous system.
Yellow fever	Yellow fever	Guinea pig, Monkey. Mouse	Intraabdominal	Blood Liver Spleen	Degenerative lesions in liver with intranuclear inclusion colonies.

* See section on viruses - Table 26.

V. BACTERIAL FOOD POISONING

(Reference: Diag. Proc. & Reag., 1941.)

Food poisoning may be due to a number of different causes. Bacteria and their toxins, certain metallic compounds, foods which are inherently poisonous, and food allergy may be responsible for the symptoms accompanying food poisoning.

When bacterial food poisoning is suspected, the bacteriologist besides examining the food considered responsible, for all the bacteria present, must also look especially for certain bacteria or their toxins known to be responsible in the past for such symptoms. Several groups have been implicated as definitely responsible for food poisonings while the presence in large numbers of less definitely implicated organisms is considered by some investigators to be responsible for food poisoning also. In the latter instances gross contamination of food by such normally non-pathogenic organisms as Esch. coli or Proteus may be held to be responsible for the outbreak. The symptoms may be due to a liberation of poisonous substances by the bacteria or the products resulting from the decomposition of the food. In beginning a study of a case of food poisoning, therefore, direct smears and cultures of the food involved should be made as soon as possible in order that an approximate idea be obtained of the number and types of bacteria present.

The bacteria which have been repeatedly found to be responsible for outbreaks of food poisoning are: (1) Clostridium botulinum, the toxin of which gives rise to clinical symptoms quite distinct from those produced by the other food poisoning organisms, (2) certain members of the Salmonella group, (3) dysentery bacilli, (4) staphylococci and (5) streptococci.

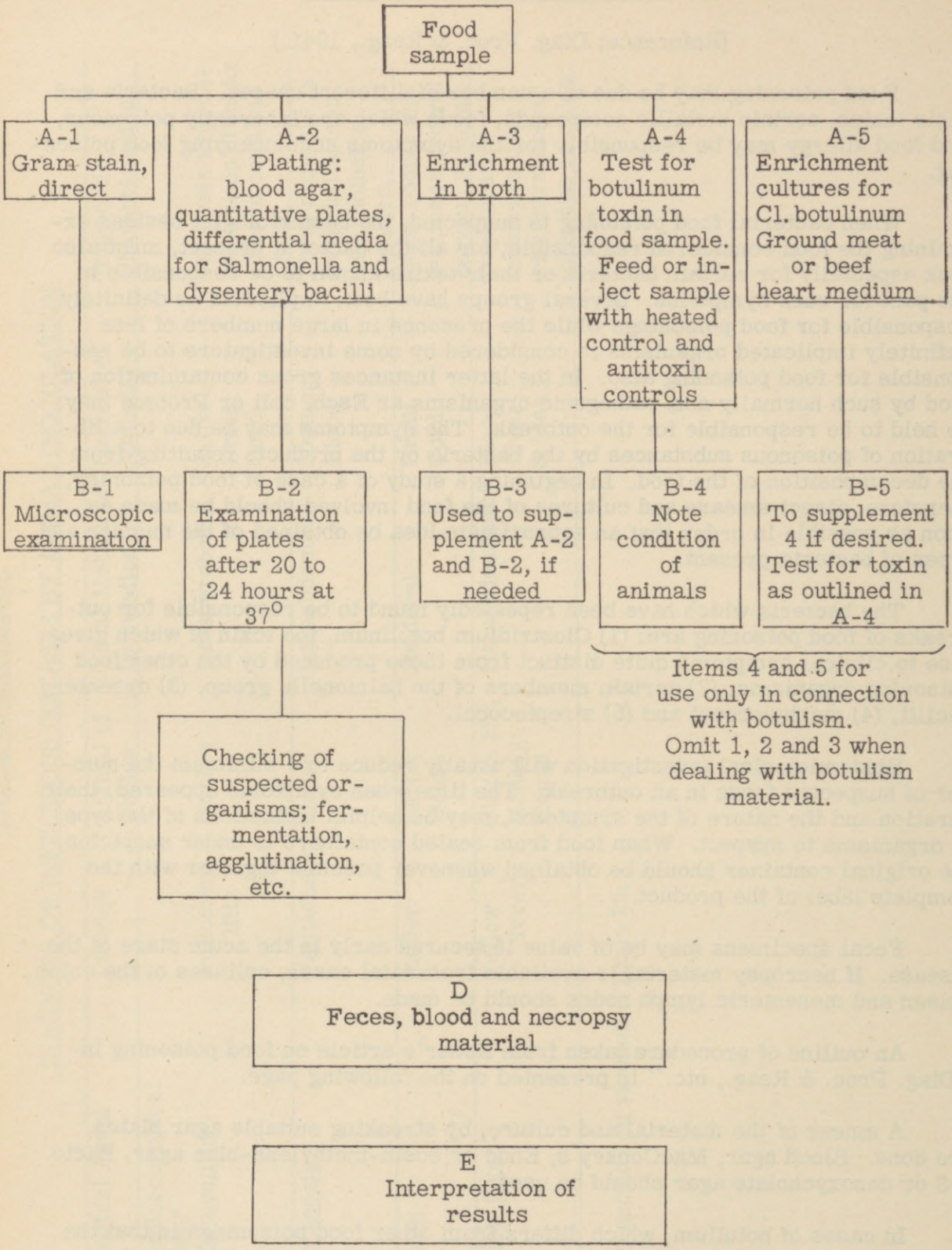
Epidemiological investigation will usually reduce to a minimum the number of suspected foods in an outbreak. The time when symptoms appeared, their duration and the nature of the symptoms, may be helpful indications of the type of organisms to suspect. When food from sealed containers is under suspicion, the original container should be obtained whenever possible together with the complete label of the product.

Fecal specimens may be of value if secured early in the acute stage of the disease. If necropsy material is available from fatal cases, cultures of the colon, spleen and mesenteric lymph nodes should be made.

An outline of procedure taken from Koser's article on food poisoning in "Diag. Proc. & Reag., etc." is presented on the following page.

A smear of the material and culture, by streaking suitable agar plates, are done. Blood agar, MacConkey's, Endo or eosin-methylene-blue agar, Bacto S-S or desoxycholate agar should be used.

In cases of botulism, which differs from other food poisonings in that the toxin is formed entirely in the food before ingestion, the liquor from the foodstuff or washings of the original container should be used if available. Gross contamination can be eliminated by centrifuging the material and using the supernatant fluid for animal inoculation. If sufficient material is available the fluid


Food sample
A-1 Gram stain, direct
A-2 Plating: blood agar, quantitative plates, differential media for Salmonella and dysentery bacilli
A-3 Enrichment in broth
A-4 Test for botulinum toxin in food sample. Feed or inject sample with heated control and antitoxin controls
A-5 Enrichment cultures for Cl. botulinum Ground meat or beef heart medium
B-1 Microscopic examination
B-2 Examination of plates after 20 to 24 hours at 37°
B-3 Used to supplement A-2 and B-2, if needed
B-4 Note condition of animals
B-5 To supplement 4 if desired. Test for toxin as outlined in A-4
Items 4 and 5 for use only in connection with botulism. Omit 1, 2 and 3 when dealing with botulism material.
Checking of suspected organisms; fermentation, agglutination, etc.
D Feces, blood and necropsy material
E Interpretation of results

may be sterilized by filtration through a Berkefeld or other suitable filter. A heated control is prepared by heating a small portion of the fluid for 10 minutes in a boiling water bath. Botulinus toxin is destroyed by this heating.

Portions of the sample and the heated control should be administered to animals - guinea pigs or white mice being injected subcutaneously. An alternative procedure, suitable for guinea pigs, is to feed them portions of the sample from a pipette. This requires a somewhat larger sample, and is perhaps not as delicate a test for minute amounts of toxin, but it has the advantage of eliminating the occasional infections caused by miscellaneous bacteria in badly spoiled samples which were not previously filtered.

The number of animals to be used will vary depending upon the amount of the sample and whether specific antitoxins for types A and B are on hand. (Types C, D and E may be disregarded by the average laboratory since specific antitoxic sera are not usually available for these types, and since types A and B are those encountered in the great majority of botulism outbreaks in this country.)

0.5 to 1.0 cc. quantities of the fluid are injected subcutaneously into the animals. The same amount of heated control is injected into a control animal and also into animals protected with 1.0 cc. of antitoxins A and B.

When the demonstration of the presence of botulinum toxin in the foodstuff may not be successful it may be possible to demonstrate the presence of the organism by means of an enrichment culture for Cl. botulinum. Three whole meat tubes (Appendix) are inoculated with portions of the sample. Two of these should be heated at 80 degrees C. for 15 minutes to destroy vegetative cells and all three should be incubated in the anaerobic jar at 37 degrees C. for 3 to 4 days. Examination of these cultures by smear may reveal the presence of a gram-positive spore-forming rod. The fluid from the cultures may then be examined for the presence of botulinum toxin.

With a strong toxin the injected animals often die within a few hours. Weak toxin may give rise to rather inconspicuous signs, such as salivation and flaccid or atonic abdominal muscles. In such instances death may not occur for 3 or 4 days or even longer. Death of the unprotected animal receiving the fluid, survival of the animal receiving the heated fluid and the survival of the animals receiving one type of antitoxin but not of that receiving the other type of antitoxin, is proof of the presence of botulinum toxin of the type indicated by the protection obtained with antitoxin.

Where cultures of Cl. botulinum are obtained from suspected foods or their containers, it is necessary to rule out contamination of either by soil after the ingestion of the food. The Cl. botulinum isolated may have been derived from the soil.

The plates streaked directly with the specimen should be examined after 24 hours of incubation for the predominating type of bacterium and for the presence of Salmonella, Shigella, Staphylococcus and Streptococcus colonies. For the identification of these organisms see the sections on the identification of the gram-negative rods and gram-positive cocci.

Salmonella aertrycke (S. typhi-murium), Salmonella enteritidis, Salmonella choleraesuis, Shigella paradysenteriae and Shigella sonnei are the chief offenders of the gram-negative rods.

Stool cultures done early in the sickness are often of value when the etiologic agent is a Salmonella or Shigella organism. Cultural examination of stool specimens should therefore be started as soon as possible.

In cases of botulism, toxin can sometimes be demonstrated in the blood or in the bowel contents by animal injection together with the use of specific antitoxins. It is necessary to filter the bowel contents to get rid of the bacteria which are present before using the specimen for toxin tests.

If a member of the Salmonella or Shigella groups has been isolated from the suspected food, this finding is highly significant. These organisms are not ordinarily encountered in foodstuffs and there is good evidence that they produce characteristic food poisoning symptoms. The incubation period is usually 15 to 24 hours, but may be longer occasionally. Additional information is obtained if it has been possible to isolate a similar Salmonella or Shigella from specimens of feces or from necropsy material.

The finding of staphylococci on the plates made directly from the foodstuff, particularly in large numbers may be indicative of a causative role played by this organism in the particular case of food poisoning. However, the Staphylococcus unlike the Salmonella and Shigella organisms is ubiquitous (pathogenic staphylococci may be isolated from the noses of 40% of an average group of individuals) and its presence may have been due to contamination before or during the process of collection of food samples. Thus a correct interpretation of their presence is more difficult than in case of the Salmonella or Shigella organisms.

Staphylococci which have been found to be definitely responsible for food poisonings have been shown to produce a heat stable "enterotoxin." At present there is no simple cultural or serological procedure enabling us to detect easily those strains of staphylococcus capable of producing this type of toxin.

The kitten intraabdominal injection method of Dolman and his associates, and the better cat intravenous injection method of Hammon have been employed in the detection of the enterotoxin. Filtrates of shallow semi-solid agar cultures in Petri dishes or Kolle flasks grown for about two days in an atmosphere containing 20-30% carbon dioxide are heated in a boiling water bath for 30 minutes for the production of the enterotoxin. (See section on gram-positive cocci for the intravenous cat method of testing for the presence of enterotoxin.)

The decision of assigning to staphylococci the causative role in food poisoning rests upon the finding of considerable numbers in the suspected food. If a long period of time has elapsed since the occurrence of symptoms and the culturing of the foodstuff or if the latter has been held at a temperature allowing bacterial multiplication for a significantly long period of time the finding of this ubiquitous organism becomes less significant.

In most cases of staphylococcus food poisoning the first symptoms appear in 2-1/2 to 3 hours after consumption of the toxic food.

In several instances streptococci have been held responsible for food poisoning. Recent evidence indicates that living cultures of alpha type streptococci and not filtrates are necessary to give rise to symptoms of food poisoning in man. There is no laboratory method available for distinguishing the strains of streptococci specifically responsible for this condition. Since these organisms are also of widespread occurrence, one can rely only upon the circumstantial evidence afforded by the finding of considerable numbers of streptococci in the foodstuff and the absence, or relative absence, of other types.

VI. EXAMINATION OF WATER.

(Reference: Standard Methods of Water Analysis,
Eighth edition, 1936, Fifth printing - 1941.)

The bacteriological analysis of water includes not only the estimation of the total number of viable organisms, but also tests for the presence of specific bacteria, usually of the colon-aerogenes group.

Water samples are usually collected in sterile, wide-mouthed, glass-stoppered bottles. The specimen should be kept cold and sent immediately to the laboratory. The reader is referred to "Standard Methods for the Examination of Water and Sewage, Eighth edition," published by the American Public Health Association, for a complete discussion of methods of obtaining samples of water for bacteriological examination.

For a total count determination, prepare 1:10, 1:100 and 1:1000 dilutions of the specimen in sterile distilled water. Transfer 1 cc. of the undiluted specimen and of each of the dilutions to two series of Petri dishes. To each plate in one series add 10 cc. of melted litmus-lactose agar which has been cooled to 43 degrees C. Incubate at 37 degrees C. for 24 hours. To the second series add 10 cc. of melted Bacto-nutrient gelatin which has been cooled to 43 degrees C. Incubate at 20 degrees for 48 hours. Count the plates having 25 to 250 colonies, multiply by the dilution and then average. If the number of colonies growing at 37 degrees C. approximate the number at 20 degrees C., pollution by sewage may be suspected, as the counts normally have a ratio of about 1 to 30. There should be not more than 100 colonies per cc. of water in the agar plates incubated at 37 degrees C. Litmus and lactose are included in the plating medium in order that a rough estimation of the degree of pollution may be obtained. The organisms indicative of pollution (fecal streptococci and members of the coli-aerogenes group) ferment the lactose to give a pink color. In reporting the water examination, the medium used for the total count should be stated, i.e., whether gelatin or agar, and the temperature of incubation given.

SAMPLE PLATE COUNT

Materials needed:

1. Sterile Petri dishes.
2. Sterile 1 cc. pipettes.
3. Sterile 10 cc. pipettes.
4. Sterile test tubes of medium size, plugged with cotton.
5. The media (agar and gelatin) melted and cooled in a water bath to 43 degrees C.

PROCEDURE

1. Into each of 4 sterile test tubes pipette exactly 9 cc. of sterile tap water using strict aseptic technique.

2. Make a 1:10 dilution in the first tube by adding exactly 1 cc. of the sample with a sterile pipette. After flaming the mouth of the tube and replacing the cotton plug, mix the contents by rolling the tube rapidly between the hands

with the tube kept in an upright position. Aseptic technique must be used. Label the tube #1.

3. Make a 1:100 dilution in the second tube by transferring with a fresh sterile pipette 1 cc. from tube #1 to a tube containing 9 cc. of sterile water. Label the latter tube #2.

4. Make a 1 to 1000 dilution in the third tube by similarly transferring 1 cc. from tube #2. Label this tube #3.

5. Make a 1 to 10,000 dilution in the fourth tube by pipetting 1 cc. from tube #3 to tube #4. A fresh pipette must be used to make each of these dilutions.

6. Place 1 cc. of the undiluted specimen in a sterile Petri dish, labelled #1. Then with a fresh pipette successively place 1 cc. from tube #1 in Petri dish #2, 1 cc. from tube #2 in Petri dish #3, 1 cc. from tube #3 in Petri dish #4, 1 cc. from tube #4 in Petri dish #5.

7. Pour melted medium into each of the Petri dishes, rotate to obtain an even distribution and mixture of medium and sample, allow to harden and incubate. (Agar plates at 37 degrees C. and gelatin plates at 20 degrees C.) The melted agar must not be warmer than 43 degrees C. or some of the bacteria in the sample will be injured by the heat and will not produce colonies.

8. At the end of 24 hours, count the plates incubated at 37 degrees C. At the end of 48 hours, count the plates incubated at 20 degrees C. Select plates showing an even distribution. Count at least two plates and count every colony on the plate.

CALCULATION

Multiply the colonies counted by the dilution. Take the average of the counts and report as number of bacteria per cc.

Example: 462 colonies are counted in plate of 1:10 dilution.
36 in the plate of 1:100 dilution.

462 x 10 equals 4,620
36 x 100 " 3,600
8,220 divided by 2 equals 4110 bacteria per cc.

The so-called presumptive test for the detection of pollution is one in which the formation of acid and gas in lactose broth (Bacto Lactose Broth) in 24 hours is determined. A positive test is presumptive evidence of the presence of the coli aerogenes group in raw water, sewage, etc. In doing this test, five 10 cc. quantities of the suspected water and one each of 1 cc. and of 0.1 cc. are inoculated into lactose-broth fermentation tubes. (The fermentation tube must contain at least twice as much medium as the portion of sample to be tested.) Of these not more than one of the 10 cc. portions should show the presence of gas after 24 hours' incubation at 37 degrees C. The absence of gas after 48 hours incubation constitutes a negative test. The presumptive test may be confirmed by using brilliant-green-bile-lactose broth (Bacto Brilliant Green Lactose Bile 2%.) The Brilliant green serves to inhibit the growth of organisms

other than coliform bacteria while the bile stimulates the growth of the coli-aerogenes organisms. In making transfers from the lactose broth fermentation tubes showing gas, the tube should first be gently shaken or mixed by rotating and at least a 3 mm. loopful transferred to the confirmatory medium (Brilliant green-lactose bile.) It is permissible to transfer larger quantities. The inoculated tubes should be incubated for 24 hours at 37 degrees C. The formation and presence of gas in any amount in the inverted vials in the fermentation tubes at any time within 24 hours constitutes a confirmed test.

Confirmation of the presumptive test may be done also by streaking some of the growth from a positive fermentation tube on a selective solid medium, such as MacConkey's, Endo's or eosin-methylene-blue agar. The appearance of typical coli-aerogenes colonies constitutes a confirmation of the presumptive test.

The so-called "Complete" test is made by fishing a typical coliform colony into lactose broth and on to an agar slant. Gas formation after 48 hours of incubation at 37 degrees C. by a non-spore-forming gram-negative rod (smear made from 24 hour agar slant) is a completed positive test.

It may be desirable, at times, to differentiate between the Esch. coli and Aerobacter aerogenes. To accomplish this four tests may be used, namely, indole, methyl red, Voges-Proskauer and sodium citrate tests (See Appendix for methods.) The possible interpretations of various combinations of results are presented in Table No. 36.

If an estimation of the numbers of coli-aerogenes organisms present in a sample of water is desired, a number of each of several amounts of the sample are cultured in lactose broth and after confirmation of "positive" tubes, the approximate number of coli-aerogenes organisms calculated as indicated in the following example:

Results of tests in amounts designated.				Indicated number of organisms of the coli-aerogenes group.	
10 cc.	1 cc.	0.1 cc.	0.01 cc.	per cc.	per 100 cc.
+	-	-	-	0.1	10
+	+	-	-	1.0	100
+	+	+	-	10.0	1,000
+	+	+	+	100.0	10,000
+	+	-	+	10.0	1,000
Totals				121.1	12,110
Average of five tests				24.0	2,400

In order that results as reported may be checked and carefully evaluated, it is necessary that the report should show not only the average number of organisms per cc., but also the number of samples examined; and, for each dilution, the total number of tests made, and the number (or per cent) positive.

TABLE 36.

COLI-AEROGENES GROUP-REACTION CLASSIFICATION

Reaction combinations.				Possible interpretation when isolated from water by the standard method.		Common source.
Indol.	M. R.	V. P.	Citrate	Usually	Occasionally	Applies to pure strain members of C-a group only.
+	+	-	-	Esch. coli	-	Predominate in feces about 50 per cent of total group in sewage
-	+	-	-	" "	Non-members of group	Minority form in feces
+	+	-	+	Mixture	Intermediate strain sometimes considered non-typical Esch. coli	Minority form in soil and sewage, rarely feces
-	+	-	+	Intermediate strain	Mixtures or slow secondary reacting A. aerogenes	Soil, minority forms in sewage and feces
+	+	+	+	Mixture	Atypical	Soil, sewage
+	-	-	+	Always mixture		
+	-	+	+	Mixture	A. cloacae	Soil, minority forms in sewage and feces
-	+	+	+	A. aerogenes	Mixture	Majority forms in soil and on vegetables
-	-	+	+	" "		Up to 50 per cent of total group in sewage
-	-	-	+	Extraneous form	A. aerogenes	Minority forms in feces

The American Public Health Association's "Standard Methods for the Examination of Dairy Products" recommends the use of Difco's "Violet Red Bile Agar" for the detection of coliform bacteria in milk. This medium, however, may well be used for the direct plate count of coliform bacteria in water also. Fifteen cc. of medium and not more than 1.0 cc. of sample (or dilution of sample) are mixed in each Petri dish of 90 mm. diameter. After solidification of the mixture, 3 or 4 cc. of agar are poured over it to form a film of medium which covers the entire surface of the solid medium in order to eliminate the formation of surface colonies. The plates are incubated at 37 degrees C. for 18 to 20 hours and at the end of this time are examined by transmitted light. Organisms of the coli-aerogenes group due to their ability to ferment lactose, form purplish red subsurface colonies, 1 to 2 mm. in diameter and are generally surrounded by a reddish zone of precipitated bile. The plates should not be incubated longer than 24 hours, inasmuch as the organisms whose growth has been suppressed may develop and confuse the count. Best results are obtained if plates are not too heavily seeded -- the inoculum being diluted so that not more than 150 colonies will develop per plate.

In summary, three recommended procedures are provided by the American Public Health Association for the detection of pollution of water:

1. The determination of gas production in lactose broth resulting from the direct inoculation of water. (Presumptive test):

The formation of gas in 24 hours, in any amount, constitutes a positive presumptive test. The presence of gas in 48 hours, but not in 24 hours, constitutes a doubtful test and must be confirmed.

2. The use of differential or selective media (fluid or solid) inoculated from "presumptive positive" tubes of lactose broth. (Confirmed test):

The presence of typical lactose fermenting colonies on the MacConkey's, Endo's or eosin-methylene-blue plate constitutes a positive confirmed test. The formation of gas in the inverted tube of a liquid confirmatory medium within 24 hours constitutes a positive confirmed test.

3. The identification of gram-negative, non-sporulating, aerobic organisms capable of producing gas when reinoculated into lactose broth. (Completed test).

If there are more than 100 colonies per cc. of water in the agar plates incubated at 37 degrees C. the potability of the water may be questionable.

The approximate numbers of coli-aerogenes organisms may be determined by culturing varying amounts of the sample (the dilution method of counting) or by the direct plating of the sample in a differential and selective solid medium. An example of such a medium which is very efficient although its use is not recommended as yet by the American Public Health Association, is Difco's "Violet-Red Bile Agar."

There is a group of organisms designated as "slow lactose fermenters," the presence of which in water may indicate sewage pollution. In order to detect the presence of this group of organisms, incubation at 37 degrees C. for at least five days may be necessary. Standard methods require only 48 hours of incubation; and do not provide for the detection of these slow gas producing organisms.-(Interpretation of the results of a water analysis should be based on a consideration of the source of the water, its manner of collection and shipment to the laboratory and the sanitary survey.)

VII. BACTERIOLOGICAL EXAMINATION OF MILK.

(Reference: Standard Methods for the Examination of Dairy Products, Eighth edition, 1941, A.P.H.A.; Standard Milk Ordinance and Code of the United States Public Health Service.)

Bacteria in milk are derived from the udder of the cow and from the environment. While some of the organisms present in the udder may be pathogenic for man (Br. abortus, M. tuberculosis, Strep. hemolyticus) most of the pathogens present in milk are derived from human sources. This is true of Bact. typhosum, Strep. hemolyticus and C. diphtheriae. Bacteria in milk derived from the environment come from (1) the skin of the udder, flank, and hind quarters, (2) milk vessels and utensils, (3) the persons and clothing of the milkers and (4) dust in the milking shed. The number of bacteria in milk depends upon the care with which it is collected, the temperature at which it is kept and the length of time it is held before examination.

With intelligent care for sanitary precautions the ordinary farmer may be able to produce milk with as few as 10,000 bacteria per cc. Total counts which run into six or seven figures indicate careless handling.

The technique employed for the bacteriological examination of milk is given in full in "Standard Methods for the Examination of Dairy Products" of the American Public Health Association.

The number of bacteria in milk may be determined in two ways: (1) by plate counts, the standard method which gives the number of viable bacteria and (2) by direct counts, which give the total number of living and dead bacteria. Direct counts are valuable in field work for grading fresh milk delivered at central stations and are also a check on the quality of pasteurized specimens.

The Plate Count

The plate count is done by first preparing dilutions of the well-shaken milk sample using water blanks containing 9 cc. of sterile water. One cc. amounts of 1:100, 1:1000, 1:10,000 dilutions are plated in sterile Petri dishes. These dilutions may be varied from 1:10 to 1:1,000,000 or more, depending upon the quality of the milk sample. After 48 hours of incubation at either 32 degrees C. or 37 degrees C., plates showing 30 to 300 colonies are counted. (Tests have shown that an incubation temperature slightly under 30 degrees C. for raw milk samples and slightly above 31 degrees C. for pasteurized samples gives a more nearly accurate determination of the number of organisms present in milk. The counts so obtained are definitely higher than on the same samples incubated at 37 degrees C. The counts obtained at 37 degrees C. and at the lower temperatures with high quality milk show relatively little difference, but low quality samples often show much higher counts at 31 degrees C.)

Difco's dehydrated Bacto-tryptone-glucose-extract-agar enriched with milk or other agars of the same composition should be used as the standard plating medium. Skim milk is added to the medium when the dilution of the milk specimen is greater than 1:10. (Suspend 24 grams of Bacto Tryptone Glucose Extract Agar in 1000 cc. of cold distilled water. Boil for a minute or two to dissolve the medium and sterilize in the autoclave for 20 minutes at 15 pounds

pressure (121 degrees C.). When the medium is to be made with skim milk, 10 cc. of skim milk are added to one liter of medium just before sterilization. If the medium is subjected to excessive sterilization, frequent remelting, prolonged holding in the liquid state, or if the medium is not in complete solution before addition of skim milk, a precipitate may develop. Holding the sterile complete milk medium at 45 degrees C. or less for periods longer than 30 minutes encourages the formation of a flocculation in the medium. This may be avoided by shorter holding periods at 45 degrees C., by raising the temperature for holding to 48 to 50 degrees C., or the flocculation may be dispersed by heating the medium to a boil. If it is not practical to follow the above directions in detail, and a troublesome precipitate persists, the complete medium may be prepared by the addition of sterile skim milk to sterile liquid tryptone glucose extract agar, under aseptic conditions, just prior to pouring plates.)

A lens magnifying one and one half diameters is used in counting and all recognizable colonies are included. In order to insure uniformity of counting conditions, illumination equivalent to that provided by the Quebec Colony Counter (A.J.P.H., 1937, 27, 809) is recommended. Reports are recorded in round numbers only, to two significant figures. A record of the dilutions used and the number of colonies developed on each plate that is counted is kept but reports are rendered in round numbers only, i.e., in case there are 252 colonies on the 1:100 plate report it as "Standard plate count 25,000 per cc." If two or more counts are averaged, do not give a fictitious idea of the accuracy of the standard plate count by using more significant figures than are found in the numbers averaged, lowering the count where the figure to be dropped is 1, 2, 3, 4, and raising it where the figure to be dropped is 5, 6, 7, 8, or 9. If plates developing less than 30 colonies must be used, report the count merely as "less than 300", if the 1:10 dilution has been used; "less than 3,000", if the 1:100 dilution has been used, etc.

The Direct Count

The direct microscopic count (Breed's method) consists of an examination with the aid of a compound microscope of stained films of milk and cream dried on glass slides. It offers the most rapid routine technique for obtaining a general opinion of the bacterial condition of the sample. It is used chiefly in routine control work for making rapid estimates of the number of bacteria present rather than for making time consuming counts of these numbers. Where large numbers of bacteria are present and are uniformly distributed on the film, the examination of a single field will indicate the general character of the sample. More microscopic fields must be examined where none or only a few bacteria are present, as uneven distribution of bacteria in clumps may deceive the person making the examination into thinking that the milk is better or worse than it really is. Normally it is possible to determine whether the milk is in excellent, good, unsatisfactory, or very unsatisfactory condition without counting, and hence no counts are required except in so far as they are needed in borderline cases to establish the correct assignment to a grade. Microscopic counts also permit one to note the types of bacteria present.

In the examination of samples of pasteurized milk or cream, the fact that these products have been heated sufficiently to kill many, but not all, of the bacteria must be kept in mind. Moreover, thermophilic or even thermoduric bacteria may have increased in number during the heating of these products. Contamination from equipment after pasteurization may also have added its quota of

living bacteria. If pasteurized milk or cream has been stored at low temperatures, it must also be remembered that low temperature (psychrophilic) bacteria may have grown in these stored products. Since there can be any combination of the above possibilities, cautious interpretation of observations is essential.

Comparisons show that there is no constant ratio between the total number of individual bacteria present and the plate count. Hence, even the most frequent ratio between the standard plate count and the individual bacterial count (1:4) is not recognized as an accurate basis for the interpretation of one count in terms of another, since the majority of ratios vary widely from the most frequent ratio.

In the direct microscopic count, the sample to be examined is shaken at least 25 times. One hundredth cc. of milk is transferred to a clean glass slide and spread evenly over an area of 1 square centimeter with a clean stiff wire. The smear is dried within 5 to 10 minutes, avoiding excessive heat which will crack the film. It is dipped in xylol to remove the fat (at least one minute), drained and dried. It is then immersed in 90 per cent alcohol for 1 to 2 minutes. It is finally stained with methylene blue.

The diameter of the field of the microscope is determined with a stage micrometer ruled in 0.1 and 0.01 mm. divisions. Immersion oil is used between the slide and the objective. When the draw tube of the microscope is adjusted so that the diameter of the circle in the ocular measures 0.146 mm. on the stage, the total number of bacteria in 30 fields multiplied by 20,000 gives the number in one cc. of milk. The number of leukocytes may also be counted. These when abundant, point to infection of the cow's udder. (mastitis.)

Methylene Blue Reduction Method

The methylene blue reduction method, frequently called the reductase test, is based on the fact that the color imparted to milk by a small quantity of reversibly reducible dye, such as methylene blue, will disappear more or less quickly. Visual reduction of methylene blue takes place over a narrow oxidation-reduction potential range which is negative to the electro-potential values of fresh, aerated milk. The evidence is that this negative potential is attained in the incubated milk as a result of the consumption of the dissolved oxygen by growing bacteria. The methylene blue reduction time depends, therefore, on the oxygen consuming power of the bacteria which grow during incubation and, consequently, is indirectly a quantitative index of the bacterial content of the milk at the start of incubation. This relation has been established empirically by the use of the agar plates and microscopic counts as well as by keeping quality and other tests.

Methylene blue thiocyanate tablets, certified by the Commission on Standardization of Biological Stains should be used. One tablet is dissolved in 200 cc. of sterile or freshly boiled distilled water. The dye is stored in amber glass bottles in the dark and is prepared weekly.

One cc. of the dye solution is mixed with 10 cc. of milk and the tube stoppered. When ready to start a batch of tubes, they are transferred from ice water or other refrigerant to a water bath which will bring them to a temperature of 37 degrees C. within an interval of five minutes. When the contents of the tubes have reached a temperature of 37 degrees C., the tubes are inverted a few times

to assure uniform creaming. Agitation after this, which would disturb the cream layer, should be avoided. The tubes are incubated at 37 degrees C., plus or minus 0.5 degrees C., either in a water bath or in an incubator.

The methylene blue reduction time is the interval between the placing of the tubes in the 37 degrees C. water bath or incubator, immediately after their inversion, and the nearly complete disappearance of the blue color from the milk.

The exact end point of reduction is not always easily determined as many of the better class milks reduce unevenly throughout the tube.

Reduction may be considered complete when four-fifths of the visual portion of the contents of the tube have turned white.

"Within the limits of 1 and 10 hours any classification of milk based on the methylene blue reduction test is necessarily an arbitrary one. A herd milk that reduces in two hours or less undoubtedly has a high bacterial content. One that requires 8 hours for reduction probably contains comparatively few bacteria other than those in the milk at the time of its withdrawal from the udder. The following classification is presented merely as a possible guide. This classification is not intended to carry with it the inference that all milk that decolorizes in less than 8 hours is unacceptable for use as market milk.

Class 1. Excellent, not decolorized in 8 hours.
Class 2. Good, decolorized in less than 8 hours but not less than 6 hours.
Class 3. Fair, decolorized in less than 6 hours but not less than 2 hours.
Class 4. Poor, decolorized in less than 2 hours."

(Standard methods for the examination of Dairy Products, Eighth edition, 1941.)

A substitute for methylene blue, resazurin, appears to be gaining favor for use in the reductase test. Several techniques are employed in this test, the chief advantage of which appears to be in the shorter time in which results are obtained.

For further details of the reductase test see "Standard Methods for the Examination of Dairy Products, Eighth edition, 1941."

Detection of Coliform Organisms

Since milk is an excellent medium for bacterial growth, unless it is tested within 3 to 4 hours after production, or has been produced and cooled under such satisfactory conditions that the total count is low, i.e., less than 10,000 organisms per cc., it becomes impossible to determine the significance of counts of coliform (Escherichia-Aerobacter) organisms.

Organisms of the coliform group are practically eliminated from milk and cream by pasteurization. For this reason, where 1 cc. samples of freshly pasteurized milk give positive results from the bottled milk, either improper pasteurization or contamination after pasteurization may be suspected.

The presumptive test for the detection of coliform organisms in milk consists of the test for gas production within 48 hours at 37 degrees C. in a fermentation tube containing Brilliant-Green Bile (2 per cent) or Formate Ricinoleate Broth.

Direct plating media such as Violet Red Bile Agar may also be used. The appearance in this agar of typical dark-red colonies of at least 0.5 mm. in diameter may be considered presumptive evidence of the presence of coliform organisms.

The completed tests consist of the demonstration of organisms of this group by showing that the fermentation tubes in which gas appeared, or the typical colonies appearing in the direct plating agar media, contain gram-negative, non-spore-forming bacilli, which when inoculated into a lactose broth fermentation tube, form gas within 48 hours upon incubation at 37 degrees C.

The presumptive test using liquid medium is done by inoculating a series (5 tubes of each dilution used are recommended) of Brilliant Green Bile (2 per cent) or of Formate Ricinoleate Broth fermentation tubes with decimal multiples or fractions of 1 cc., such as 10 cc., 1 cc., 0.01 cc., etc., of the milk to be tested. In order to be certain of obtaining a definite result, it is essential that the dilutions be such that at least one positive and one negative tube result be obtained. To satisfy this requirement, it may be necessary to plant three or even more dilutions. (See section on media in Appendix for directions for use of these media.)

When, however, the purpose of the test is merely to determine whether a specific density of organisms is exceeded, only one or two dilutions may be required. In pasteurization control, for example, 5 tubes, each inoculated with 1 cc. (or 10 cc.) of sample are recommended in those cases where previous experience has shown that results are likely to be negative.

The tubes are incubated at 37 degrees C. for 48 hours. The formation, within this period, of gas constitutes a positive "Presumptive Test."

When solid medium is employed in the presumptive test, not more than 1 cc. of sample is placed in a 100 mm. x 15 mm. Petri dish. Ten to 15 cc. of Violet Red Bile Agar, or Desoxycholate Agar which has been liquefied and cooled to a temperature of 40 degrees to 44 degrees C., is added. The mixture is completed by tilting and rotating the dish. After solidification of the mixture, 3 or 4 cc. of agar are poured over it to form a film of medium which covers the entire surface of the solid mixture. The purpose of the cover is to eliminate the possibility of the occurrence of surface colonies of coliform organisms, for the appearance presented by such colonies is often so atypical that they may not readily be recognized.

When the agar cover has solidified, the plates are placed in an inverted position in the 37 degrees C. incubator for a period of 20-24 hours. The appearance, at this time, of typical dark-red colonies of at least 0.5 mm. diameter constitutes a positive presumptive test. These colonies should be counted and the number recorded.

To perform the completed test the procedure described for the examination of water is used.

To perform the completed test from positive selective agar plates it may be necessary to purify the culture obtained from fishing the deep colony. Such purification may be effected by transferring material from the typical dark-red colony to a lactose broth fermentation tube which is incubated at 37 degrees C.

As soon as possible after gas appears, the streaking of Endo or eosin-methylene-blue agar may be executed and from the isolated colonies, lactose and an agar slant may be inoculated for the performance of the "Completed test."

Results should be recorded as observed, indicating the amount tested and the result from each.

TESTS FOR PATHOGENS

Beta hemolytic streptococci:

Milk should be examined for the presence of beta hemolytic streptococci under any one of the following circumstances: (a) Where milk supplies are thought to have caused septic-sore-throat or scarlet fever epidemics, (b) Where routine examination of animals in herds producing raw milk is to be made, and (c) Where pasteurized milk supplies are to be examined for the presence of streptococci associated with human diseases.

Two procedures are available for the original isolation of hemolytic streptococci, viz., blood agar plates and Burri agar slants. The blood agar plate method is usually used although the Burri slant method is preferable where a large number of samples are to be examined in a routine way as the technic is less complicated and test tubes are used instead of Petri dishes.

Plate method: The milk is diluted and plated as outlined in the standard agar plating procedure using Tryptone Blood Agar.(2.0 per cent agar, 0.5 per cent tryptone and 0.5 per cent Na Cl. Just prior to pouring the plates 5 per cent defibrinated horse blood is added to the agar which has been allowed to cool to 45 degrees C.) The plates are incubated at 37 degrees C. for 48 hours and examined for beta hemolytic colonies. Uninoculated blood agar plate controls as a test of the sterility of the blood should be included.

Burri slant method: Tryptone agar slants (no blood) allowed to dry until no water of condensation is present are employed. A platinum loop 1.0 mm. in diameter, made from wire 0.3 mm. in diameter, is flamed and after cooling used to withdraw a loopful of milk from the sample. It is touched to the surface of the slant in three places beginning at the bottom. The inoculum is then streaked by drawing the loop in a zigzag way across the surface of the slant, beginning at the bottom and continuing to the top.

Definite colonies are usually visible on the slants after incubation at 37 degrees C. for 48 hours. Colonies that have the appearance of streptococcus colonies are examined by the Gram procedure. Colonies that are found to be streptococci are subsequently tested on blood agar plates and if found to be hemolytic, reserved for more complete identification.

(These two types of procedure, recommended for use in "Standard Methods, etc.," might be easily combined, it seems, by streaking either blood agar slants, or sectored blood agar plates, following the streaking procedure used in the Burri method. The blood agar used as the basic medium in this manual could be employed.)

Various species of streptococci may be encountered in ordinary milk supplies. The streptococci in samples drawn aseptically from the udder are generally alpha or gamma streptococci. In market milk, Streptococcus lactis is commonly found. Another common species is the streptococcus of bovine mastitis (Streptococcus agalacteae) which appears as an alpha, alpha prime or sometimes a beta hemolytic colony. It can be readily differentiated by cultural and serological tests from other species of streptococci.

The most significant species from a public health standpoint are the beta-hemolytic streptococci of Lancefield's group A.

The finding of a gram-positive, beta-hemolytic streptococcus in a freshly drawn quarter sample should be interpreted as a presumptive positive finding and is sufficient to warrant the elimination of a cow from the herd.

Confirmation of the identity of the streptococcus may be carried out by determining its cultural characteristics and the serological group to which it belongs.

Before a relationship between a milk supply and an epidemic can be regarded as established, it should be shown that the strains from throat cultures secured from patients and strains secured from the suspected milk give identical reactions.

The beta-hemolytic streptococci which survive pasteurization temperatures (Strep. durans, Strep. zymogenes) are not regarded as pathogenic for man.

Tubercle bacilli:

Because milk usually contains relatively few tubercle bacilli it is desirable to concentrate the organisms present. This is usually done by centrifuging or by allowing the milk to stand for 24 hours or longer in the refrigerator. By either method the fat or cream rises to the surface and most foreign substances, such as bits of dirt, manure, fragments of tissue that may have originated in tuberculous ulcers, and leukocytes settle to the bottom. Most of the bacteria present, tubercle bacilli among them, will be found in these two layers. The examination, therefore, should be made of these two portions, either separately or by mixing them together and examining the mixture.

If large quantities of milk are available, pint or quart samples are placed in sterile bottles or cylinders which are allowed to stand in the refrigerator for 24 hours. The cream and sediment layers are collected by siphoning. These are mixed and centrifuged in 250 cc. sterilized bottles at high speed for 30 minutes. The top and bottom layers are collected, mixed together and the mixture used for direct microscopic examination, for culture and for animal inoculation.

Direct microscopic examination of milk or cream for acid-fast rods should be carried out in the usual manner except for the removal of fatty substances by rinsing the fixed films, before staining, with a good fat solvent such as ether or xylol. The Ziehl-Neelsen method is as reliable a method as any. The microscopic demonstration of acid-fast rods does not, of course, prove the presence of tubercle bacilli.

Tubercle bacilli may be cultivated directly from milk after treating it with an agent capable of destroying the non-acid-fast organisms present. However, acid-fast organisms which appear on culture should not be regarded as tubercle bacilli without confirmatory evidence. The average worker will succeed better with the animal inoculation procedure.

Guinea pigs are the best experimental animals for detecting tubercle bacilli in milk. They are not susceptible to avian bacilli, however; hence if this type is sought, other animals, such as rabbits or chickens, should be used. Animals weighing at least 350 grams are preferred to smaller animals since the former are less apt to succumb to the effects of extraneous organisms that may be in the milk.

The guinea pig is inoculated intraperitoneally using quantities up to 5 cc. The quantity injected should depend on the quality of the milk and its age. Dirty milk and old milk with high bacterial counts naturally are more likely to cause death from peritonitis than better grades, and smaller quantities should be injected.

If milk samples have been collected especially for examination for tubercle bacilli, and particularly if they must be shipped, it is well to preserve the milk by adding 1 per cent boric acid. The tubercle bacilli are not harmed by this treatment, while multiplication of other organisms is prevented.

All animals that die should be carefully autopsied soon after death. Caseous masses in the great omentum should be looked for; also the characteristic lesions of the liver, spleen and lungs. Tubercle bacilli are not always easily found in smears of these lesions. In case the nature of the lesion is not clear it is best to inoculate other quinea pigs with them. In this case the material is inoculated into the muscles of the thigh to permit tracing of the infection through the lymphatics until it reaches the visceral organs.

Surviving animals are destroyed for autopsy not sooner than 8 weeks after inoculation.

The fact should always be kept in mind that lesions simulating those of tuberculosis can be produced in experimental animals by injection of any acid-fast organism provided large numbers are used. When suspended in a fatty material, such as cream, the power of such organisms to produce lesions is greatly increased. If it is suspected that lesions are pseudo-tuberculous, it is an easy matter to settle the question by injecting other guinea pigs. The true tuberculous infection will, of course, appear in them, whereas the pseudo-disease will not. Furthermore, the saprophytic-acid-fast organisms grow easily and rapidly on culture media and may be identified in this way.

Brucella species:

The sample of milk to be tested is allowed to stand in the refrigerator for 24 hours in order to allow the cream to rise to the surface. The cream layer up to 2 cc. is injected intraperitoneally or subcutaneously into a healthy guinea pig. At the end of six weeks the pig is killed and the tissues examined for lesions characteristically produced by bacteria of the Brucella group: cultures may be made from the lesions and the pigs blood serum may be tested for the pres-

ence of Brucella agglutinins.

One per cent boric acid or 1:25,000 crystal violet may be added as a preservative to milk which is to be shipped.

The culture method offers a rapid, accurate and quantitative means of detecting the presence of these organisms in milk drawn directly from the udder of the animal. 0.1 to 0.2 cc. quantities of the cream layer are streaked on the surface of Difco's Bacto-Tryptose Agar plates containing crystal violet in a final dilution of 1:700,000. The inoculated plates are incubated in the candle-jar for 5 days at 37 degrees C. and examined for Brucella colonies. Suspicious colonies should be transferred to agar slants and identified as Brucella by means of the agglutination test using an agglutinating serum of known titer and also a normal serum.

The direct isolation of bacteria belonging to the Brucella group from market milk is difficult if streptococci are also present in the cream. Although gram-positive, these organisms are not inhibited in their growth by 1:700,000 crystal violet. Streptococci appear to affect the medium in such a manner that the growth of bacteria belonging to the genus Brucella is inhibited.

(For a description of the procedure for differentiation of the species of Brucella, the reader is referred to "Standard Methods for the Examination of Dairy Products," Eighth edition, 1941, pp. 106 to 108 and to Section III and the Appendix of this manual.)

Note:

The Standard Milk Ordinance and Code classifies and defines milk as:

(a) Grades A, B, C and D raw.
(b) Grades A, B, and C pasteurized.

Grade A pasteurized milk is the grade usually sold for drinking purposes. It must have a colony count of not over 30,000 per cc., and must be prepared from grade A (50,000 per cc.), or grade B (200,000 per cc.) raw milk in plants meeting strict sanitary requirements.

Other grades of milk are based on definite sanitary requirements for the production, distribution and bacterial content. The allowable colony counts for raw milks are greater than for the corresponding grade of pasteurized milk. The sanitary requirements are progressively less rigid and the allowable colony counts greater for grades B, C and D, respectively.

APPENDIX

VIII MEDIA AND SOLUTIONS

A great variety of artificial culture media is employed for the cultivation of bacteria. Levine and Schoenlein (Monographs on Systematic Bacteriology, Vol. II, Williams and Wilkins, Baltimore, 1930), have compiled a list of about 2500 such media. These media supply food for the bacteria, are adjusted with respect to reaction (hydrogen-ion concentration or pH), moisture content, osmotic pressure and consistency and may be prepared free from bacteria. The addition of agar or gelatin is the usual means of increasing the consistency of these media. The availability of media made solid by means of these substances permits ready separation of the members of a mixture of bacteria.

The composition of some media is so adjusted that bacteria give different types of growth on them. These are the so-called "differential" media. Other media permit the growth of some bacteria while retarding the growth of others. These are the so-called "selective" media. The widespread use of sulfonamides has made advisable the incorporation of p-aminobenzoic acid in media to be used in the culture of specimens received in the diagnostic laboratory. A small concentration (0.005%) of this substance is used to neutralize the growth inhibiting properties of the sulfonamides without interfering appreciably with the growth promoting properties of media.

The colorimetric adjustment of the pH of media and sterilization procedures are described in the section on techniques and special procedures.

Complete dehydrated media are prepared by the Difco Laboratories of Detroit, Michigan and by the Baltimore Biological Laboratory. Such media are relatively easy to prepare and are generally satisfactory. When they are not available in the complete form they may be prepared from their constituents.

One of the most important of these constituents is peptone, a product of protein hydrolysis. Not all peptones are equally utilizable by bacteria and some are prepared for specific purposes. Since the appearance of the bacterial colony may be influenced by the type of peptone used it is important that in the preparation of media (especially blood agar) peptones be employed that have been standardized in this respect. Among the peptones that have been found satisfactory for this purpose are Difco's Bacto-Tryptose, Bacto-Proteose Peptone No. 3 and Neopeptone and Wilson's Thiopeptone (distributed by the Baltimore Biological Laboratory).

Where peptones are not available it is possible to prepare media which are excellent for the support of growth of fastidious bacteria by digestion of animal or plant protein (see below--LXVIII and LXIX).

Some of the media found most useful are listed below.

I. BASIC BROTH MEDIUM: (For blood cultures and routine use.)

Distilled water	1,000 cc.
Beef extract	3 Gm.
Tryptose	20 Gm.
NaCl	5 Gm.
Dextrose	1 Gm.
0.5% p-amino-benzoic acid	10 cc.

Combine the ingredients and dissolve by heating. Adjust to pH 7.4-7.5. Boil for a few minutes. If necessary filter through paper. Dispense in bottles or tubes and autoclave at 15 lbs. for 20 minutes. Bacto Tryptose Phosphate broth to which 0.005% p-amino benzoic acid has been added, may be used.

I. (A) BASIC BROTH MEDIUM PLUS 0.1% AGAR: (For blood cultures.)

Add 1 gram of agar for every liter of medium. Dispense 40 cc. quantities in tubes (200 x 25 mm.)

II. BASIC AGAR MEDIUM: (Agar for slants, for pouring plates and for use as a Base for Blood, Chocolate or "Combination" blood agar.)

Distilled water	1,000 cc.
0.5% p-amino-benzoic acid	10 cc.
Agar	15 Gm.
Beef extract	3 Gm.
*Bacto-Tryptose	10 Gm.
*Bacto Proteose Peptone #3	10 Gm.
NaCl	5 Gm.
Dextrose	0.3 Gm.
Dextrin	0.5 Gm.
Nicotinamide	1.0 Gm.

*(2% Wilson's Thiopeptone may be used instead of the Bacto Tryptose-Proteose Peptone #3 combination).

Combine the ingredients and dissolve by heating. Adjust to pH 7.4 - 7.5. Boil for a few minutes. If necessary, filter through paper. Dispense in flasks or tubes, autoclave at 15 lbs. for 20 minutes. Final pH should be 7.3.

To prepare blood agar from the above base, melt the latter with steam or in a boiling water bath, cool to 45-50 degrees C. (or until comfortable when pressed against the cheek) add 5% sterile defibrinated, citrated, or oxalated horse or human blood. Aseptically pour about 12-13 cc. quantities into sterile Petri dishes and allow to harden. A few of these plates are incubated for sterility while the rest are inverted and placed in a Petri dish can which is kept in the refrigerator. In laboratories where only a few blood agar plates are employed, the basic agar medium may be distributed in 12 cc. quantities in test tubes. When a blood

agar plate is needed, the test tube is heated in boiling water to melt the agar. The agar is cooled to a temperature of 45-46 degrees C. by standing it in warm water. Six tenths of a cc. of sterile defibrinated blood is added to the agar which is immediately mixed in the tube and then poured into a sterile Petri dish where it is allowed to harden before use.

To prepare blood agar slants, pipette aseptically 3 to 4 cc. quantities of the blood agar into sterile plugged test-tubes, slant and allow to harden. All slants should be incubated for 24 hours in order to test them for sterility. These also are kept in the refrigerator.

The agar base may be distributed, before sterilization, in 12 cc. quantities in test-tubes, for use in pour-plate cultures of blood. For the preparation of plain agar slants,about 4 to 5 cc. are distributed in test tubes and after autoclaving, allowed to harden in a slanted position.

This blood agar base is especially suitable for the preparation of a "combination" blood agar possessing the growth supporting properties of chocolate blood agar and at the same time permitting the detection of hemolysis and the green coloration produced by such organisms as pneumococci and alpha streptococci. The use of such a "combination" blood agar permits the employment of one blood agar medium for the cultivation of almost all of the common pathogens including N. gonorrhoeae and H. influenzae. It is prepared by chocolating the blood agar by heating at 85 to 95 degrees C. for five minutes, centrifuging while hot to clear the medium and, after cooling to 45-50 degrees C. adding 5% blood to the clear supernatant. (Since the coagulum forms a compact mass at the bottom of the container, the supernatant need not be removed before the second addition of blood). Plates and slants are prepared in the usual manner.

III. INFUSION BROTH: (For blood cultures and routine use.)

To finely ground, fat free, veal, beef, beef heart or pork add twice its weight of distilled water, (i.e., to 500 Gm. of meat add 1 liter of water) and infuse overnight in the refrigerator. Add 1% neopeptone and 0.5% sodium chloride the next morning, mix and bring the temperature of the whole to between 60 and 70 degrees C. Allow to stand at this temperature for 1/2 hour. Boil for 10 or 15 minutes or heat in flowing steam (autoclave without pressure) for 1/2 hour. Strain through gauze and filter through paper. Make up to original water volume with distilled water. Adjust the pH to 7.8. Autoclave at 20 pounds steam pressure for 1/2 hour. Filter through paper. Adjust pH to 7.4-7.6, distribute in final containers and autoclave at 20 pounds for 15 minutes. 0.005% p-amino-benzoic acid should be added to this medium if it is to be used for routine culturing of hospital specimens.

IV. INFUSION AGAR: (For routine use.)

Add 1.5% agar to the infusion broth. Autoclave. Stir while hot and adjust the reaction to pH 7.6. Steam in the autoclave without pressure for 1/2 hour. Filter through a layer of absorbent cotton. Adjust the pH to 7.6. Distribute in final containers and autoclave at 20 pounds for 15 minutes.

V. BROTH FOR FERMENTATION TESTS: (Phenol Red Broth.)

Bacto-Tryptose	10 Gm.
Sodium chloride	5 Gm.
Dipotassium phosphate	1 Gm.
Bacto-Phenol Red	0.018 Gm.
Distilled water	1 liter.

The final reaction of the medium should be 7.3 to 7.5.

The above basic medium may be obtained in dehydrated form from the Difco Laboratories Inc., Detroit, Michigan.

Carbohydrate, polyhydric alcohol, glucoside or other fermentable compound is added to the phenol red broth in 1% concentration. Five cc. quantities are distributed in Durham fermentation tubes (a test tube containing a smaller inverted tube) and autoclaved at 12 lbs. steam pressure for exactly 10 minutes. Since maltose is easily hydrolysed in an alkaline broth medium when subjected to heat, it should be sterilized in 10% aqueous solution by filtration and then added aseptically to the basic medium to give a final 1% solution. (If sterilization of the maltose by filtration is not possible, the 10% aqueous solution should be heated in the autoclave for 10 minutes at 12 pounds pressure.) The basic broth is added in 4.5 cc. quantities to the Durham fermentation tubes and sterilized by autoclaving. Five tenths of a cc. of the sterile 10% maltose solution is then added to each tube. Incubation to test for sterility of the finished medium should be carried out.

If the Phenol-red-broth base is not available, extract broth (see below) may be used as a base for fermentation studies. This medium will adequately support the growth of members of the coli-typhoid-dysentery group. Brom-cresol-purple may be added to the broth before sterilization, to give a purple color.

V. (A) Difco also prepares a newer Phenol red broth base with the following formula:

Proteose Peptone No. 3	10 Gm.
Bacto Beef Extract	1 Gm.
Sodium chloride	5 Gm.
Bacto Phenol Red	0.018 Gm.

Sixteen grams of the dehydrated product is added to 1 liter of distilled water. The final pH of the medium is 7.4 and carbohydrates are added to it to give a concentration of 0.5%.

This newer medium is said to support the growth of such fastidious organisms as streptococci, pneumococci and meningococci.

VI. EXTRACT BROTH: (Nutrient broth-Difco.)(For culture of less fastidious bacteria.)

Distilled water	1,000 cc.
Meat extract	3 Gm.
Sodium chloride	5 Gm.
Peptone	10 Gm.

Combine the ingredients and dissolve in water. Adjust to pH 6.8-7.2. If necessary, filter through paper. Sterilize in the autoclave at 15 pounds for 15 minutes.

Difco prepares the dehydrated medium without salt as Bacto-Nutrient broth.

VII. EXTRACT AGAR: (For culture of less fastidious bacteria.)

Add 1.5% granulated agar to extract broth. Dissolve by heating in flowing steam. Check the pH which should be 7.0-7.2. Filter if necessary through absorbent cotton. Sterilize by autoclaving at 15 pounds for 15 minutes.

VIII. HEATED BLOOD AGAR: (Chocolate agar.)(For the Neisseria and Hemophilus groups.)

Melt the basic agar medium #II or infusion agar. Add 5% defibrinated blood, mix well, heat to 90-95 degrees C. Allow to cool to a temperature of about 45-50 degrees C. Mix and pour aseptically into sterile Petri dishes, if plates are wanted, or pipette into sterile test tubes for slants.

IX. DIFCO CHOCOLATE BLOOD AGAR PROTEOSE NO. 3: (For culturing Neisseriae)(Not Hemophilus)

Bacto-Proteose No. 3 Agar	9 Gm.
Water	200 cc.
Bacto-Hemoglobin	2 Gm.

Suspend the agar in 100 cc. of cold water. Dissolve the medium by steaming for a few minutes. Mix well and autoclave for 15 to 20 minutes.

Dissolve the Bacto-Hemoglobin in 100 cc. of water at 50 degrees C. When solution is nearly complete, filter through coarse, moistened cheesecloth to remove undissolved particles. Autoclave the Bacto-Hemoglobin solution for 20 minutes.

After cooling both the agar and the solution of hemoglobin to 50 to 60 degrees C., mix them in equal quantities under aseptic conditions, avoiding air bubbles, and pour into sterile Petri dishes.

This medium will not support the growth of all strains of N. gonorrhoeae. It is not suitable for the culturing of H. influenzae (Pfeiffer's bacillus). For this purpose use freshly prepared chocolate agar (Medium VIII) or "combination" blood agar. (See Medium II).

X. PEPTONE SOLUTION: (For indol test)(Also for culture of less fastidious bacteria.)

Distilled water	1,000 cc.
Sodium chloride	5 Gm.
Tryptone or Tryptose peptone	10 Gm.

Dissolve the ingredients in water and sterilize in the autoclave at 15 lbs. pressure for 15 minutes. The final pH should be 7.3.

XI. NITRATE PEPTONE WATER: (For detection of reduction of nitrate to nitrite.)

This medium is the same as medium # X except that 0.02% potassium nitrate is added.

XII. NUTRIENT GELATIN: (For the study of gelatin liquefaction.)

Beef extract	3 Gm.
Peptone	5 Gm.
Gelatin	120 Gm.
Distilled water	1,000 cc.

Combine the ingredients and heat slowly in a double boiler to 65 degrees C. until the ingredients are dissolved. Make up the lost weight with distilled water. Adjust the reaction to pH 7.0. Heat to boiling while stirring vigorously. Make up lost weight with distilled water and filter through absorbent cotton. Dispense in 5 cc. amounts in small tubes and autoclave. The final reaction should be pH 6.6 to 7.0.

XIII. SEMISOLID AGAR MEDIUM: (For shake tube cultures.)

Semisolid agar is prepared by adding 0.3% agar to the basic broth medium (Medium #I).

XIV. SEMISOLID AGAR FOR FERMENTATION TESTS:(With members of the Neisseria group.)

Semisolid agar for fermentation tests may be used conveniently for the study of fermentation of sugars by members of the Neisseria group of organisms.

To phenol-red-broth (Medium V or V(A)) 0.3% agar and carbohydrates are added as described above (See Medium V) to give a final concentration of 1.0% or 0.5%. After autoclaving at 12 pounds for 10 minutes, the medium is allowed to cool to 44-46 degrees C. 10% sterile serum (human, horse, rabbit) is added aseptically. At this time the test carbohydrate may also be aseptically added if its addition to the medium has not been made prior to the autoclaving of the medium. A 10% sterile aqueous solution of the carbohydrate is added in a volume 1/10 that of the finished medium.

XV. SHALLOW LAYER BROTH FOR FERMENTATION TESTS: (With members of the Neisseria group.)

Instead of a semisolid medium, a shallow layer of a suitable broth medium may be used in the study of fermentation by Neisseria organisms.

Five cc. quantities of phenol-red-broth (Medium V or V(A)) are distributed in 50 cc. Erlenmeyer flasks and sterilized. To these, sterile carbohydrate solution and sterile serum are added aseptically to give 1 per cent concentration. The carbohydrate may be added to each flask as 0.5 cc. of a 10% solution. Each flask will contain 0.05 cc. of serum.

XVI. COOKED MEAT MEDIUM: (For anaerobes).

Add to a tube of basic broth medium (Medium #I) enough ground lean beef heart or veal to occupy about half the column of liquid and autoclave. The final reaction should be pH 7.2 to 7.6.

XVII. BORDET GENGOU MEDIUM: (Pertussis Blood agar)(For whooping cough diagnosis.)

Peeled, sliced potatoes	100 Gm.
Glycerin	8 cc.
Water	200 cc.

Steam in the autoclave or boil until soft. Make up to volume, strain through gauze and allow to stand for sedimentation. Syphon off the supernatant fluid.

To 50 cc. of this extract, 150 cc. of 0.75% sodium chloride and 5 Gm. of agar are added.

Let the mixture stand for 15 minutes to saturate the agar. Heat until the agar is dissolved (in the autoclave), dispense in amounts convenient for storage (50 cc. in small flasks or 10 cc. in test tubes), and autoclave at 15 pounds for 20 minutes.

When whooping cough plates are needed, the potato agar base is melted, cooled to 45 degrees C. enriched with sterile blood (15 cc. of blood to 50 cc. of medium) and poured into sterile Petri dishes.

XVIII. SABOURAUD'S AGAR: (For fungi.)

Maltose (technical)	40 Gm.
Neopeptone	10 Gm.
Distilled water	1,000 cc.
Agar	20 Gm.

Dissolve the mixture in the autoclave. Filter through paper. Dispense 20 cc. quantities in large 200 x 25 mm. test tubes. Autoclave at 15 pounds pressure for 10 minutes, remove from the autoclave, slant, allow to harden and store in the refrigerator. The final reaction should be pH 6.6.

XIX. MALT AGAR: (Difco-dehydrated)(For fungi).

Malt extract, Difco	30 Gm.
Agar	15 Gm.

To prepare the medium for use, 45 grams of Difco's dehydrated Bacto-Malt agar are suspended in 1000 cc. of cold distilled water. Boil for a minute or two to dissolve the medium. The medium is then sterilized in the autoclave for 20 minutes at 15 pounds pressure. The final pH of the medium will be 5.5.

XX. POTATO-CARROT AGAR: (For demonstrating color characteristics of fungi.)

Carrots	20 Gm.
Potatoes	20 Gm.
Agar	15 Gm.
Distilled water	1,000 cc.

1. Wash and peel the vegetables and cut them into small pieces, then add them to 700 cc. of water and boil the mixture down to 500 cc. Filter through paper.

2. Dissolve the agar in 500 cc. of water by heating.

3. Mix the vegetables and the agar.

4. Measure the mixture into test tubes.

5. Autoclave the tubes for 20 minutes at a pressure of 15 lbs.

6. Slant the tubes and leave them until the medium is cooled.

XXI. CORN MEAL AGAR:

This medium is used in the differentiation of various species of Monilia and Cryptococcus. It is also useful when one is studying the spore forms of the dermatophytes. Its value is partly due to a minimal nutrient content; the growth, while scant, shows the characteristics of fructification.

Yellow corn meal	40 Gm.
Agar	15 Gm.
Distilled water	1,000 cc.

1. Add the corn meal to 500 cc. of water and keep heated to 65 degrees C.. for one hour. Filter through paper.

2. Dissolve the agar in the remaining 500 cc. of water by heating.

3. Mix the corn meal and the agar.

4. Filter through cotton. This is a slow process, and the agar will cool and harden unless the flask is placed in a steam bath or sterilizer.

5. Measure the mixture into test tubes.

6. Autoclave the tubes for 20 minutes at a pressure of 15 lbs.

7. Slant the tubes and leave them until the medium is solid.

The Difco Laboratories prepare a suitable dehydrated corn meal agar.

XXII. DORSET'S EGG MEDIUM: (For tubercle bacilli.)

Fresh eggs	4
NaCl solution (0.85%)	25 cc.

Scrub the eggs, clean with soap and water, and allow them to dry. Place the eggs in a wire basket and dip them into 95% alcohol allowing them to remain a few seconds. Remove the basket and the eggs from the alcohol and ignite the alcohol remaining on the basket and eggs. Break the shell aseptically and remove the whites and yolks to a sterile container. Add 25 cc. of sterile 0.85% sodium chloride solution. Mix thoroughly. Distribute 10 cc. quantities in sterile test tubes and coagulate and sterilize in the autoclave using the procedure described above for the preparation of Loeffler's serum slants.

XXIII. PETRAGNANI'S MEDIUM: (For tubercle bacilli. Am. Rev. Tub., 1934, Vol. 29).

120 test tubes (150 x 18 mm.)	
Skimmed milk	450 cc.
Potato flour	18 Gm.
Asparagin	2.6 Gm.
Peeled and thinly sliced potatoes	225 Gm.
Eggs	12
Egg yolks	3
Sterile C. P. glycerin	35 cc.
2% aqueous malachite green (certified)	30 cc.

Place the sliced potatoes, potato flour, milk and asparagin in a double boiler and cook for two hours, stirring constantly until the mixture becomes sticky, after which occasional stirring will suffice. Sterilize the eggs by rubbing with a sponge soaked in 70% alcohol, and drop the egg yolks and egg white along with the 3 extra yolks into a sterile liter Erlenmeyer flask. Break the egg yolks with a sterile glass rod and shake well. Use a sterile rubber stopper while shaking. Add the glycerin and malachite green and shake. Cool the potato milk mixture to 45-50 degrees C. and add the egg-glycerin mixture slowly. Mix well. Filter through sterile gauze into a sterile beaker. The medium should be neutral to litmus.

It is distributed aseptically in sterile test tubes and carefully inspissated. This may be done in the autoclave using the following procedure:

Raise the temperature to 45 degrees C. in the first half hour.
" " " " 65 " " " " second half hour.
" " " " 80 " " " " third half hour.

Hold at 80 degrees C. for 20 minutes. Allow to cool and then substitute sterile rubber stoppers for the cotton plugs.

An alternate autoclave method for coagulation and sterilization is that described for the preparation of Loeffler's serum slants.(See Medium XXXII).

XXIV. GLYCEROL EGG YOLK MEDIUM: (For the tubercle bacillus.)(Corper: J.Lab. & Clin.Med., 1938, 23, 1195.)

Prepared from fresh egg yolks (separated from the white which has been found to be a poor nutrient for supporting the growth of small numbers of tubercle bacilli) and sufficient pure glycerol (4 Gm. to 100 Gm. of egg yolk), and 33 cc. of water.

The tubed mixture is sterilized in a slanted position by inspissating for one hour on three successive days at 85 degrees C. It also can be sterilized by a single autoclaving if provision is made to avoid the presence of bubbles in the completed medium. (See below under preparation of Loeffler's slants.)

XXV. GLYCEROL POTATO MEDIUM: (For the tubercle bacillus.)

1. Scrub large white potato under running water.
2. Cut cylinders by means of an apple corer.
3. Cut the skin off the ends of the potato cylinders.
4. Make two wedge shapes from each cylinder by cutting through obliquely.
5. Soak the potatoes over night in a 1:1000 Na_2CO_3 solution.
6. Drain.
7. Cover with a 5.0% glycerol solution for 24 hours.
8. Place the pieces in test tubes one inch or more in diameter.
9. Add a little of the glycerol solution, or water, to each tube.
10. Sterilize in the autoclave at 15 pounds for 20 minutes.

XXVI. MILK WITH INDICATOR: (For the study of acid and clot formation in milk.)

Add 1 cc. of 1.6 per cent brom-cresol-purple (in 95 per cent alcohol) per liter of skim milk. Dispense in tubes in 5 cc. quantities and sterilize by heating in streaming steam for 20 minutes on each of 3 successive days.

XXVII. CALCIUM LACTATE MILK: (Used especially in the study of fungi.)

Dispense fresh skim milk in 10 cc. quantities in test tubes. Sterilize by heating in streaming steam on 3 successive days, 30 minutes each day. Add aseptically, to each tube 0.5 cc. of 10 per cent calcium lactate solution which has been autoclaved for 30 minutes. The lactate solution should be fresh enough to show no precipitate. The milk should be cool when the lactate is added.

XXVIII. WHOLE MILK: (For anaerobes.)

Shake fresh whole milk in order to distribute the cream uniformly. Dispense 7 cc. quantities in test tubes and autoclave at 12 pounds for 10 minutes. Allow to cool without shaking. Prior to inoculation the milk should be heated in a boiling water-bath for 10 to 15 minutes and rapidly cooled in order to remove dissolved oxygen.

XXIX. AVERY BROTH FOR PNEUMOCOCCI: (Artificial mouse.)

To basic broth (Medium #I) adjusted to pH 7.6 to 7.8 aseptically add a solution of 20% sterile glucose to increase the glucose concentration from 0.1 to 1.0% and also add 5% sterile defibrinated rabbit, horse or sheep blood. The medium is distributed aseptically in sterile test tubes in 4 to 5 cc. amounts. For 90 cc. of the basic broth medium, 5 cc. of 20 per cent sterile glucose solution and 5 cc. of sterile blood are needed.

XXX. GLUCOSE CYSTINE-BLOOD AGAR: (For P. tularensis.)

To beef infusion agar (Medium IV) add 0.1% cystine and heat in flowing steam for two hours. After cooling the medium to 50 degrees C. add 5 to 8 per cent of sterile defibrinated or whole rabbit blood and heat to 60 degrees C. for two hours. Add 1 per cent glucose from a sterile 50 per cent solution and distribute aseptically into test tubes.

XXXI. BACTO-CYSTINE HEART AGAR: (Difco) (For P. tularensis.)

Beef heart infusion	500 Gm.
Proteose peptone	10 Gm.
Glucose	10 Gm.
Sodium chloride	5 Gm.
Cystine	1 Gm.
Agar	15 Gm.
Distilled water	1000 cc.

This medium is prepared in dehydrated form by the Difco Laboratories and is used by some workers for the cultivation of P. tularensis. It is not as good as Medium XXX for this purpose.

16.8 Gm. are dissolved in 300 cc. of distilled water. The reaction is adjusted to pH 7.3 and the medium is autoclaved for 20 minutes at 15 pounds pressure. It is cooled to 60-70 degrees C. and 18 cc. of whole or defibrinated rabbit blood is added. After thorough mixing it is distributed aseptically into sterile test-tubes.

When used with Bacto-Hemoglobin, the medium is prepared for use by suspending 11.2 grams of Bacto-Cystine-Heart-Agar in 100 cc. cold distilled water. This is a double strength agar. Boil for a minute or two, or preferably heat in the Arnold or flowing steam to dissolve the medium. This solution is sterilized in the autoclave for 15 minutes at 15 pounds pressure. At the same time, 2 grams of Bacto-Hemoglobin are dissolved in 100 cc. of distilled water. This solution is strained through coarse gauze to remove the larger undissolved clumps, and is then sterilized in the autoclave for 15 minutes at 15 pounds pressure. The sterile, double strength cystine heart agar and hemoglobin solution are cooled to 50-60 degrees C., and are then mixed in equal portions. The mixture is then dispensed, under aseptic conditions, into sterile tubes or plates as desired.

XXXII. LOEFFLER'S BLOOD SERUM MEDIUM: (For the cultivation of C. diphtheriae.)

3 volumes of beef, horse, hog or human serum
1 volume of 1% dextrose broth [Basic Medium (#1) containing 1% instead of 0.1% dextrose] The reaction is adjusted to pH 8.0 with sodium hydroxide.

The medium is distributed in 3 to 5 cc. amounts, avoiding the formation of air bubbles. The medium is then coagulated and sterilized in the autoclave as follows: The tubes are placed in a slanting position in the autoclave and are covered with paper to avoid too sudden contact with steam and protect them from dripping condensation water. The door and air outlet valve of the autoclave are closed. The pressure is gradually raised to 15 lbs., without letting any of the air escape. The pressure is maintained for at least 10 minutes, or until the temperature reaches at least 100 degrees C. The air outlet valve is opened so slightly that the pressure will not vary more than 1/2 pound - thus allowing the condensed water and some of the confined air to escape. The valve is closed and the sterilization process continued for 20 minutes at 15 pounds pressure. After sterilization is completed, the source of steam is cut off and the autoclave allowed to cool slowly until the pressure is nil. The final reaction of the medium (the water of syneresis may be tested) should be between pH 7.6 and 7.8.

If serum is not available, Difco's dehydrated Bacto-Loeffler's blood serum may be conveniently employed. This is a dehydrated mixture of beef blood serum and dextrose broth. This dehydrated medium is dissolved in warm (42 to 45 degrees C.) distilled water. The solution, which will not be clear, is distributed in test tubes and coagulated and sterilized as above. This medium is probably less efficient than one prepared from dextrose broth and fresh serum, with regard to the support of growth of diphtheria organisms.

XXXIII. CYSTINE-TELLURITE BLOOD AGAR: (For diphtheria.)

To 15 cc. of basic agar medium (Medium #II) melted and cooled to 45 degrees C. add 0.75 cc. (5%) of blood or serum, 2.25 cc. of a sterile 0.3% aqueous

potassium tellurite* solution, and a few granules (enough to cover the point of a pen-knife) of powdered cystine. Agitate the mixture and pour into a Petri dish.

XXXIV. HEMOPEPTONE WATER: (For Hemophilus organisms.)

Distilled water	1000 cc.
Peptone	20 Gm.
Sodium chloride	5 Gm.

Heat slowly to dissolve the peptone. Adjust the reaction to pH 7.6. Add 20 cc. of defibrinated blood. Heat to 95 degrees C. or just to boiling. Filter through paper. Sterilize by filtering through a sterile Berkefeld or other suitable bacteriological filter. Tube aseptically in sterile test tubes. Incubate at 37 degrees C. for 8 days to test sterility.

XXXV. NITRATE HEMOPEPTONE WATER:

Hemopeptone water to which 0.02 per cent potassium nitrate is added before filtering. This medium is used to differentiate H. influenzae from H. pertussis. The former reduces nitrates to nitrites, the latter does not.

XXXVI. SODIUM HIPPURATE BROTH: (For the study of streptococci.)

To the basic broth medium (Medium #I) add exactly 1 per cent of sodium hippurate. Distribute into tubes and with a "Non-Run" wax pencil or other means mark the level of the medium on each tube. Sterilize in the autoclave.

When the culture has grown for 48 hours or longer restore the volume of medium in each tube by the addition of distilled water up to the mark on the tube. Test for hydrolysis of sodium hippurate with the ferric chloride reagent of Ayers and Rupp. (See section on procedures).

* The tellurite must be titrated. In the above formula the final concentration is 0.0375 per cent, but the concentration refers to a particular lot of tellurite. It is necessary to test (titrate) each lot of tellurite unless the package is accompanied by a "directions" sheet giving the proper amount to use. Arthur H. Thomas Co., Philadelphia, Pa., stock such titrated potassium tellurite. (The titration is done by preparing small amounts of medium containing varying amounts of tellurite and cystine. Those concentrations are selected which yield the most luxuriant growth of C. diphtheriae of darkest colony color with a maximum inhibition of other organisms.).

XXXVII. TARTRATE MEDIUM: (For differentiation of Salmonellae).

Agar	20 Gm.
Peptone (Difco)	10 Gm.
Sodium potassium tartrate	10 Gm.
Sodium chloride	5 Gm.
Phenol red (0.2% alcoholic solution)	12 cc.
Distilled water	1000 cc.

Melt the ingredients in flowing steam in an Arnold sterilizer. Adjust the reaction to pH 7.6-7.8 and tube in 5 to 10 cc. amounts. Sterilize in the autoclave at 15 pounds pressure for 20 minutes and allow to cool and solidify in an upright position. (Difco Laboratories prepare this medium in dehydrated form).

The medium is inoculated by stab and incubation carried out for 24 to 48 hours.

The reactions obtained in this medium are indicated in Table 37.

TABLE 37.

The Reactions Obtained with Tartrate Medium.

Acid reaction (Yellow)	Alkaline reaction (Red)
S. aertrycke	S. schottmulleri (Para B)
S. enteritidis	S. paratyphi (Para A)
S. suipestifer	S. alkaligenes
S. abortivo-equinus	B. subtilis
E. typhosa	
Proteus vulgaris	
Esch. coli	

XXXVIII. BUFFERED DEXTROSE-PEPTONE SOLUTION: (For methyl red and Voges Proskauer tests.)

For the methyl red and Voges Proskauer tests, Difco's Bacto M.R.-V.P. medium may be conveniently used and has been found by a number of workers to be superior to laboratory-made media for these tests.

The Difco formula is:

Buffered peptone	7 Gm.
Bacto-Dextrose	5 Gm.
Dipotassium phosphate	5 Gm.

Seventeen grams of the medium are dissolved in 1000 cc. of distilled water and the medium then distributed in 10 cc. quantities in test tubes. It is sterilized in the autoclave for 20 minutes at 15 pounds pressure.

Incubation of the inoculated broth should be at 37 degrees C. for 48 hours. After incubation the culture is divided into two 5 cc. portions. One portion is used for the methyl red test and the other for the Voges Proskauer test. When the incubation temperature is 30 degrees C., the Voges Proskauer test should be done after 24 or 48 hours of incubation and the methyl red test after 5 days. (See section on procedures for the performance of these tests).

If Difco's dehydrated medium is not available, the Voges Proskauer test may be done on a culture in peptone water (Medium X) containing 2% glucose.

A suitable medium for the methyl red test and one that may be used for the Voges Proskauer test also is the following:

Proteose peptone.....	5 Gm.
Dextrose (C.P.).....	5 Gm.
Potassium phosphate(K_2HPO_4)..	5 Gm.
Water to make.....	1000 cc.

In eight-tenths of the water (800 cc.), dissolve the other ingredients by heating on a water bath for 20 minutes with occasional stirring. Filter through paper, cool to 20 degrees C. and make up to 1 liter. Dispense 10 cc. amounts in 19 by 150 mm. test tubes and sterilize in flowing steam by the intermittent method (20 minute periods on three successive days).

XXXIX. TETRATHIONATE BROTH BASE: (J. Path. Bact., 1936, 42, 455)(Enrichment medium for E. typhosa.)

Proteose peptone No. 2, Difco.....	5 Gm.
Bacto-Bile Salts.....	1 Gm.
Calcium carbonate.....	10 Gm.
Sodium thiosulfate.....	30 Gm.
H_2O.....	1000 cc.

Dissolve the ingredients in water and bring to a boil.

Two cc. of iodine solution (6 Gm. iodine crystals and 5 Gm. potassium-iodide in 20 cc. of water) is added to 100 cc. of the medium at 45 degrees C. or below, and tubed in 10 cc. quantities, exercising care to obtain an even distribution of the insoluble material. The medium should not be heated after the iodine has been added. The complete medium containing iodine should be used the day it is prepared; the base medium without the iodine can be stored indefinitely.

The prepared broth is inoculated by adding 1 to 3 grams of stool, sewage, urine or other infected material to 10 cc. of the medium and mixing with a swab, glass rod, or pipette to suspend the particulate matter. A loosely packed cotton plug may be passed through the inoculated broth to carry the coarser particles of fecal material to the bottom of the tube. The inoculated medium is incubated for 12 to 24 hours and streaked upon Bismuth sulfite and MacConkey's or Endo's or eosin-methylene blue agar.

A suitable dehydrated tetrathionate broth base may be obtained from the Difco Laboratories.

XL. SELENITE-F ENRICHMENT MEDIUM: (For E. typhosa).

Sodium hydrogen selenite (anhydrous)	0.4%
Sodium phosphates (anhydrous)	1.0%
Peptone	0.5%
Lactose	0.4%
pH 7.0	

In preparing the medium it is advisable to determine experimentally the exact proportions of monosodium phosphate and disodium phosphate which, together with the particular kind of peptone used and a particular lot or make of selenite, will give a final pH of about 7.0.

Dissolve the ingredients in distilled water. Sterilize gently, 30 minutes in flowing steam (no pressure) being sufficient. It is important that the medium should not be autoclaved.

Satisfactory dehydrated selenite-F enrichment medium may be obtained from the Baltimore Biological Laboratory.

XLI. SODIUM THIOGLYCOLLATE BROTH: (Brewer's medium for anaerobes).

Pork infusion solids	1.0%
Peptone (Thio)	1.0%
Sodium chloride	0.5%
Sodium thioglycollate	0.1%
Agar	0.05%
Dextrose	1.0%
Methylene blue	0.0002%

The medium is tubed in 15 cc. amounts in 6 x 3/4 inch test tubes, making a column of medium 7 cm. high. After autoclaving for 20 minutes at 15 pounds it is stored at room temperature. The loss of anaerobiasis by the medium during storage is indicated by the return of the color of the methylene blue, giving the aerobic portions of the medium a green color. If the color extends over 1-1/2 inches below the surface, the medium should be heated and then cooled before using in order to restore anaerobic conditions.

Satisfactory dehydrated sodium thioglycollate medium may be obtained from the Baltimore Biological Laboratory.

XLII. MALONATE BROTH: (Leifson: J. Bact., 1933, 26, 3.)(For differentiation of Esch. coli and Aerobacter aerogenes).

Ammonium sulphate ($(NH_4)_2SO_4$)	2.0 Gm.
Dipotassium phosphate (K_2HPO_4)	0.6 Gm.
Potassium dihydrogen phosphate (KH_2PO_4)	0.4 Gm.
Sodium chloride	2.0 Gm.
Sodium malonate	3.0 Gm.
Indicator (0.5% alcoholic solution of brom-thymol-blue)	5.0 cc.
Distilled water	1000 cc.

NOTE: 2.1 Gm. of malonic acid may be substituted for the sodium malonate.

Adjust the reaction in a glass vessel with N/1 NaOH until the indicator gives a green color (about pH 6.8). Distribute in 5 cc. amounts in test tubes and sterilize in the autoclave at 15 pounds for 15 minutes.

XLIII. CITRATE AGAR: (Simmons: J. Infect. Dis., 1926, 39, 209) (For differentiation of E. coli & A. aerogenes).

Distilled water	1000 cc.
Agar	20.0 Gm.
Sodium chloride	5.0 Gm.
Magnesium sulphate ($MgSO_4$)	0.2 Gm.
Ammonium dihydrogen phosphate ($NH_4H_2PO_4$)	1.0 Gm.
Dipotassium phosphate (K_2HPO_4)	1.0 Gm.
Sodium citrate	2.0 Gm.

Adjust the reaction to pH 6.8 and add 5 cc. of 0.5% alcoholic solution of brom-thymol-blue. Distribute in 6 cc. amounts in test tubes and sterilize in the autoclave at 15 pounds for 15 minutes. Solidify in a slanting position.

XLIV. DOUBLE SUGAR SLANT: (Russell). (For identification of intestinal bacteria.)

Beef extract	3 Gm.
Peptone	10 Gm.
Lactose	10 Gm.
Dextrose	1 Gm.
Sodium chloride	5 Gm.
Agar	15 Gm.
Phenol red (0.02% aqueous sol.)	50 cc.
Distilled water	1000 cc.

Mix ingredients and dissolve by boiling. Restore the volume with distilled water. Adjust the reaction to pH 7.4-7.6. Filter through cotton. Tube, placing 10 cc. in each tube. Sterilize in the autoclave at 15 pounds for 15 minutes. Slant to make a deep butt of agar and allow to harden.

Russell's double sugar slant is employed in the identification of gram-negative organisms belonging to the coli-typhoid-paratyphoid-dysentery groups. Alkaline reactions on this medium turn the indicator red and acid reactions change it to yellow.

Inoculation is made by smearing over the surface of the slant and stabbing the butt. The fermentation of the sugars can be detected by changes in color of the indicator. Gaseous fermentation is indicated by splitting of the agar or formation of bubbles in the butt.

A properly inoculated tube showing, after incubation, a red or cerise slope and a yellow butt with or without gas formation indicates fermentation of the dextrose. Some strains of typhoid may require as long as 30 to 40 hours to produce a characteristically alkaline slant. A tube showing a yellow slant and butt with or without gas indicates fermentation of the lactose. A tube showing no change indicates that neither dextrose nor lactose has been fermented.

The following typical reactions are obtained in this medium:

	SLANT	BUTT
Escherichia & Aerobacter	Yellow	Yellow & gas
Salmonella	No change	Yellow & gas
Eberthella & Shigella*	No change	Yellow
Alkaligenes fecalis	No change	No change

* An occasional strain of Newcastle will produce gas on prolonged incubation.

XLV. LEAD ACETATE AGAR: (For the detection of H_2S production).

To each 10 cc. of agar (Basic Medium #II) melted and cooled to 50 degrees C. add aseptically 1 cc. of a sterile (autoclaved) 0.5% aqueous solution of the basic lead acetate.

The solid medium is inoculated by stab.

XLVI. KLIGLER IRON AGAR: (For the detection of H_2S production and for differential study of the gram-negative intestinal bacteria.) (Combines the principles of Russell's double sugar slants and lead acetate agar into one medium.) Jour. Bact., 1927, 13, 183.

Bacto-Tryptone	20 Gm.
Lactose	10 Gm.
Dextrose	1 Gm.
Sodium chloride	5 Gm.
Ferric ammonium citrate	0.5 Gm.
Sodium thiosulphate	0.5 Gm.
Agar	15 Gm.
Phenol Red	0.025 Gm.
Water	1000 cc.

Dissolve the ingredients by heating. Adjust the reaction to pH 7.4. Distribute in test tubes and autoclave at 15 pounds for 15 minutes. Allow to solidify in a slanting position in a manner that will give a generous butt. Best reactions are obtained on freshly prepared media. If the medium is not used the same day as sterilized, melt the agar and allow to solidify before inoculation.

Tubes of Kligler iron agar medium are inoculated by smearing over the surface of the slant and stabbing into the butt. The inoculated tubes are observed after incubation at 37 degrees C. for 18 to 36 hours.

.In addition to showing the fermentation reactions obtained with Russell's double sugar, Kligler iron agar indicates whether or not hydrogen sulfide is formed. This is indicated by a blackening of the medium.

A satisfactory dehydrated Kligler iron agar may be obtained from Difco Laboratories.

Typical reactions of various bacteria in this medium are indicated in Table 38.

XLVII. BASIC AGAR (MEDIUM #II) CONTAINING THIONIN OR BASIC FUCHSIN:
(For differentiation of Brucellae.)

To one liter of medium #II add either 5.0 cc. of a 0.1% solution of thionin to give 1:200,000 or 10 cc. of a 0.1% solution of basic fuchsin to give a 1:100,000 solution of the dye. The basic dye solutions are prepared by dissolving 0.1 gram in 100 cc. of sterile distilled water at 70 degrees C. These stock solutions may be stored indefinitely. Before adding to the medium, the dye solutions should be heated in flowing steam for 20 minutes, shaken well and while still hot added to the medium. The dyes and melted agar are thoroughly mixed and poured immediately into Petri dishes. The plates should be placed in the incubator until the water of condensation disappears and then used. They should be inoculated within 24 hours after pouring, as the dyes become reduced in the medium on standing.

The surface of the plate is streaked with a heavy suspension of Brucella prepared from a slant culture. Duplicate plates should be streaked, one set receiving aerobic incubation and the other being incubated in 10% carbon dioxide.

TABLE 38.

TYPICAL REACTIONS OF VARIOUS BACTERIA ON KLIGLER IRON AGAR

	Slant	Butt	
		Fermentation	H_2S
Escherichia coli	y	y.g	-
Aerobacter aerogenes	y	y.g	-
Eberthella typhosa	n.c	y	+
Salmonella paratyphi (Para A)	n.c	y.g	-
Salmonella schottmulleri (Para B)	n.c	y.g	+
Salmonella enteritidis	n.c	y.g	+
Salmonella typhimurium (aertrycke)	n.c	y.g	+
Salmonella suipestifer	n.c	y.g	+ -
Proteus vulgaris	n.c	y.g	+ -
Shigella dysenteriae (Shiga)	n.c	y	-
Shigella ambigua (Schmitz)	n.c	y	-

(continued on next page)

TABLE 38 (continued)

TYPICAL REACTIONS OF VARIOUS BACTERIA ON KLIGLER IRON AGAR

	Slant	Butt	
		Fermentation	H_2S
Shigella alkalescens	n.c	y	-
Shigella sp. (Newcastle type)	n.c	y*	-
Shigella paradysenteriae (Flexner)	n.c	y	-
Alkaligenes fecalis	n.c	n.c	-

* An occasional strain of Newcastle will produce gas on prolonged incubation.
y = yellow color (acid)
g = gas
n.c = no change.

The plates are incubated for 72 hours and observed for inhibition of growth by either thionin or basic fuchsin or by both dyes.

XLVIII. CRYSTAL VIOLET AGAR: (For inhibition of gram-positive organisms.)

1.4 cc. of a 0.1% solution of crystal violet is added to each liter of basic Medium #II before sterilization of the medium. (Dye dilution - 1:700,000). The sterile medium is cooled to about 50 degrees C., poured into sterile Petri dishes, allowed to solidify and then inoculated.

The addition of 1:5,000,000 crystal violet to "combination" blood agar (See Medium II) facilitates the isolation of N. gonorrhoeae from specimens containing gram-positive organisms. One per cent of a sterile 1:5,000,000 solution of crystal violet of high dye content may be added to the medium before pouring. The crystal violet solution may be sterilized by autoclaving at 10 to 15 pounds steam pressure for 15 minutes.

XLIX. EOSIN METHYLENE BLUE AGAR: (Levine: Iowa State College of Agric. and Mech. Arts Bull., 62, 1921, 117). (For differentiation of bacteria of intestinal origin).

Peptone	10 Gm.
Agar	15 Gm.
Lactose	10 Gm.
Dipotassium phosphate	2 Gm.
Eosin Y	0.4 Gm.
Methylene blue	0.1 Gm.
Distilled water	1000 cc.

The peptone, dipotassium phosphate and agar are dissolved in 1000 cc. of

water and the loss due to evaporation made up with distilled water. Add the lactose (50 cc. of a 20% solution), the eosin (20 cc. of a 2% aqueous solution) and the methylene blue (20 cc. of a 0.5 per cent aqueous solution), mix thoroughly, and sterilize in the autoclave at 15 pounds for 15 minutes.

Satisfactory dehydrated eosin-methylene-blue agar may be obtained from the Difco Laboratories.

(If the dehydrated product is not available, the medium may be prepared by adding to 100 cc. of the melted basic agar medium (Medium #II), 5 cc. of a sterile 10 per cent lactose solution, 2 cc. of a 2 per cent aqueous solution of eosin and 2 cc. of a 0.5 per cent aqueous solution of methylene blue).

It is permissible to add all of the ingredients to the stock agar at the time of preparation, place in tubes or flasks and sterilize. Plates may be prepared from this stock. Decolorization of the medium occurs during sterilization but the color returns after cooling. (See Table 39.)

L. MacConkey's AGAR: (For differentiation of bacteria of intestinal origin). (J. Hyg., 1905, 5, 333.)

Water	1000 cc.
Agar	17 Gm.
Peptone	20 Gm.
Sodium chloride	5 Gm.
Lactose	10 Gm.
Bile salts - Bacto	3 Gm.
Neutral red, 1% aqueous solution	5 cc.*

* This amount may vary with different lots and brands of dye.

The agar is dissolved in one half of the water by autoclaving for 30 minutes. The other ingredients with the exception of the dye are dissolved in the remainder of the water by heating in a water bath. The two solutions are combined and adjusted to pH 7.4. The medium is dispensed in convenient amounts and autoclaved for 20 minutes.

When ready for use, the neutral red is added and 15 to 20 cc. amounts are distributed in Petri dishes. The plates should be used within 4 or 5 days after pouring. The basic medium may be stored in bulk in a refrigerator and melted in steam at 100 degrees C., the neutral red added and plates poured when required.

A suitable dehydrated MacConkey's agar may be obtained from the Difco Laboratories.

LI. ENDO'S AGAR: (Standard Methods for the Examination of Water and Sewage, Eighth Edition, 1941.)(For differentiation of bacteria of intestinal origin).

1. Preparation of stock agar. Add 5 Gm. of beef extract, 10 Gm. of peptone and 30 Gm. of agar to 1000 cc. of distilled water. Boil until the agar is dissolved and make up lost weight, due to evaporation, with distilled water.

TABLE 39.

DIFFERENTIATION OF BACT. COLI AND BACT. AEROGENES ON LEVINE'S EOSIN METHYLENE BLUE AGAR
(Levine, Bull. 62, Iowa Eng. Exp. Sta., 1921.)

	Bact. coli (1)	Bact. aerogenes (2)
Size	Well isolated colonies are 2-3 mm. in diameter.	Well isolated colonies are larger than coli; usually 4-6 mm. in diameter or more.
Confluence	Neighboring colonies show little tendency to run together.	Neighboring colonies run together quickly.
Elevation	Colonies slightly raised; surface flat or slightly concave, rarely convex.	Colonies considerably raised and markedly convex; occasionally the center drops precipitately.
Appearance By Transmitted Light	Dark, almost black centers which extend more than 3/4 across the diameter of the colony; internal structure of central dark portion difficult to discern.	Centers deep brown; not as dark as Bact. coli and smaller in proportion to the rest of the colony. Striated internal structure often observed in young colonies.
Appearance By Reflected Light	Colonies dark, button-like, often concentrically ringed with a greenish metallic sheen.	Much lighter than Bact. coli, metallic sheen not observed except occasionally in depressed center when such is present.

(1) Two other types have been occasionally encountered. One resembles the type described, except that there is no metallic sheen, the colonies being wine colored. The other type of colony is somewhat larger (4 mm.), grows effusely, and has a marked crenated or irregular edge, the central portion showing a very distinct metallic sheen. These two varieties constitute about 2 or 3 per cent of the colonies observed.

(2) A small type of aerogenes colony, about the size of the colon colonies which shows no tendency to coalesce, has been occasionally encountered.

Adjust the reaction so that the pH reading after sterilization will be 7.4.

Clarify if desired.

Add 10 Gm. of lactose and dissolve.

Place in small flasks or bottles, 100 cc. to each, and sterilize in the autoclave at 15 pounds for 15 minutes.

2. Preparation of plates. Prepare a 3 per cent solution of certified basic fuchsin in 95 per cent ethyl alcohol.

Allow to stand 24 hours and filter.

Melt lactose agar as prepared above and to each 100 cc. add 1 cc. of the 3 per cent basic fuchsin solution and 0.125 Gm. of anhydrous sodium sulfite dissolved in 5 cc. of distilled water. The sulfite solution must be freshly prepared.

Mix thoroughly, pour plates with usual precautions against contamination and allow to harden.

The medium should be light pink when hot and almost colorless when cool. As batches of fuchsin differ somewhat in dye content, it is possible that the medium made up according to this formula may be too highly colored before incubation or may not give the proper reaction when seeded with colon bacilli. In such case, the strength of the basic fuchsin solution may be varied.

A satisfactory dehydrated Endo medium is prepared by the Difco Laboratories.

LII. DESOXYCHOLATE AGAR: (Leifson: J. Path. and Bact., 1935, 40, 581)(For differentiation of organisms of intestinal origin.)

Distilled water	1000 cc.
Peptone	10 Gm.

Boil for a short time and filter through paper. Neutralize if necessary. Add the following:

Agar	17 Gm.
Sodium hydroxide (N)	2 cc.

Heat in flowing steam until the agar is dissolved. Add the following substances in the order given:

Sodium desoxycholate	1 Gm.
Sodium chloride	5 Gm.
Dipotassium phosphate	2 Gm.
Lactose	10 Gm.
Ferric ammonium citrate (green scales)	2 Gm.

Adjust the reaction to pH 7.2-7.4 and add:

Neutral red (certified), 1% aqueous solution	3.3 cc.

Tube and sterilize for 15 minutes in flowing steam (no pressure), longer if distributed in flasks. Autoclaving is not necessary because gram-positive

spore-forming bacteria do not grow in the medium. The medium should be heated as little as possible and only sufficiently to kill vegetative cells. Store in the dark because neutral red is decolorized by light. Iodine green may be substituted for neutral red and is not affected by light. Wilson P. peptone is not suitable for this medium; proteose or Fairchild peptone may be used.

Dehydrated desoxycholate agar may be obtained from the Baltimore Biological Laboratory.

LIII. DESOXYCHOLATE-CITRATE AGAR; (Leifson: J. Path. and Bact., 1935, 40, 581) (A differential and coli-restricting medium for organisms of intestinal origin.)

To ground fresh lean pork or beef add 3 times its weight of distilled water and acidify the mixture to pH 5.0-6.0 by the addition of HCl. Boil for 1 minute, strain off the meat and filter the fluid through paper. Add 1 per cent of peptone (Wilson P or proteose peptone) and when dissolved readjust the reaction to pH 7.0. Boil for 1 minute and filter through paper. (It is important that the medium be free from visible fats and lipoids).

To 950 cc. of the above infusion add 20 Gm. of agar and 10 cc. of N/1 NaOH. Heat in flowing steam until the agar is dissolved. Restore weight or volume with distilled water. Add and dissolve in the order given:

Sodium desoxycholate (20 per cent aqueous solution).....	25 cc.
Sodium citrate as $2Na_3C_6H_5O_7.11H_2O$.....	25 Gm.
(or as $Na_3C_6H_5O_7.2H_2O$.....	20.6 Gm.)
Lactose.....	10 Gm.
Ferric ammonium citrate (green scales).....	1 Gm.
Lead as $PbCl_2$(0.033 per cent aqueous solution).....	1 cc.
(or as $Pb(CH_3COO)_2.3H_2O$)(0.05% aqueous sol.).	1 cc.

Adjust the reaction to pH 7.2-7.3. (In titrating the medium use phenol red as an indicator. Brom-thymol-blue is unsatisfactory since it is affected by the medium and does not indicate the correct pH). Add:

Neutral red (certified, 1% aqueous solution).....	2 cc.

Distribute into tubes or small flasks and sterilize for 15 to 30 minutes in flowing steam. Store in the dark. Iodine green may be substituted for neutral red and is not affected by light.

Dehydrated desoxycholate-citrate agar may be obtained from the Baltimore Biological Laboratory.

LIV. BISMUTH SULFITE AGAR; (Wilson & Blair, J. Path. & Bact., 1926, 29, 310-311)(For isolation of typhoid-paratyphoid organisms).

(a) Agar Base:

Agar, granulated or powdered.....	20 Gm.
Beef extract.....	5 Gm.
Peptone.....	10 Gm.
Hot water to make.....	1 Kg.

(1) Dissolve the ingredients by autoclaving for 15 minutes.

(2) Store in a refrigerator if not used at once.

(b) Bismuth sulfite mixture:

Bismuth ammonium citrate scales	6 Gm.
Sodium sulfite,anhydrous	20 Gm.
Dextrose	10 Gm.
Sodium phosphate, anhydrous (Na_2HPO_4)	10 Gm.
Water	200 cc.

(1) Dissolve the bismuth ammonium citrate scales in 50 cc. of boiling water; the sodium sulfite in 100 cc. of boiling water; and the dextrose in 50 cc. of boiling water.

(2) Mix the solution of bismuth ammonium citrate and sodium sulfite; boil; admix the sodium phosphate while boiling.

(3) Allow the mixture to cool; then admix the dextrose solution.

(4) Add water to make up lost weight and store in a well-stoppered pyrex vessel in a dark cupboard at room temperature.

(c) Iron citrate brilliant green solution:

Iron citrate (Ferric citrate)	1 Gm.
Water	100 cc.
Brilliant green, 1% solution	12.5 cc.

(1) Dissolve the iron citrate in the water with heat and add the brilliant green solution.

(2) Store in a well stoppered Pyrex vessel in a dark cupboard at room temperature.

(3) To 1,000 cc. of hot melted agar base, add with thorough mixing:

Bismuth sulfite mixture	200 cc.
Iron citrate brilliant green solution	45 cc.

(4) Pour immediately into porous-top Petri dishes, 15 to 20 cc. to each.

(5) Keep the plates at room temperature for 1 to 2 hours and then store in a refrigerator until required. It is advisable to use these plates within 4 days after preparation.

A good dehydrated bismuth sulfite agar medium may be obtained from the Difco Laboratories. They describe the results obtained with their preparation as follows:

As surface and subsurface colonies on bismuth sulfite agar are strikingly characteristic, it is possible to use the medium both as a smear plate and as a

poured plate in the isolation of E. typhosa. Smear plates are prepared by pouring 15 to 20 cc. quantities of the medium into sterile Petri dishes (90 mm.) and allowing the medium to solidify with the cover removed to obtain a dry surface. In preparing poured plates the inoculum is placed in the sterile Petri dish and the melted medium at 45 degrees C. is added and mixed in the usual manner.

The following technique is recommended for the isolation of typhoid organisms from fecal specimens.

Smear Plate

Smear or streak the surface of a plate with a heavy inoculation of the fecal material in such a way that on some portion of the plate the inoculation will be light, permitting the development of discrete colonies.

Poured Plate

(a) Transfer about 2 or more grams of the fecal material to a test tube, add 12 to 15 cc. of water, mix well, being careful to break up all the larger particles of the material.

(b) Insert a loosely-packed cotton plug, about 1 inch long, into the tube, and slowly force it down through the fecal mixture by means of a glass rod or pipette, so that all the gross particles are carried to the bottom of the tube on the cotton plug, and an opaque fluid rises through the cotton. A second cotton filtration may be necessary, since it is essential that the supernatant fluid be free from gross particles. Such solid particles in the medium may support growth of the extraneous organisms, giving pseudo-blackening which may be mistaken for typhoid colonies.

Some workers may prefer to allow the gross solid particles of fecal suspension to settle by gravity instead of removing them by filtration with cotton. In such cases it is not advisable to allow the suspension to stand longer than 30 minutes in order to obtain a supernatant fluid free from gross particles. Other methods of preparing fecal suspensions that will give a liquid free from gross solid suspended material without removing typhoid, may also be employed.

(c) Transfer about 5 cc. of the prepared fecal suspension to one Petri dish and 1 drop to a second dish. Add 20 cc. of bismuth sulfite agar, cooled to 45 degrees C., to each dish, and mix thoroughly. It is necessary to use at least 20 cc. of the medium to each 5 cc. of inoculum, for dilution of the medium beyond this point will allow the development of extraneous fecal forms.

(d) Incubate at 37 degrees C. and observe after 24 hours for typical colonies as described below. Frequently typical colonies develop within 24 hours incubation; however, in all cases the plates should be incubated for at least 48 hours to allow the development of all typhoid strains, before considering the specimen negative. Specimens containing only a small number of typhoid organisms should show isolated colonies from the 5 cc. inoculum, while those specimens containing increasingly large numbers of typhoid organisms should show isolated colonies from the 1 drop inoculum in the poured plate or on the smear plate.

Description of Colonies

Smear Plate

The typical discrete surface typhoid colony is black and is surrounded by a black or brownish-black zone which may be several times the size of the colony. By reflected light, preferably daylight, this zone exhibits a distinctively characteristic metallic sheen. Plates heavily seeded with typhoid organisms may not show this reaction except possibly near the margin of the mass inoculation. In these congested areas, E. typhosa frequently appears as small light green colonies. This fact emphasizes the importance of inoculating plates in such a manner as to have some sparsely populated areas with discrete typhoid colonies.

Poured Plate

Well isolated subsurface typhoid colonies are circular, jet black, and well defined. The size of the black colony may vary from 1 to 4 mm. in diameter depending upon the particular strain, length of incubation, and position of the colony in the agar. Only those colonies growing very close to the surface or on the surface will show a decided black metallic sheen. Plates containing typhoid too numerous to permit the development of individual colonies give a black plate or a plate dotted with black areas. Plates with about three hundred to a thousand typhoid colonies will exhibit this appearance. When typhoid develops in a plate in still larger numbers, typical blackening does not occur and the appearance is that of a negative plate.

Ordinarily typhoid will develop well isolated colonies showing typical round jet black colonies with or without sheen, from either the 5 cc. or 1 drop inoculation of cotton-filtered fecal suspension using the poured plate method. However, the typhoid organisms developing from the specimens containing large numbers of this organism may be so numerous that the blackening cannot occur typically and the plate may appear dotted black or greenish gray. From such heavily seeded specimens the direct smear on bismuth sulfite agar from feces should demonstrate typhoid, while the poured plate should give positive results from specimens containing lesser numbers of typhoid.

Description of Colonies of Other Organisms Which Grow Upon Bismuth Sulfite Agar

Salmonella schottmuelleri (Paratyphoid B) and Salmonella enteritidis grow luxuriantly upon Bacto-Bismuth Sulfite Agar forming black surface and subsurface colonies slightly more moist, but otherwise similar to those produced by E. typhosa.

Salmonella paratyphi (Paratyphoid A), Salmonella aertrycke, Salmonella suipestifer, and Salmonella morgani develop upon Bacto-Bismuth Sulfite Agar yielding flat or only slightly raised green colonies.

Generally, the members of the dysentery group other than Flexner and Sonne are inhibited. The Flexner and Sonne strains that do develop upon this medium produce brownish raised colonies with depressed centers and exhibit a crater-like appearance.

Coli is usually completely inhibited. Occasionally a strain will be encount-

ered that will develop small black, brown, or greenish glistening surface colonies. This color is confined entirely to the colony itself and shows no metallic sheen. Likewise, a few strains of aerogenes may develop on this medium forming raised, mucoid colonies. These may exhibit a silvery sheen, appreciably lighter in color than that produced by typhoid. Subsurface colonies of the coliform group, when they develop, are green or brown in color, generally lenticular in shape, and not at all to be confused with the typical round black typhoid subsurface colony. There are some members of the coliform group capable of producing hydrogen sulfide that may develop on the medium, giving colonies similar in appearance to typhoid. These may readily be differentiated in that they produce gas from lactose in differential media--Bacto-Russell Double Sugar Agar or Bacto-Kligler Iron Agar, for example.

The isolation and purification of E. typhosa for agglutination or fermentation studies may be readily accomplished by fishing characteristic black colonies from smeared or poured plates of bismuth sulfite agar, and subculturing them upon Bacto-MacConkey Agar. The purified colonies thus obtained may then be fished to differential tube media such as Bacto-Russell Double Sugar Agar, Bacto-Kligler Iron Agar or other satisfactory differential media for partial identification. Agglutination tests may be made from the fresh growth on the differential tube media or from the growth on agar slants inoculated from the differential media. The growth on the differential tube media may also be used for inoculating carbohydrate media for fermentation studies. It is a common practice among many bacteriologists to fish colonies typical of E. typhosa directly from bismuth sulfite agar onto the differential tube media. This may be permissible if the colonies are discrete and well isolated, but it must be remembered that although coliform bacteria are inhibited they are not destroyed by the medium.

To prepare the medium for use, suspend 5.2 grams of Bacto-Bismuth Sulfite Agar in 100 cc. of cold distilled water. Bring to a boil as rapidly as possible and allow it to simmer for 1 or 2 minutes. The medium should not be sterilized in the autoclave or by fractional sterilization, since heating for a longer period than is necessary to dissolve the medium destroys the selectivity of the medium. A uniformly correct medium may be obtained at all times merely by dissolving the powder in water. Upon a medium prepared in this way, reactions typical of those described by Wilson and Blair are routinely obtained. The characteristic precipitate present in the medium should be evenly dispersed by twirling the flask just prior to pouring plates. Best results are obtained when the medium is dissolved and used immediately. If it is necessary to prepare the medium several days before using, it should be poured into plates and stored in a cold moist atmosphere to prevent drying. The melted medium should not be allowed to solidify in flasks and be remelted. The final reaction of the medium will be pH 7.7 $\pm$.

LV. BACTO SS AGAR: (A restrictive, selective medium recommended for the isolation of members of the Shigella, Salmonella, and Eberthella groups from stools and other materials suspected of containing these organisms).

Bacto beef extract	5 Gm.
Proteose peptone	5 Gm.
Bacto Lactose	10 Gm.
Bacto-Bile Salts No. 3	8.5 Gm.
Sodium citrate	8.5 Gm.
Sodium thiosulfate	8.5 Gm.
Ferric citrate	1 Gm.
Agar	17 Gm.
Bacto-Brilliant green (D Bg-1)	0.33 mg.
Bacto-Neutral Red	0.025 Gm.

Bacto-SS agar supports well the growth of nearly all the different types of dysentery organisms. The more fastidious Shiga strains do not develop as readily as do strains of Flexner, Sonne, Newcastle, Schmitz and alkalescens. Shigella, Salmonella, Eberthella and other organisms not fermenting lactose form opaque, transparent or translucent uncolored colonies, which generally are smooth. The few lactose fermenting organisms which may develop on the medium are readily differentiated due to the formation of a red color in the colony. At times, isolated coliform colonies may not show a definite red color, being pink or nearly colorless with a pink center. Occasionally an aerogenes type will develop a rather large white or cream colored opaque characteristic mucoid colony.

The medium may be heavily inoculated because of its marked growth restricting properties. Since the medium inhibits but does not destroy contaminating organisms, the center of each colony must be carefully fished for identification purposes. If selected colonies are not well isolated they should be purified by sub-culturing on some non-selective medium such as MacConkey agar. The medium may be obtained in dehydrated form from Difco Laboratories.

LVI. FORMATE RICINOLEATE BROTH: (Stark & England, J. Bact., 1935, 29, 26) (For the detection of coli-aerogenes in water and milk).

Add 5 Gm. of peptone, 5 Gm. of lactose, 5 Gm. of sodium formate and 1 Gm. of sodium ricinoleate to 1000 cc. of distilled water.

Heat slowly on a water bath with constant stirring until dissolved. Add distilled water to make the volume to 1000 cc.

Adjust the reaction so that the pH reading after sterilization will be 7.3-7.5.

Distribute in fermentation tubes and sterilize at 11 to 13 pounds for 15 minutes.

The dehydrated medium may be obtained from the Difco Laboratories.

Formate ricinoleate broth is used for the confirmation of the presumptive test for members of the coliform group in the bacteriological examination of water and for the presumptive test for members of the coliform group in the bacteriological examination of milk according to "Standard Methods of Water Analysis" and "Standard Methods for the Examination of Dairy Products", respectively, of the American Public Health Association.

When quantities greater than 1 cc. of sample are to be planted, the strength of the medium must be adjusted in accordance with the following tabulation:

TABLE 40.

Concentrations of Dehydrated Medium Required to Maintain the Optimum Concentration of Ingredients.

Inoculum	Amount medium in tube	Vol. medium and inoculum	Dehydrated medium per 1000 cc.
1 cc.	10 cc. or more	10 cc. or more	16 Gm.
0.1 cc.	10 cc. or more	10 cc. or more	16 Gm.
Loop	10 cc. or more	10 cc. or more	16 Gm.
1 cc.	5 cc.	6 cc.	19.2 Gm.
10 cc.	10 cc.	20 cc.	32 Gm.
10 cc.	15 cc.	25 cc.	26.6 Gm.
10 cc.	20 cc.	30 cc.	24 Gm.
10 cc.	30 cc.	40 cc.	21.3 Gm.

LVII. BRILLIANT GREEN LACTOSE PEPTONE BILE BROTH: (For the detection of members of the Coli-aerogenes group in water and milk).

Dissolve 10 Gm. of peptone and 10 Gm. of lactose in not more than 500 cc. of distilled water. Add 200 cc. of fresh ox bile or 20 Gm. of dehydrated ox bile dissolved in 200 cc. of distilled water. No dehydrated ox bile should be used which has a pH of less than 7.0. Make up with distilled water to a total of at least 975 cc. and adjust the reaction to a pH of 7.1-7.4. Add 13.3 cc. of a 0.1 per cent solution of brilliant green (certified dye) in water, make up to a total of 1000 cc. and filter through cotton. Distribute in fermentation tubes and sterilize at 15 pounds pressure for 15 minutes. The reaction after sterilization should be not less than pH 7.1 and not more than pH 7.4.

Difco Laboratories prepare a suitable dehydrated brilliant green lactose peptone bile broth (Brilliant green bile 2%).

When the medium is to be inoculated with an amount greater than 1 cc., care must be taken to preserve the correct concentration of dye and bile in the medium after dilution with the sample. The following table (Table 41) indicates the quantity of dehydrated medium to use per 1000 cc. of distilled water to maintain the correct concentration of dye and bile:

TABLE 41.

Concentration of Dehydrated Medium Required to Maintain the Optimum Concentration of Bile (2%) and Dye (1:75,000).

Inoculum	Amt. medium in tube	Vol. medium and inoculum	Dehydrated medium to 1000 cc.
1 cc. or less	10 cc.	10 cc.	40 Gm.
10 cc.	20 cc.	30 cc.	60 Gm.
10 cc.	30 cc.	40 cc.	53 Gm.

LVIII. TRYPTONE-GLUCOSE-EXTRACT-MILK AGAR: (Standard medium for the routine plate counting of milk).

Agar	1.5 per cent
Beef extract	0.3 per cent
Tryptone	0.5 per cent
Glucose	0.1 per cent
Distilled water	Q.S.

Reaction.....pH 6.6 to 7.0
Preferred reaction.....pH 7.0

One per cent skim milk is to be added just before final sterilization in all cases where dilutions greater than 1:10 are to be made.

Prepare nutrient agar by adding 3 Gm. of beef extract, 5 Gm. of tryptone, 1 Gm. of glucose and 15 Gm. of agar to 1000 cc. of distilled water. Dissolve the agar by boiling over a free flame and stirring to prevent burning on the bottom of the container, or by exposing the mixture of ingredients in a flask or other suitable container to the action of flowing steam. Make up lost weight with distilled water. Where the dilutions that are to be made are greater than 1:10, add 10 cc. of good quality skim milk just before final sterilization. The milk may be kept in stock by storing in sterile condition in test tubes, bottles or flasks.

An equivalent amount of spray process skim milk powder may be substituted for the skim milk. Dissolve 10 Gm. milk powder in 100 cc. of water. Use 10 cc. of this reconstituted milk per liter of agar. Care should be taken where powder is used to avoid troublesome precipitates.

Before final sterilization, the medium is brought to a boiling temperature, stirring frequently. The lost weight is restored with hot distilled water and the medium is clarified if this is deemed advisable.

The medium is distributed in suitable containers and sterilized in the autoclave at 20 pounds pressure for 15 minutes. The pH of the medium should be checked just before use.

A satisfactory dehydrated tryptone-glucose-extract agar may be procured from the Difco Laboratories. The use of this medium is described in the section on the bacteriological examination of milk.

LIX. VIOLET RED BILE AGAR: [Solid medium for detection of the coliform (Escherichia-Aerobacter) group.]

Peptone	10 Gm.
Lactose	10 Gm.
Bile Salts	1 Gm.
Yeast extract	5 Gm.
Agar	15 Gm.
Neutral red	0.05 Gm.
Crystal violet	0.004 Gm.
Distilled water	1000 cc.

In the interest of uniformity it is recommended that this medium be used in the dehydrated form as purchased from the Difco Laboratories. The use of this medium is described in the section on the bacteriological examination of water.

LX. STANDARD LACTOSE BROTH: (For the detection of coliform organisms).

This medium is prepared by adding to standard nutrient broth (0.3% beef extract, 0.5% Bacto-tryptone), 0.5% lactose. The peptone used may be either Bacto-peptone or Bacto-tryptone.

The dehydrated lactose broth produced by the Difco Laboratories may be used. Sterilization of the medium is carried out by autoclaving at 15 pounds for 15 minutes.

LXI. DIEUDONNE MEDIUM: (Centralbl. f. Bakteriol., Orig. I, 1909) (For the cultivation of V. comma).

To seventy parts of ordinary 3 per cent agar, neutralized to litmus, there are added thirty parts of a sterile mixture of equal parts of defibrinated beef blood and normal sodium hydrate. The latter is sterilized by steam before being added to the agar. This pure alkali agar is poured out in plates and allowed to dry several days at 37 degrees C., or five minutes at 60 degrees C.

The material to be examined is smeared upon the surface of these plates. If the blood-alkali mixture is prepared beforehand and allowed to stand for four or five weeks, the plates may be used immediately after pouring.

LXII. ARONSON'S MEDIUM FOR ISOLATION OF V. COMMA: (Deutsche med. Wchnschr., 1915, 41, 1027).

Thirty-five grams of agar are added to 1 liter of water and soaked overnight.

Add 10 Gm. of meat extract, 10 Gm. of peptone, 5 Gm. of sodium chloride and heat in steam for four to five hours. The particles are allowed to settle by letting the hot agar stand and the clear supernatant agar poured into flasks to hold 100 cc. each.

The following solutions are previously made and sterilized for one-half hour

in flowing steam:

1. 10% sodium carbonate.
2. 20% sucrose.
3. 20% dextrin.
4. Saturated solution of basic fuchsin.
5. 10% sodium sulfite (Sterilized by being brought to a boil).

To 100 cc. of agar add 6 cc. of the 10% solution of sodium carbonate and heat for 15 minutes at 100 degrees C. The agar, because of the alkalinity, becomes brown and cloudy. While hot add 5 cc. of the 20% solution of cane sugar, 5 cc. of the 20% solution of dextrin, 0.4 cc. of the saturated solution of basic fuchsin and 2 cc. of the 10% sodium sulfite solution. The flask is allowed to stand to let the coarser particles settle and plates are poured with the clear supernatant fluid. By adding 0.25% nutrose to Aronson's medium, Teague and Travis were able to improve it considerably.

Cholera strains give large red colonies in from 15 to 20 hours, whereas, the colon colonies are smaller and colorless.

LXIII. SCHUFFNER'S MODIFICATION OF VERWOORT'S MEDIUM: (J.A.Vet.Med. Assoc., 1939, p. 95)(For the cultivation of Leptospira).

To 1.5 liters of distilled water add 1.5 grams of Bacto-peptone. Boil.

Add 6 cc. of a phosphate solution prepared by dissolving 0.35 Gm. monobasic potassium phosphate (KH_2PO_4) and 1.33 Gm. dibasic sodium phosphate (Na_2HPO_4) in 100 cc. of distilled water. Boil. Add 300 cc. of Ringer's solution (0.8% NaCl, 0.02% $CaCl_2$, 0.02% KCl, 0.02% $NaHCO_3$). Continue boiling. Add 150 cc. of Sorenson's phosphate buffer of pH 7.4 (120.6 cc. M/15 Na_2HPO_4 plus 29.4 cc. M/15 KH_2PO_4). Boil until precipitation is complete (about 30 minutes). Cool in refrigerator overnight. Filter. Check the pH which should be 6.8-7.2. Bottle and autoclave at 15 pounds for 15 minutes. Before use, add 8-10% sterile rabbit serum; tube in 2.5 to 3 cc. amounts and inactivate for 30 minutes at 56 degrees C. Incubate to determine sterility. The serum should be obtained from a rabbit deprived of food for 24 hours.

LXIV. MUELLER'S STARCH AGAR MEDIUM: (Cultivation of the meningococcus).

To make 1000 -cc.

Add 17 grams dry shredded agar to 500 cc. of tap water in a two liter flask. Autoclave at 15 pounds for 15 minutes to dissolve. While still hot, add the following solution which may be prepared while the agar is being autoclaved:

Beef heart or meat infusion*	300 cc.
Casein hydrolysate**	17.5 Gm.
Starch paste***	100 cc.
Para-amino-benzoic acid, 1.0%****	5 cc.
Water	75 cc.

Adjust pH to 7.6

Mix and distribute at once either into test tubes, (about 20 cc. each for pours, 5 cc. for slants) or flasks of 120 to 200 cc. Autoclave not more than 10 minutes at 10 pounds. Over autoclaving spoils the medium. The flasks can be used to pour plates at once. The tubes may be melted in boiling water and used as needed.

* MEAT INFUSION: 1 pound meat, (chopped lean beef or beef heart) 500 cc. water. Suspend meat in water, bring to active boiling, strain through cheesecloth and filter through paper. Autoclave in 200 cc. quantities, 10 minutes at 10 pounds, and store in ice box in stoppered bottles with a few cc. of chloroform.

** CASEIN HYDROLYSATE: Difco product supplied under trade name "Casamino Acids, Lot #S-64123." This consists of a complete hydrochloric acid hydrolysate of casein from which the greater part of the acid has been removed by vacuum distillation, and the remainder neutralized with sodium hydroxide. The resulting solution has been decolorized with charcoal and dried. The material contains considerable salt. The quantity to be used must be determined, at present, for each lot, and will be specified on the label.

*** STARCH PASTE: Suspend 1.5 grams ordinary starch (corn starch or laundry starch, not "soluble starch") in 10 cc. of cold water. Pour slowly into 90 cc. of boiling water while stirring and bring to active boil.

**** PARA-AMINO-BENZOIC ACID: Suspend 1 gram in 75 cc. of water. Add strong sodium hydroxide drop by drop, with shaking, until dissolved, (about 0.3 grams NaOH required). Dilute to 100 cc. The solution keeps well.

LXV. PHYSIOLOGICAL SALINE:

NaCl	8.5 Gm.
Distilled water	1000 cc.

LXVI. STERILE BUFFERED GLYCEROL:

(1) Citric acid--21 Gm. to 1,000 cc. double distilled water.

(2) Anhydrous Na_2HPO_4--28.4 Gm. to 1,000 cc. double distilled water.

(3) Take 9.15 cc. of (1) and 90.85 cc. of (2) to make 100 cc. of buffer solution of pH 7.4.

(4) Mix equal parts of (3) and neutral C.P. glycerine; fill cork-stoppered specimen bottles half full and sterilize at 15 lbs. of steam pressure for 30 minutes. (Equal parts of Simm's solution (See below) and neutral C.P. glycerine may also be used).

LXVII. SIMM'S SOLUTION: (Modification of Tyrodes).

Solution A.

Sodium chloride	160.00 Gm.
Potassium chloride	4.00 Gm.
Calcium chloride	2.94 Gm.
Magnesium chloride	4.06 Gm.
Distilled water, qs to	1000.00 cc.

Autoclave at 15 lbs. for 15 minutes.

Solution B.

Sodium bicarbonate	20.20 Gm.
Disodium phosphate	4.26 Gm.
Dextrose	20.00 Gm.
Phenol red	1.00 Gm.
Distilled water, qs to	1000.00 cc.

Filter through a Berkefeld "N" filter.

Both A and B are stored in the refrigerator.

Add 50 cc. of solution A to 900 cc. of double distilled water and autoclave the whole. After it has cooled add 50 cc. of solution B without further autoclaving. Keep this solution in the refrigerator. Use sterile precautions throughout.

LXVIII. BLOOD CLOT PEPSIN DIGEST: (From Zinsser and Bayne-Jones, 8th Ed., 1939, p. 878).

A simple and very cheap way of obtaining an excellent base for broth and agar is one described by Ten Broeck as used in China. It has the advantage of cheapness. Obtain blood clot at slaughterhouse, mixing 2000 cc. of the total clotted shed blood with six fresh pig stomachs. Grind them together and to three parts of this add two parts of water. Add concentrated hydrochloric acid to about 4 per cent, bringing the pH to between 2 and 3. Incubate from thirty-six to thirty-eight hours. Strain through gauze. To each part of this digest add 3 to 4 parts of water, heat to 85° - 100° C.; add concentrated sodium hydroxide up to pH 6. Filter, add 0.5 per cent dextrose and adjust reaction. Agar can be added to this to the desired percentage.

LXIX. VEGETABLE BACTERIOLOGICAL MEDIA: (Brewer, J.H., J. Bact, 1943, 46, 395-396).

Cotton-seed meal, peanut meal, soy-bean meal, various whole and sprouted grains, beans and seeds are digested with papain as follows:

Materials: 650 gms. vegetable meal
30 gms. papain
5 gms. Na_2S
4,000 ml. water

Dissolve the papain in about 500 ml. of water. Dissolve the sodium sulfide in 100 ml. of water, add it to the papain solution and allow to set for fifteen minutes. Then add the papain solution to the mixture of vegetable meal and water. The mixture is adjusted to pH 5 with hydrochloric acid and incubated overnight at 37° C. Clarify by filtration. The filtrate is adjusted to pH 7.6 and heated to boiling and filtered. The medium may then be distributed into flasks or bottles, autoclaved, stored and diluted as used. The yield of concentrated medium is about 3,500 ml. and contains 7-10% of total solids. For use, the concentrated medium may be diluted with water to about 14,000 ml. and 0.5% of NaCl added, resulting in a finished broth containing 2-3% solids.

The cost is only a fraction of that of meat-infusion broth and no added peptone is required.

An especially satisfactory single vegetable source is soy-bean meal. The growth of fastidious organisms such as streptococci, pneumococci and gonococci is supported. This is not found to be true for acid digests, peptic or pancreatic digests of the same vegetable material.

IX. STAINS AND MICROSCOPIC PREPARATIONS.

For the examination of stained bacteria it is advisable to use clean new slides. New slides may be adequately cleaned by immersing them in 95% alcohol and rubbing them dry. The material to be examined is spread in a thin film on a clean slide. To make smears of cultures from a solid medium, a small drop of water or saline is placed on the slide and with the needle a minute amount of the growth is added to give a very faint turbidity. The drop is then spread and allowed to dry in the air. If a fluid culture is to be examined, a drop of the culture is smeared on the slide without adding water or saline and allowed to dry in the air.

When the smear is dry, it is fixed to the slide by passing it through the flame three or four times without over-heating and the slide is allowed to cool before staining. Smears may also be fixed by flooding them with absolute ethyl alcohol, with methyl alcohol or glacial acetic acid. After fixing with these reagents the latter may be rinsed off and the smear allowed to dry before staining. As a rule, however, fixation by heat is adequate.

For the examination of living bacteria, especially for the determination of motility, the hanging-drop method may be employed. A small loopful of the fluid culture or of a saline suspension of the organism is placed in the center of a clean thin cover-slip. Small drops of oil are placed on opposite sides of the concavity of the hollow ground slide and the cover-slip is inverted over the concavity of the slide so that the drop of fluid culture or bacterial suspension hangs from the coverslip without touching the slide. The light is reduced with the diaphragm and the edges of the hanging drop found with the low power objective. The high power objective is turned into place, the light again adjusted, and after finding the edge of the drop with this objective, the slide is moved around and the bacteria observed for motility. The oil-immersion objective may be used also after placing a drop of cedar oil on the cover-slip. Hanging drop preparations must be sterilized before cleaning and care must be taken not to break the coverslip because of the danger of smearing the preparation over lens and stage. Young broth or peptone water cultures should be used. If a hollow ground glass slide is not available, the coverslip may be ringed with vaseline and inverted over an ordinary slide.

The following stains are most commonly used in the bacteriological laboratory:

LOEFFLER'S ALKALINE METHYLENE BLUE

REAGENTS

Methylene blue (90% dye content, certified*).....	0.3 Gm.
Ethyl alcohol (95%).....	30.0 cc.
KOH (0.01% aqueous solution).....	100.0 cc.

Dissolve the methylene blue in the alcohol.

Mix the methylene blue solution with the KOH and filter.

* Found to be satisfactory by the Commission of Standardization of Biological stains of the Society of American Bacteriologists.

Flood the fixed smear with the stain and allow it to act for 1 minute. Wash with water and blot dry with blotting or filter paper. (Use the blotting paper once only). No coverslips need be used for examination under the oil-immersion objective when stained bacteria are examined.

GRAM STAIN
Kopeloff and Beerman modification (Modified).
(J. Infect. Dis., 1922, 31, 480)

REAGENTS

Solution A

Gentian or crystal violet..... 1 Gm.
Distilled water..... 100 cc.

"

Solution B

Sodium bicarbonate..... 1 Gm.
Distilled water..... 20 cc.

Just before use, mix 30 drops of sol. A with 8 drops of sol. B.

"

Iodine solution

Iodine..... 2 Gm.
Normal NaOH..... 10 cc.

After the iodine is dissolved make up to 100 cc. with water.

"

Counterstain

Basic fuchsin..... 0.1 Gm.
Distilled water..... 100 cc.

TECHNIQUE OF STAINING. Stain for 2-5 minutes with the mixture of A and B (alkaline crystal violet solution). Rinse with water. Do not blot. Add the iodine solution and allow to stand 2-5 minutes. Rinse with water. Decolorize with 50% acetone in ethyl alcohol, adding drop by drop to the slide while tilted until the drippings are almost colorless (usually less than 10 seconds). Dry in air and counterstain for 10 to 30 seconds with the basic fuchsin. Wash in water, dry by blotting and examine with the oil immersion lens.

NEISSER'S STAIN (For C. diphtheriae).

REAGENTS

Solution #1

Dissolve 1.0 Gm. of methylene blue in 20 cc. of 95% alcohol. Add 950 cc. of distilled water and 50 cc. of glacial acetic acid. Filter.

"

Solution #2

Dissolve 1 Gm. of Bismarck brown in 500 cc. of boiling distilled water. Filter.

TECHNIQUE OF STAINING. Stain the fixed smear with solution #1 for 1-2 minutes. Rinse with water. Stain for 30 seconds with solution #2. Rinse with water, blot and dry.

BECK'S STAIN (For C. diphtheriae).

REAGENTS

Solution #1

Gentian violet (Saturated alcoholic solution)..... 10 cc.
Acetic acid (4 and 1/3 per cent)..... 90 cc.

"

Solution #2

Bismarck brown..... 0.48 Gm.
Distilled water..... 125 cc.

Heat the water to boiling, add the Bismarck brown, boil for 2 minutes, filter and cool.

TECHNIQUE OF STAINING. Apply solution #1 for 1-2 minutes. Wash with water and apply solution #2 for 30 seconds. Wash in water and dry.

Most bacteria are stained brown by this method. The Corynebacteria show deep purple polar bodies in a brown rod. Some streptococci may retain the purple stain.

ZIEHL-NEELSEN'S STAIN (For acid-fast bacilli).

REAGENTS (a) Carbol fuchsin

Mix 1 Gm. of basic fuchsin in 10 cc. of absolute alcohol or 10 cc. of a saturated solution of basic fuchsin in 95% alcohol with 90 cc. of a 5% aqueous solution of carbolic acid.

" (b) 3% acid alcohol

Mix 3 cc. of concentrated HCl with 97 cc. of 95% alcohol.

" (c) Loeffler's alkaline methylene blue (See above).

TECHNIQUE OF STAINING. Cover the fixed smear with carbol fuchsin and steam gently over a flame for 5 minutes. Renew the stain repeatedly to prevent drying on the slide. Wash with water. Decolorize with acid alcohol until thin areas are colorless. Wash with water. Counterstain with Loeffler's alkaline methylene blue, 30 to 60 seconds. Wash with water and blot dry. Acid fast organisms appear as red rods against a blue background.

NOTE: The carbol fuchsin may be allowed to act, without steaming, overnight. The slide is placed in a Koplin jar containing the carbol fuchsin and allowed to stand at room temperature overnight or longer. It is washed, decolorized and treated with methylene blue as above.

CAPSULE STAIN (Hiss)(J. Exp. Med., 1905, 6, 317).

Mix the organism or specimen with a drop of serum on a slide. Spread, allow to dry in the air and fix gently with heat.

Stain with 1 per cent aqueous solution of gentian violet, steaming gently over the flame for a few seconds. (A mixture of 5 cc. of a saturated alcoholic solution of gentian violet or fuchsin and 95 cc. of distilled water may be used).

Wash off the dye with a 20 per cent aqueous solution of copper sulfate.

Blot (but do not wash with water), dry and examine.

The capsule appears as a faint blue halo around a dark purple cell body.

DORNER'S NIGROSIN SOLUTION
(For the negative demonstration of bacteria)

Ten grams of nigrosin (certified) are boiled in 100 cc. of distilled water for 30 minutes in an Erlenmeyer flask. One-half cc. of formalin is added as a preservative and the solution filtered twice through double filter paper and stored in serological test tubes, about 5 cc. to the tube.

A loopful of the bacterial suspension is mixed on the slide with the same quantity of the nigrosin solution, and spread. After drying, the slide can be examined without a cover glass.

SPORE STAIN (Dorner's method).

Solutions:

A. Carbol fuchsin--(freshly filtered).
B. Saturated aqueous solution of nigrosin.

TECHNIQUE OF STAINING. Make a heavy suspension of the organism in 2 to 3 drops of distilled water in a small test tube. Use the growth of the culture on an agar slant for this emulsion. Add an equal quantity of freshly filtered carbol-fuchsin. Allow the mixture to stand in a boiling water bath 10 to 12 minutes. On a slide, mix one loopful of the stained preparation with one loopful of a saturated aqueous solution of nigrosin. Smear as thinly as possible and dry rapidly.

The spores are stained red, the bodies of the bacteria are almost colorless and stand out against the dark gray background of nigrosin.

WRIGHT'S STAIN (Blood stain)

REAGENTS Methylene blue hydrochloride (90% dye content).. 0.9 Gm.
Sodium carbonate, 0.5% aqueous solution.....100 cc.

Heat in a steam sterilizer at 100 degrees C. for one hour, in containers in which the solution is not over 6 cm. deep. Cool and filter. To the filtrate add:

Eosin Y (dye content about 85%)..... 1.0 Gm.
Distilled water..... 500.0 cc.

Mix thoroughly and filter. Save precipitate, and dissolve for use as follows:

Wright's stain (dry)..... 0.1 Gm.
Methyl alcohol, absolute, neutral, acetone free.... 60.0 cc.

Allow stain to stand a day or two; then filter. Always filter before using.

TECHNIQUE OF STAINING. Cover the dried preparation for 1 minute with the alcoholic solution of the stain. Dilute by dropping upon the stain an equal quantity of distilled water. A metallic film forms on the surface. Leave the diluted stain on for 3 to 15 minutes. Wash in distilled water by flooding the slide, taking care to float off the metallic film to prevent adherence of a precipitate to the slide.

Wright's stain may be purchased in powder form or in solution ready for use. The commercial preparations are often better than those made up in a bacteriological laboratory.

DIFFERENTIAL STAINING OF GRAM POSITIVE AND GRAM NEGATIVE BACTERIA IN TISSUE SECTIONS

(Brown and Brenn. J. Bact., 1931, 21, 21)

Paraffin sections are prepared as usual for staining.

1. Stain in freshly filtered alum-hematoxylin (Harris) for 2 to 5 minutes.

2. Wash in acid alcohol (3% HCl in 95% alcohol) until light pink.

3. Wash in ammonia water (1 cc. of aqua ammoniae in 100 cc. water) until blue.

4. Wash in water.

5. In a small vial mix 5 drops of 5% aqueous solution of sodium bicarbonate (containing also 0.5% phenol as a preservative) with about 0.75 cc. of 1% aqueous solution of gentian violet. Immediately pour the mixture onto the slide and stain for 2 minutes.

6. Wash quickly with water.

7. Cover with iodine solution (iodine, 1 Gm.; potassium iodide, 2 Gm.; water, 200 cc.) for 1 minute.

8. Wash with water. Blot.

9. Decolorize in 1 part of ether plus 3 parts of acetone, dropping it onto the slide until no more color comes off.

10. Blot.

11. Stain for 5 minutes with rosanilin hydrochloride (0.005 Gm. per 100 cc. water).

12. Wash in water. Blot but do not allow the sections to dry.

13. Pass through acetone.

14. Decolorize and differentiate by dropping over the section a solution of 0.1 Gm. of picric acid in 100 cc. of acetone until the section becomes a yellowish pink. This is the most critical stage of the process and should be carried out by holding the slide over a white plate or dish. Most of the rosanilin should be decolorized from the tissue but the gram-negative bacteria should remain red.

15. Pass successively through acetone, equal parts of acetone and xylol, and xylol.

16. After clearing in xylol mount in balsam.

(Beginning with step 5 it is best to work with only one slide at a time).

Cell nuclei should be stained dark reddish-brown; cytoplasm, yellowish; gram-positive bacteria, deep violet or almost black; gram-negative bacteria, bright red. Leukocytes generally stand out plainly with a dusky yellowish cytoplasm. Basophilic granules stain red. Red blood cells may be yellow or red, depending upon the degree of decolorization in picric acid. Cartilage stains pink, striated muscle and fibrin generally stain yellow but may retain more or less of the red stain.

TRACHOMA INCLUSION BODIES
(Rices Method of Demonstration)
(Gradwohl-Clin. Lab. Methods and Diagnosis, 2nd. Ed., 1938, p. 931)

Smears from trachomatous lids are fixed in 95% alcohol. Place several drops of Lugol's solution on slide and drop a thin cover-slip over the solution. It can be found under low power and observed under high power. When the preparation begins to dry, the cover-slip can be flooded off with water, the slide washed slightly in tap water and the smear can again be flooded with Lugol's solution and a cover-slip applied. This can be repeated an indefinite number of times.

The inclusions show up as amber colored bodies, while the cell itself is slightly yellowish. The nucleus can be made out. The amber colored bodies are the so-called von Prowazek-Halberstaedter inclusions found in the epithelial cells in trachoma.

LUGOL'S SOLUTION (Used in Rice's staining method above).

Iodine	1 Gm.
Potassium iodide	2 Gm.
Water	300 cc.

LUDFORD AND LEDINGHAM'S MODIFICATION OF SCHRIDDE'S METHOD
(For the demonstration of cell inclusions)

Fix small pieces of tissue for two days, in 1 part of formalin to 10 parts of

Muller's fluid ($K_2Cr_2O_7$--2 parts; Na_2SO_4--1 part; and H_2O--100 parts). Transfer to Muller's fluid for three days, then to 2% OsO_4 for three days. Pass through several changes of water over a period of 6 to 8 hours. Stain the sections with either iron-hematoxylin or Altmann's aniline-acid-fuchsin. If the inclusions are coated with fats or lipoid, as are the Bollinger bodies of fowl pox, they may be bleached by placing the slides for one-half hour or more in a mixture consisting of 1 part hydrogen peroxide and 4 parts 80% alcohol. When the blackening due to osmic acid is removed, stain with iron-hematoxylin, differentiate with acid-alcohol and counterstain with Altmann's aniline-acid-fuchsin.

REGAUD'S METHOD (For the demonstration of cell inclusions)

Fix small pieces of tissue for four days in a mixture consisting of 3% solution of $K_2Cr_2O_7$--80 cc. and commercial formalin--20 cc.; use fresh fluid each day. Transfer to 3% $K_2Cr_2O_7$ for eight days. Pass through several changes of tap water over a period of 6 to 8 hours, stain with iron-hematoxylin, and counterstain with Altmann's aniline-acid-fuchsin.

BEAUVERIE STAIN (For ascospores)

REAGENTS — Carbol fuchsin

Basic fuchsin	10 Gm.
Ethyl alcohol 95 per cent	100 cc.
5 per cent aqueous phenol	900 cc.

Add the dye, a little at a time, to the alcohol. Let stand for 24 to 48 hours. Shake frequently. Add the phenol to the dissolved dye very slowly, shaking thoroughly after each addition. Filter through paper.

TECHNIQUE OF STAINING. Fix smear with heat. Flood with carbol fuchsin and steam for 2 minutes. Decolorize well with glacial acetic acid. Wash in water. Counterstain with 1% aqueous thionin.

Vegetative parts of cells will be blue; ascospores will be red.

SPIROCHETE STAINING (Fontana-Tribondeau method)
(Depends upon the deposition of a silver salt in the organism and the reduction of the compound with formalin).

REAGENTS — Solution A: Formalin.

Acetic acid (glacial)	1 cc.
40 per cent formalin	10 cc.
Distilled water	100 cc.

" — Solution B: Tannic acid.

Tannic acid	5 Gm.
1 per cent phenol	100 cc.

REAGENTS (continued)

Solution C: Silver nitrate.

5 per cent silver nitrate solution..... 50 cc.

Reserve a few cc. of the silver nitrate solution. To the remainder add, drop by drop, concentrated ammonia solution until the sepia precipitate which forms redissolves. Shake and stir constantly during the addition of the ammonia. Then add, drop by drop, some of the reserved silver nitrate solution until there occurs a slight clouding, which persists on shaking. This solution will remain useful for several months. Occasionally pour it into a clean receptacle, and if it has become clear, add a few more drops of 5% silver nitrate.

TECHNIQUE OF STAINING. On a clean slide, make a film (thin) of fluid from a chancre or other material containing spirochetes, and let this dry in the air. Cover the film with solution A for 1 minute. Wash thoroughly with distilled water. Cover with solution B and heat until the fluid steams. Wash with distilled water. Cover with solution C and heat until fluid steams. Let this act for 30 seconds. Wash with water. Dry in air or blot. (The stained film should be of a dark maroon color. The spirochetes are stained dark brown or black.)

MACCHIAVELLO'S STAIN (For rickettsiae in tissue culture).

REAGENTS

Basic fuchsin..... 1% in distilled water
Citric acid..... 0.5% in distilled water
Methylene blue..... 1% in distilled water

TECHNIQUE OF STAINING. Apply the basic fuchsin for 4 minutes. Wash in tap water. Run citric acid on and off rapidly. Apply methylene blue for 20 seconds. Wash in water and blot.

The cells are stained blue and the rickettsiae red.

CASTANEDA STAIN (For rickettsiae in tissue culture).

REAGENTS

STAIN

(a) Buffer solution

KH_2PO_4..... 1 Gm. in 100 cc. distilled water.
$Na_2H\ PO_4.\ 12H_2O$..... 25 Gm. in 100 cc. distilled water.
(mixing these gives a pH of 7.5)
Add 1 cc. of formalin as a preservative.

(b) Dye solution

Methyl alcohol..... 100 cc.
Methyl blue..... 1 Gm.

To 20 cc. of (a), add 1 cc. of formalin and 0.15 cc. of (b).

COUNTERSTAIN

Safranine "O" (National Aniline & Chemical Co.) 0.2% 1 part.
Acetic acid..... 0.1% 3 parts.

Use clean slides and make very thin smears.

TECHNIQUE OF STAINING. The smear is stained for 3 minutes. The stain is then poured off, without washing, and the preparation is counterstained with safranine, which is allowed to remain on the slide from one to four seconds (never more than five seconds) in order to differentiate the preparation. The smear is then washed with running water and dried on filter paper.

STAIN FOR DEMONSTRATION OF NEGRI BODIES.

See section on Procedures - the diagnosis of rabies infection in the animal brain.

TABLE 42.

Solubilities at 26 degrees C. of the dyes commonly used in bacteriology.

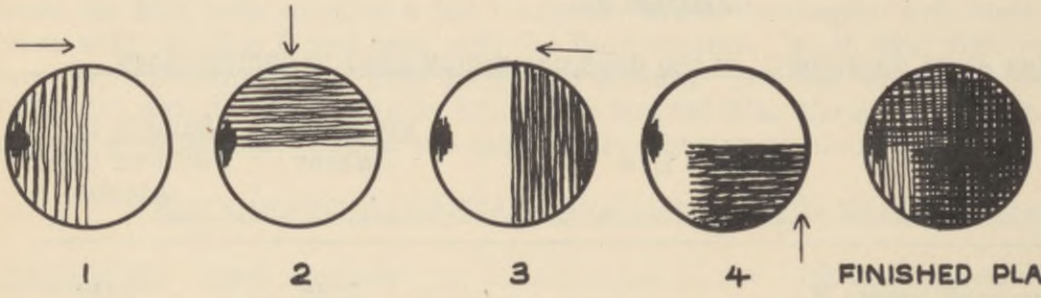
Color Index Number	Name of Dye	Per Cent* Water	Soluble in: 95 Per Cent Alcohol
381	Bismarck brown Y	1.36	1.08
382	Bismarck brown R	1.10	0.98
20	Chrysoidin Y	0.86	2.21
21	Chrysoidin R	0.23	0.99
370	Congo red	.....	0.19
681	Crystal violet (chloride)	1.68	13.87
768	Eosin Y (Na salt)	44.20	2.18
678	Fuchsin, basic, new	1.13	3.20
	Fuchsin, acid	.....	.....
	Gentian violet, see methyl and crystal violet	.....	.....
133	Janus green	5.18	1.12
657	Malachite green (oxalate)	7.60	7.52
680	Methyl violet	2.93	15.21
922	Methylene blue (chloride)	3.55	1.48
825	Neutral red (chloride)	5.64	2.45
7	Picric acid	1.18	8.96
739	Pyronin G	8.96	0.60
676	Rosanilin	0.39	8.16
	Pararosanilin	0.26	5.93
779	Rose bengal (Na salt)	36.25	7.53
841	Safranin	5.45	3.41
248	Sudan III	0	0.15
920	Thionin	0.25	0.25
925	Toluidin blue O	3.82	0.57

* These figures are for grams per hundred cubic centimeters.

X. TECHNIQUES AND SPECIAL PROCEDURES.

TECHNIQUE OF STREAKING A PLATE: In order to obtain isolated colonies on a plate the procedure of spreading the material on the agar must be such as to adequately dilute specimens containing either a large or small number of organisms. The following procedure usually results in the procurement of isolated colonies.

The inoculum is placed on one side of the agar near the edge and lightly smeared over one half of the agar with the end of a flexible platinum needle. As many streaks as possible are made without flaming the needle or retracing the streaks. The entire surface of the agar is streaked working from the edge to the middle of the agar in four steps as indicated in the following diagrams:



In the fourth step the streaks are not made completely across the plate and are more widely spaced.

CANDLE JAR TECHNIQUE: Incubation in an atmosphere containing carbon dioxide, moisture and a lowered oxygen tension provides optimum conditions for the growth of most pathogenic bacteria on blood agar. These conditions may be easily obtained by means of the "candle-jar" procedure. Any container capable of admitting Petri dishes and of being closed "air-tight" may be used. A candle is placed on the Petri dishes, lighted, and the jar tightly closed. The flame burns until the oxygen tension is reduced and an adequate amount of carbon dioxide produced. Museum jars fitted with covers and rubber gaskets may be made "air-tight" with vaseline, one of the stop-cock greases, or Cello-Seal (Fisher Scientific Co.) A one gallon wide-mouthed glass jar fitted with a metal screw top, manufactured by the Illinois Owens Glass Co., of Toledo, Ohio, may be conveniently used. By screwing a suitable stop-cock into the cover, this jar may also be used for anaerobic culture by the Rosenthal technique. (See below).

ANAEROBIC INCUBATION:

1. A simple practical method for the procurement of anaerobic conditions is Mueller's modification of Rosenthal's anaerobic method. (J. Bact., 1941, 41, 301-303).

The reaction upon which this method is based is as follows:

1. $Cr + H_2SO_4 = CrSO_4 + H_2$
2. $4CrSO_4 + 2H_2SO_4 + O_2 = 2Cr_2(SO_4)3 + 2H_2O$

The same reaction serves both to displace air by generating hydrogen and to absorb residual oxygen by the chromous compound.

a. Material needed:

Any sealable container, which is acid proof and may be provided with a gas outlet, will do - glass jar or desiccator - Mason fruit jar with threaded cap and gas outlet. A sealing mixture of petrolatum with 10% beeswax, Celloseal, etc. Powdered chromium. Sulphuric acid. Indicator tube (5 cc. of sterile glucose broth containing 0.1 cc. of Loeffler's alkaline methylene blue). Tubes and plates can be supported on an acid-proof stand.

b. Method:

1.5 grams of chromium powder, 0.5 gram Na_2CO_3 and 15 cc. of 15% H_2SO_4 (by volume) are used per liter of jar capacity. Place the chromium and sodium carbonate in the jar and add the sulfuric acid with a pipette. Put the lid on and leave the gas outlet open until the initial vigorous evolution of hydrogen has subsided and only slight effervescence continues. Close the gas outlet and incubate.

On opening the jar, flame should be avoided since the jar is saturated with hydrogen.

Since there is no vacuum at any stage of the process, Petri plates may be inverted in the jar.

The indicator tube should remain decolorized throughout the period of incubation.

c. Appraisal:

This is a simple efficient method of anaerobic culture suitable for the small laboratory.

2. Gas displacement:

Where facilities permit, a method based upon the displacement of air by hydrogen in which the air is first removed by means of a vacuum pump may be conveniently employed. (Diagram - next page).

a. Material needed: (See diagram - next page).

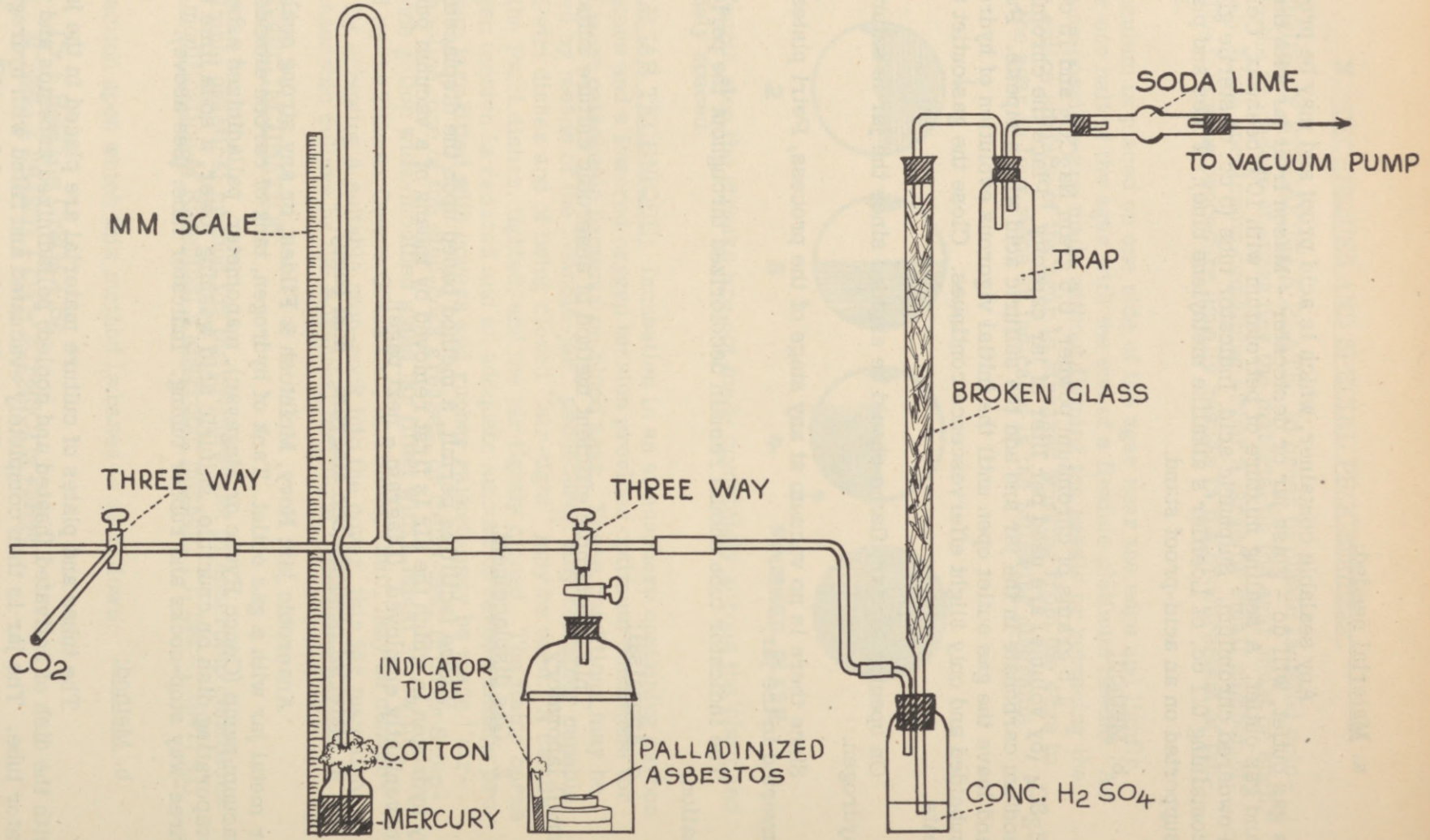
Anaerobic jar: Novy, McIntosh & Fildes, or any strong sealable glass or metal jar with a gas outlet. Tank of hydrogen, tank of carbon dioxide, a good vacuum pump (Cenco Hyvac or Megavac), manometer, palladinized asbestos in an evaporating dish or crucible, sulfuric acid washing tower, a soda lime tube, two three-way stop-cocks and rubber tubing. Indicator tube. (See above).

b. Method:

The tubes and plates of culture material are placed in the jar along with the dish of activated (heated and cooled) palladinized asbestos and the indicator tube. The jar is then completely evacuated and filled with hydrogen. It is again evacuated and incompletely filled with hydrogen and 5 to 10% carbon dioxide, using the manometer as a gauge. A slight vacuum of about 30 or 40 mm.

GAS DISPLACEMENT PROCEDURE FOR ANAEROBIC CULTURE

METHOD II



of mercury is allowed to persist in order that the expansion of the gas, on placing the jar in the incubator may not force the lid off. Petri dishes containing soft agar may not be inverted in this procedure and should be provided with porcelain tops of which only the exteriors are glazed.

The above method is one of several modifications of the gas displacement method of McIntosh and Fildes (Lancet, 1916, 190, 768). Smillie (J. Exper. Med., 1917, 26, 59) is believed to have introduced the method of heating the catalyst by means of an electric current.

c. Appraisal:

The gas displacement method is a reliable and convenient method for routine anaerobic culture work. Where much anaerobic work is done it is the method of choice. Because of the amount and expense of the equipment needed, it is not a practical method for the smaller laboratory in which a less elaborate outfit may be more conveniently employed.

NOTE: Mixtures of hydrogen and oxygen are extremely explosive when ignited, and the use of anaerobic jars involving such mixtures should be attended with care to avoid danger of explosion. In the methods involving enclosure of the catalyzer within a wire screen, no danger of explosion is incurred so long as combustion is confined within the wire screen.

3. Oxygen absorption by the use of pyrogallic acid dissolved in alkaline solution.

For the cultivation of anaerobic bacteria on agar slants a simple procedure is the following:

a. Materials needed:

Dry pyrogallic acid. Five per cent sodium hydroxide. Liquid paraffin, albolene or petrolatum.

b. Methods:

#1. The inoculated slant, with the stopper removed, is inverted into a tumbler or beaker containing about a gram of dry pyrogallic acid. A small quantity of 5% solution of sodium hydroxide is then run into the tumbler and this is covered with a thin layer of liquid paraffin,albolene or petrolatum before the pyrogallic acid has been completely dissolved.

#2. The nonabsorbent cotton plug is cut short and pushed down into the tube of inoculated solid medium. (Take care not to touch the medium). Dry pyrogallic acid is put on top of the cotton, covered with a few cc. of 10% sodium hydroxide and the tube is fitted with a tight rubber stopper. The tube must be incubated in an inverted position to prevent the chemicals from reaching the medium.

#3. Buchner's original method consists of placing the culture tube in a sealed larger tube or jar containing the alkalinized pyrogallol. The dry pyrogallic acid is placed in the larger tube, a layer of absorbent cotton is lightly packed over the pyrogallic acid. The sodium hydroxide solution is added, the culture tube

placed in the larger tube, and the large tube tightly closed with a rubber stopper. In this way, the immediate solution of the pyrogallic acid is prevented and one is allowed time to add the sodium hydroxide solution, insert the smaller tube inside the Buchner tube and tightly insert the rubber stopper in place before the solution and oxygen absorption has taken place.

c. Appraisal:

This method is extremely simple and requires very little equipment. By using slants having a large surface area and diluting the inoculum by streaking several slants in series it is possible to obtain isolated colonies.

4. Brewer's sodium thioglycollate broth and other fluid media for anaerobic cultures. (See section on media for formula).

a. Materials needed:

Brewer's or other suitable fluid media.

b. Method:

Brewer's medium is used as a fluid medium for culturing anaerobes and contains methylene blue which serves as an indicator of the suitability of the medium for anaerobic culture work. In the reduced state methylene blue loses its color.

c. Appraisal:

While this medium may be useful in detecting the presence of anaerobes, being a fluid medium it is not practical for the isolation of anaerobes from a mixed flora. If Brewer's medium is not available, another good medium (e.g. Basic Broth) may be enriched with sodium thioglycollate or thioglycollic acid (correcting any effect on the pH of the medium by the addition of acid or alkali), about 0.1% agar, and a little glucose (0.3 to 1.0%) and used in the same way. If sodium thioglycollate or thioglycollic acid are not available, cysteine or cysteine hydrochloride may be added in 0.1 or 0.2% concentration instead. The basic broth itself without enrichment may be used, if before inoculation it is heated in a boiling water bath for fifteen minutes to remove the oxygen, and then rapidly cooled. Immediately after inoculating the medium, without too much agitation, sterile petrolatum is used to cover the surface.

Another simple and fairly reliable method for growing anaerobes in a fluid medium is to use cooked meat medium (XVI in section on media). If not freshly prepared, this medium should be heated and cooled before use in order to drive out the dissolved oxygen. This medium, although very efficient with regard to the support of growth of anaerobic bacteria has the drawback of not being clear.

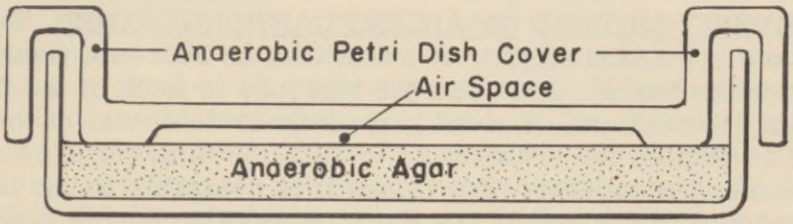
5. Semi-solid agar. (See section on media).

This agar, if heated and cooled before inoculation, may also be used for the cultivation of anaerobes especially when sufficient (0.3 to 1.0%) glucose is present. This medium is helpful in the cultivation of both anaerobic and microaerophilic organisms. If the inoculum is sufficiently dilute, isolated colonies may be obtained.

6. Brewer Petri Dish Cover with anaerobic agar. (Brewer, J.H., Science, 1942, 95, 587).

a. Materials needed:

The Brewer Petri Dish Cover and a special anaerobic agar. The cover is so designed that it touches the agar at the periphery and traps a small amount of air, less than 1 mm. in thickness, over the surface of the agar. A reducing agent in the medium uses up the oxygen in this small amount of air and an anaerobic condition develops. Methylene blue in the agar acts as an indicator and the center of the dish which is anaerobic becomes colorless, while there is a blue oxygenated ring at the periphery.



CROSS SECTION SHOWING ANAEROBIC PETRI DISH COVER IN USE

The Baltimore Biological Laboratory supplies a suitable anaerobic agar of the following composition:

Polypeptone BBL	2%
Sodium chloride	0.5%
Dextrose	1%
Agar	2%
Sodium thioglycollate	0.2%
Sodium formaldehyde sulfoxylate	0.1%
Methylene blue	0.0002%

The medium is prepared by suspending 58.0 grams of the powder in 1000 cc. of distilled water. After 5 to 10 minutes of soaking, the suspension is heated gently to boiling to dissolve the powder. It is dispensed in tubes and autoclaved for 20 minutes at 15 pounds steam pressure.

b. Method:

For use, the sterile anaerobic agar is melted, cooled to 50° C. and poured into a Petri dish and allowed to harden. For best results a porous cover may be used instead of the glass lid to obtain a dry surface. The medium may be

inoculated before pouring or by streaking the central portion of the poured plate. After the agar has solidified the Petri dish cover is replaced by the Brewer Petri Dish Cover the glass rim of which forms a seal with the moist agar.

The agar should be distributed in about 40 cc. amounts if 15 mm. Petri dishes are used and in 25 cc. amounts if 10 mm. dishes are used. It is essential that the depth of the agar in the dish be sufficient for the rim of the anaerobic cover to rest on the surface of the agar and not on the Petri dish at any point.

c. Appraisal:

The method offers a simple, convenient and clean method for growing anaerobes on a solid medium without the use of anaerobic jars. It permits the daily examination of plates without destroying anaerobiosis and does not require the use of much incubator space.

SHAUGHNESSY METHOD OF ADDING CARBON DIOXIDE: The upper fluffy portion of the sterile cotton stopper of a test tube or Florence flask is cut off and the remainder pushed as far down into the test tube or neck of the flask as possible without touching the medium. A short glass tube or shell vial of the proper size (about 10 x 35 to 40 mm. for ordinary 16 to 18 x 150 mm. culture tube) is placed open end upward on the stopper. For flasks it is desirable to use larger vials, their size depending on the size of the flask. A gelatin capsule containing a measured amount of bicarbonate solution is placed in the inner tube or vial. A number O capsule has been found satisfactory for the test tube cultures, but larger or smaller capsules may, of course, be used depending upon the amount of bicarbonate solution required for a particular purpose. A sufficient amount of sulfuric acid to cover the capsule, about 1 cc. for the test tube culture, is then placed in the vial and the culture tube sealed with a closely fitting rubber stopper. After about five minutes at room temperature the gelatin capsule begins to disintegrate and carbon dioxide begins to evolve gently.

Potassium bicarbonate is preferable to the sodium salt because of its greater solubility. At 20 degrees C. assuming normal pressure, approximately 7.2 cc. of carbon dioxide are liberated from 0.1 cc. of three-molar potassium bicarbonate solution. For liberating gas it is convenient to use an excess of acid so that it is unnecessary to measure it. Since it is desirable to use sufficient acid to cover the gelatin capsule, 1 cc. of sulfuric acid diluted 1:30 is used for a test tube culture. For a flask culture an appropriately larger amount of acid should be used.

REDUCED TENSION SLANT: The growth of the members of the Neisseria group is stimulated considerably by the addition of carbon dioxide to the atmosphere. A simple method for supplying carbon dioxide to a test tube culture is to set fire to the fluffy portion of the cotton stopper and while it is burning, push the stopper into the test tube without touching the medium and then quickly closing the tube with a rubber stopper.

CITRATE BOTTLES FOR BLOOD CULTURES: Add 0.5 cc. of 10% sodium citrate to a bottle and sterilize by dry heat or by autoclaving.

PREPARATION OF SAMPLE BOTTLES FOR CHLORINATED SWIMMING POOL WATERS: Sodium thiosulfate solution is prepared by dissolving 1.5 g. of sodium thiosulfate in 100 ml. of distilled water. One-half ml. of this solution is placed in each clean bottle. (This amount has been found sufficient to completely reduce residual chlorine in an amount up to 2 parts per million in a sample of 130 ml. of water). Sterilize for 15 minutes at 20 lbs.

Collect samples by plunging the open bottle beneath the surface, sweeping the bottle forward until filled. Do not rinse the bottle in the pool, otherwise the sodium thiosulfate will be removed.

Sample during periods of the pool's greatest use.

PREPARATION OF BACTERINS:

Whole culture bacterins: Inoculate several tubes of broth (Medium I) with the organism or organisms from which the bacterin is to be prepared. After 18 to 24 hours of incubation centrifuge the cultures and suspend the sediments in about 10 cc. of the supernatant broth to give a suspension four times heavier than that desired. If the finished bacterin is to contain 1 billion organisms per cc. the suspension at this point should, for example, contain 4 billion organisms per cc. If the finished bacterin is to contain 500 million organisms per cc. then the suspension should contain 2 billion organisms per cc. If the McFarland nephelometer scale is used for the adjustment of the suspension, the tube corresponding to a suspension of 2 billion organisms per cc. may conveniently be used as a standard. If a heavier suspension, like one containing 4 billion organisms per cc. is desired, one should dilute a small measured portion of the suspension with a measured amount of supernatant broth to the turbidity of the " 2 billion" standard and then dilute the rest of the suspension with the supernatant broth to give only one-half as much dilution.

The organisms may be killed by heat or by a chemical. In killing by heat, the suspension is usually exposed to a temperature of 60 degrees C. for one hour. This treatment will usually kill all vegetative forms (not spore containing) of pathogenic bacteria. Thymol may be conveniently used by adding 0.1 cc. of an alcoholic (95% ethyl alcohol) 5% solution of thymol for every 5 cc. of the final volume of the bacterin. Thus, if the suspension prepared above amounts to 10 cc., the final volume of the bacterin should be 40 cc. and 0.8 cc. of the thymol solution should be added to the suspension. The latter is allowed to stand in the refrigerator overnight and is then diluted with sterile 0.85% saline. In the example presented above, 29.2 cc. of saline will be required. A 1 to 10 dilution of the bacterin may be prepared using saline again to provide an additional but weaker suspension.

The bacterin should be cultured for sterility by inoculating portions into ordinary broth and into either Brewer's broth or a large broth tube containing 0.1% agar, 0.25% dextrose and an inverted vial for the support of anaerobes. Forty-eight hours of incubation of the ordinary broth and seven days of incubation of the anaerobic broth are required before the bacterin may be designated "sterile." All

bottles or vials should be labelled with the date of preparation of the vaccine, the names of the organisms contained, the number of organisms per cc. and if advisable, the name of the patient for whom it was prepared.

THE MCFARLAND NEPHELOMETER (Barium sulfate standards): To prepare these standards, use 1% sulphuric acid and a 1% solution of barium chloride.

Combine the solutions as follows:

Standard No.	1% $BaCl_2$ cc.	1% H_2SO_4 cc.	Standard No.	1% $BaCl_2$ cc.	1% H_2SO_4 cc.
1	1	99	7	7	93
2	2	98	8	8	92
3	3	97	9	9	91
4	4	96	10	10	90
5	5	95			
6	6	94			

Select 10 tubes of the same internal diameter. Put 10 cc. of each suspension of barium sulfate in each tube and seal each tube over a flame. Prepare fresh suspensions at least once a year.

The calibration of these standards in terms of the number of bacteria per cc. is accomplished by making direct counts of the organisms in suspensions matching in density several of the tubes in the series. The density of the suspensions in these tubes corresponds approximately to from 300 million organisms per cc. for the first tube to 3000 million organisms per cc. for the tenth tube, increasing by 300 million bacteria for each succeeding tube from #1 to #10.

NOTE: A bacterial standard containing 1 billion organisms per cc. can be prepared by adding 4 cc. of sterile saline to 8 cc. of triple typhoid vaccine (1,500 million bacteria per cc.).

INCUBATION OF AGGLUTINATIONS: Since incubation at 50-55 degrees C. strips H. influenzae of its capsule, when performing macroscopic test-tube type specific agglutinations with this organism, two hours of incubation at 37 degrees C. followed by overnight refrigeration should be employed. The same procedure is followed when the agglutination test is employed in the typing of pneumococci.

All other macroscopic test tube agglutination tests are incubated for 5 hours at 50-55 degrees C. and refrigerated overnight.

If a 50-55 degrees C. water-bath is not available, two hour incubation at 37 degrees C. and overnight refrigeration should be employed.

Leptospiral agglutinations are incubated at 32 degrees C. for 3 to 4 hours or at room temperature for six hours. (See section on serological procedures).

Macroscopic slide agglutination tests may be read almost immediately without incubation.

It is important that the temperature and time of incubation be the same when the patient's serum is studied repeatedly for changes in titer.

PROCEDURE FOR DIFFERENTIATION OF BRUCELLA TYPES BY DYE INHIBITION: Thionin is employed in 1:200,000 dilution in the basic agar (Medium II) (0.5 cc. of 1% solution of thionin per liter of medium) and basic fuchsin in 1:100,000 dilution (dissolve 0.1 gram of basic fuchsin in 100 cc. of distilled water at 70 degrees C. and add 10 cc. per liter of medium). The plates should be inoculated within 24 hours after pouring, as the dyes become reduced in the medium on standing.

The plates may be divided in segments if several strains are to be tested.

A heavy loopful of a broth culture is spread over each plate and the plates are incubated at 37 degrees C. for 72 hours. Care must be taken not to mistake a heavy inoculum for growth.

DIFFERENTIATION OF BRUCELLA TYPES BY H_2S PRODUCTION: For the production of H_2S by the Brucella group the basic medium (Medium II) must be modified by dissolving the ingredients in a fresh liver infusion prepared from one fourth pound of fresh liver per liter of distilled water. Differentiation of the three Brucella species by means of their hydrogen sulfide production is not clearly defined when distilled water alone is used in preparing the medium.

In studying hydrogen sulfide production by this group of organisms, slants prepared from this modified medium are inoculated with a large loopful of the culture. A strip of lead acetate paper (see below) is inserted between the cotton plug and the test tube wall so that the strip projects one inch below the bottom of the plug. The cultures are incubated at 37 degrees C. for 24 hours and the production of H_2S as evidenced by blackening of the lead acetate paper recorded as "none," "trace" or "moderate" to "marked." The strip of paper is removed and a fresh one inserted. Incubation is continued for another 24 hours and the result again recorded. This procedure is repeated until four papers have been collected.

The record is interpreted according to the following key:

Species	H_2S Production
Br. melitensis	None or trace for four days
Br. abortus	Moderate to marked - first 2 days
Br. suis	Moderate to marked - first 4 days

LEAD ACETATE PAPER: Dissolve 10 Gm. of normal lead acetate in 50 cc. of boiling distilled water. Immerse sheets of filter paper in the acetate solution until they are saturated. Allow the paper to dry and cut it into strips measuring 2-1/2 inches x 1/4 inch, and store the strips in stoppered bottles.

OXIDASE TEST FOR NEISSERIAE: The enzyme oxidase is tested for by the use of one of the dye compounds, dimethyl or tetramethyl paraphenylene diamine hydrochloride. The dimethyl compound produces a pink colony. On further oxidation the color becomes maroon and finally black. The tetramethyl compound produces a lavender color which eventually turns purple. The dimethyl compound is much cheaper than the tetramethyl compound and the latter tends to stain the medium purple as well as the gonococcus colonies, making it difficult to distinguish the "oxidase-positive" colonies.

To perform the test, from 1 to 2 cc. of a 1% aqueous solution of dimethyl paraphenylene diamine hydrochloride are dropped on each agar culture (blood agar or chocolate agar) by means of a pipette, and the plate tilted so that the entire surface is moistened. If a large series is to be examined, a "nasal" atomizer provides a simple and economical way to apply the dye compound. The dye may be applied to the individual colony with a small loop. The plate is observed for a period of five to eight minutes for evidence of change in the color of the colonies. This usually occurs in five minutes, but a freshly prepared solution may delay the reaction slightly. The Gram reaction is not affected by the treatment but if subcultures are to be made, the colonies should be picked as soon as they become pink, because the dye component is toxic for the gonococcus. After the colonies are black, subcultures fished from them usually fail to grow.

FIBRINOLYSIN TEST FOR BETA HEMOLYTIC STREPTOCOCCI (Tillett and Garner: J; Exp. Med., 1933, 58, 485): Dilute 0.2 cc. of oxalated human plasma (0.02 Gm. of potassium oxalate to 10 cc. of blood) with 0.8 cc. of physiological salt solution. Add 0.5 cc. of a young (18 to 24 hour) turbid broth culture of the streptococcus to be tested. Mix immediately and add 0.25 cc. of a 0.25% aqueous solution of calcium chloride. Mix and place in a water bath at 37 degrees C. In about 10 minutes there should be a solid coagulum. Observe frequently and note the time when the contents of the tube become completely fluid.

Plasma from individuals who have recovered recently from hemolytic streptococcus infections, is not suitable for the test.

ANTIFIBRINOLYSIN: When normal blood is used complete dissolution occurs within an hour. The presence of antifibrinolysin is demonstrated by prolonged lysing time. The time required for complete lysis of the plasma-clot is used as an index of antifibrinolysin concentration as indicated in the following scheme:

-	complete dissolution in less than one hour
1	" " " 1 to 4 hours
2	" " " 4 to 8 "
3	" " " 8 to 24 "
4	no complete dissolution in 24 hours

COAGULASE TEST FOR PATHOGENIC STAPHYLOCOCCI: When human blood is employed, 0.2% dry potassium oxalate or citrate is added to prevent clotting. For the prevention of the clotting of rabbit blood, 0.4% oxalate or citrate should be added.

The citrated or oxalated blood is centrifuged and the plasma removed with a pipette. After diluting the plasma 1:5 (1 part plasma plus 4 parts physiological saline) it is distributed in 0.5 cc. quantities in small test tubes.

In the performance of the test, 0.5 cc. of a 24 hour broth culture or a small platinum loopful of the growth obtained from agar is mixed with 0.5 cc. of the diluted plasma. The tube is placed in the 37 degrees C. water bath and examined at 15 to 30 minute intervals for evidence of clotting. A clot usually forms within two hours if the organism added is coagulase positive. Plasma for use in this test may be preserved in the lyophilized state.

OXALIC ACID PAPER TEST FOR INDOL (Holman and Gonzales; J. Bact., 1923, 8, 577): Soak filter paper in saturated oxalic acid solution. Dry and cut into strips. Hang a strip of the paper in the form of a loop over the medium in a culture tube, securing the ends of the paper between the mouth of the tube and the cotton plug. Indol is shown by the development of a pink color on the paper during growth of the culture. The paper must not be allowed to become wet.

EHRLICH'S TEST FOR INDOL:

Reagent:

Paradimethylaminobenzaldehyde.....	2 Gm.
Ethyl alcohol (95 per cent).....	190 cc.
Hydrochloric acid (concentrated).....	40 cc.

Test: To a peptone water culture, add about 1 cc. of ether. Shake, allow the ether layer to form at the surface and allow three drops of the reagent to flow down the side of the tube without disturbing the layer of ether any more than necessary. The formation of a pink layer at the junction of the ether and culture is a positive test for indol.

HEMOLYSIN TEST FOR STREPTOCOCCI: To 0.5 cc. of a 12 to 15 hour 20% serum broth culture, 0.5 cc. of a 5% suspension of washed rabbit erythrocytes is added. After incubation at 37 degrees C. for 2 hours the suspension is examined for laking of the cells. A control in the form of a mixture of 0.5 cc. of sterile broth and 0.5 cc. of the cell suspension should be run.

One may also inoculate a blood agar slant and add broth to almost the top of the slant. After 18 to 24 hours of incubation, beta hemolytic strains produce a zone of clearing in the agar which may be detected along the edges of the slant.

BILE SOLUBILITY:

Reagent: 10 per cent Bacto-Oxgall.

Test: Add 0.1 cc. of the reagent to 1 cc. of broth culture. Add 0.1 cc. of saline to 1 cc. of broth culture. Incubate both tubes at 37 degrees C. for 15 to 30 minutes. Clearing in the tube containing the bile and absence of clearing in the tube containing the saline is evidence of bile solubility.

The reagent may be prepared from fresh beef bile. The undiluted bile is

autoclaved, filtered through paper, and again autoclaved. For the test, about one-fifth volume of the bile is added to a turbid broth culture suspected of containing pneumococci.

GREEY'S POWDERED BILE SOLUBILITY TEST (J. Infect. Dis., 1939, 64, 206.)

Reagent: Dried bile (Bacto-Oxgall-Difco).

Test: The dried bile is stored in a test tube with a rubber stopper to which is attached a swab. A fair amount of the bile is transferred to the swab and dropped on the colonies so that the colonies are completely covered. The dry bile rapidly goes into solution and in half an hour or less it is absorbed leaving the surface again dry. The result of the test can then be read and if the bacteria are bile soluble their colonies will have disappeared. The bile also lakes (lyses) the normal red blood cells in the blood agar when it is applied as above but it does not affect the cells in the zone of green coloration about pneumococcus and Strep. viridans colonies.

Occasionally colonies of pneumococci are encountered which resist lysis by this method. When transferred to broth and to a second blood agar plate they become susceptible to the lytic action of the bile.

SODIUM DESOXYCHOLATE SOLUBILITY TEST FOR PNEUMOCOCCI (Leifson: J.A.M.A., 1935, 104, p. 213):

Reagent: A 10 per cent aqueous solution of sodium desoxycholate containing 1:50,000 merthiolate as a preservative.

Test: To 1 cc. of a 24 hour broth culture or salt solution suspension of organisms, 2 drops of the sodium desoxycholate solution are added. A control with 1 cc. of culture and 2 drops of salt solution or broth is prepared. If the organisms are pneumococci, clearing of the suspension or culture occurs very quickly usually in less than one minute, but the test should be held for 10 or 15 minutes before discarding as negative.

Sodium desoxycholate is easily obtained and gives quicker and more clear-cut results than does bile, but gives a precipitate in an acid reaction and will also be precipitated by sodium citrate if the latter substance is present in appreciable amounts. The basic broth contains enough fermentable carbohydrate to give an acid reaction after growth of pneumococci. Such cultures should therefore be carefully adjusted to an approximately neutral reaction (pH 6.8-7.2) before the addition of the sodium desoxycholate.

DUPONOL SOLUBILITY TEST FOR PNEUMOCOCCI (Harris, J. Lab. & Clin. Med., 1942, 27, 1591-1592):

Detergents such as sodium lauryl sulphate (Duponol WA flakes - manufactured by Dupont) or sodium lauryl sulphonate may be used instead of bile or sodium desoxycholate in both the Greey solubility test and the tube test. For the tube test,

0.1 cc. of a 0.2% solution of the detergent is added to 0.6 cc. of broth culture or saline suspension. Lysis is very rapid at room temperature.

CHOLERA-RED (NITROSOINDOL) REACTION: Add a few drops of concentrated sulfuric acid to the culture in peptone water. In the presence of nitrite and indol a pink color appears. A two to three day old culture of the cholera vibrio is used.

TEST FOR NITRITES:

Reagent:

#1. Eight grams of sulfanilic acid in 1 liter of dilute sulfuric acid (1 part of sulfuric acid in 20 parts of distilled water).

#2. Six cc. of dimethylalphanaphthylamine in 1 liter of 30% acetic acid.

Test: To five or 10 cc. of the nitrate peptone or nitrate hemopeptone culture add 0.1 cc. of solution #1. Add solution #2 drop by drop until a red color appears. In the presence of nitrites a pink or red color develops. The reagents must be added in the order given.

VOGES-PROSKAUER REACTION (Acetyl methyl carbinol test).

Reagent: 50 per cent KOH.

Test: Add 1 cc. of the reagent to a 24 hour glucose peptone or Clark and Lubs dextrose-phosphate-broth culture. (See section on media). Shake vigorously and allow to stand in the 37 degrees C. incubator. Shake at 5 minute intervals in order to thoroughly aerate the culture. A positive test is indicated by the development of an eosin-pink color. Difco's Bacto M.R.-V.P. medium may be used for this test.

A more sensitive test is that described by Vaughn, Mitchell and Levine, (J. Am. Water Works Assoc., 1939, 31, 993-1001) and Barritt, (J. Path. & Bact., 1936, 42, 441-454).

The culture is grown in Difco's M.R.-V.P. medium at 30° C. for from one to five days. To one cc. of culture, 0.6 cc. of 5.0 per cent alphanaphthol in absolute ethyl alcohol and 0.2 cc. of 40 per cent KOH are added. In from 30 minutes to 6 hours a crimson to ruby red color appears denoting a positive reaction. A coppery color is characteristic of a negative reaction.

METHYL RED TEST: To 5 cc. of a 48 hour dextrose-phosphate broth (See section on media) culture add 5 drops of methyl red solution. A positive reaction is indicated by a distinct red color, showing the presence of acid. A negative reaction is indicated by a yellow color.

The indicator solution is prepared by dissolving 0.1 gram of methyl red in 300 cc. of 95% alcohol and diluting to 500 cc. with distilled water.

Difco's Bacto M.R.-V.P. medium is best used for this test.

UREA DECOMPOSITION:

For the detection of urea decomposition the method described by Rustigan and Stuart (Proc. Soc. Exp. Biol. & Med., 1941, 47, 108-112) may be employed. The medium used by these workers contains 2% urea (Merck), 0.01% yeast extract (Difco) and M/15 primary and secondary phosphate buffers in distilled water to give a final pH of 6.8. The medium is sterilized by filtration. This test medium is inoculated from a 24 hour agar culture and incubated at 37° C. The presence of ammonia is detected by adding a loopful of the culture to a drop of Nessler's reagent on a porcelain plate. A portion of the medium without urea controls the production of ammonia from the basic medium. If Nessler's reagent is not available, alkalinazation of the medium may be used to detect decomposition of the urea. After eight hours of incubation Nessler's reagent will give a yellow to orange color with most Proteus cultures although an appreciable rise in pH may not be detected. After 24 hours of incubation, however, most strains will raise the pH of the medium beyond the range of thymol blue. Cultures of Proteus morganii, however, require at least two days of incubation to raise the pH to 8.0 - 8.2.

FERRIC CHLORIDE TEST FOR DETECTING THE HYDROLYSIS OF SODIUM HIPPURATE (Ayers and Rupp: J. Inf. Dis., 1922, 30, 388):

Reagent: Twelve grams of ferric chloride ($FeCl_3.6H_2O$) dissolved in 100 cc. of 2% hydrochloric acid in water.

Test: Transfer 0.8 cc. of culture in sodium hippurate broth (See section on media) to a small test tube (Wassermann tube) and add 0.2 cc. of the reagent. Mix immediately and observe after 10 to 15 minutes. A permanent precipitate indicates the presence of benzoic acid (positive hydrolysis).

Since sodium hippurate is first precipitated and later redissolved by the amount of reagent specified, and since benzoic acid is also redissolved by a greater excess of the reagent, it is necessary to have the reagent and the medium balanced, and to measure the amounts used in the test quite accurately. A control test of the sterile medium should always be made. If the culture is quite turbid so as to confuse the reading of the result, it should be centrifuged and the clear supernatant fluid used in the test.

AGAR IMPREGNATED SWAB FOR COLLECTING SPECIMENS:

To prevent the drying of small quantities of pus - especially specimens of gonorrhoeal urethral discharge, a swab contained in a closed tube and impregnated with an agar medium may be employed. This procedure is to be preferred to suspension in fluid media because the organisms remain viable for several hours and the morphology of the leucocyte is preserved permitting the detection of intracellular organisms. The swab is prepared by wrapping absorbent cotton around one end of an applicator and inserting the pointed opposite end into cork stoppers. The swab is dipped into melted basic agar (Medium II in Section on Media or a

similar agar medium) and the swabs attached to stoppers are loosely placed in 75 x 100 mm. test tubes. They are then autoclaved in an upright position at 15 pounds for 20 minutes. On removal from the autoclave, the stoppers are pushed into the tubes to prevent drying.

DELAYED PLANTING OF GONOCOCCUS CULTURES (Cox, McDermott and Mueller, Ven. Dis. Inf., 1942, 23, 226-227):

Method: Add 1 per cent aqueous solution of gentian violet to distilled water to make a final dilution of 1:15,000 (0.1 cc. of 1 per cent solution of gentian violet in 14.9 cc. of distilled water). Autoclave at 10 pounds for 10 minutes. Add an equal amount of sterile defibrinated horse blood to the dye to make a final solution of 1:30,000 gentian violet (15 cc. defibrinated horse blood to 15 cc. of 1:15,000 gentian violet). Test tubes 6 x 50 mm. are used with cork stoppers to fit. (No. 000xxx). Autoclave the tubes which are to contain the blood-dye solution at 15 pounds for 20 minutes. Use a 10 cc. sterile pipette to fill the tubes approximately two-thirds full with the blood-dye solution. Plug with cork stoppers which have been previously dipped in melted paraffin and kept under 70 per cent alcohol. Invert tubes and seal stoppers with hot paraffin. A similar number of tubes is used for keeping swabs sterile. Dip cork stoppers in melted paraffin. Cut round toothpicks in half, make swabs at cut ends of toothpicks, and insert pointed ends into stoppers. Place swabs attached to stoppers in test tubes and autoclave at 15 pounds for 20 minutes. Invert tubes and seal stoppers with hot paraffin.

In collecting the specimen, remove from the tube the cork to which the swab is attached. Touch the swab to the exudate, then place it in a tube containing the blood-dye and close tube with cork to which applicator is attached. When obtaining material from the cervix, dressing forceps are used for grasping the cork end of the swab.

INOCULATION OF GUINEA PIGS WITH TUBERCULOUS MATERIAL: Test the specimen for the presence of organisms other than M. tuberculosis by streaking a blood agar plate. If no growth is obtained suspend the specimen in a small amount of sterile saline (1 or 2 cc.) and inject two guinea pigs, both subcutaneously and intramuscularly, in the left thigh (in the inguinal region). Traumatize the inguinal glands at this time with the fingers.

Guinea pigs weighing about 250 grams should be used and a record should be kept of their weight, sex and appearance, the nature of the specimen, and the date of injection.

The animals should be examined weekly for enlarged glands and loss of weight and all animals that die should be autopsied and examined as described below. If the animals survive they should be killed and autopsied after 6 weeks and three months.

At autopsy the weight of the animal and the extent of involvement of the lymphatic glands and spleen and liver are noted. The inguinal gland is observed

for swelling and caseation and pathological material from it smeared for the presence of acid-fast rods.

The spleen appears to be the most vulnerable organ and when involved may be tremendously enlarged and contain many tubercles. The liver may likewise be severely affected and is peppered with conglomerate yellow necrotic tubercles. Sections of the spleen and liver should be prepared for histological examination and the tubercles examined for the presence of acid-fast rods.

In pseudotuberculosis of guinea pigs, the etiological agent is a gram-negative rod (Pasteurella pseudotuberculosis). The tubercles in this disease differ from true tubercles in that they lack giant cells.

Where facilities permit it guinea pigs may be tested by the tuberculin test prior to use and again 4 weeks or more after the injection of the specimen. (Diagnostic Procedures and Reagents, 1941, A.P.H.A., p. 290).

When other organisms are present in the specimen they must be destroyed before injection into animals or inoculation of culture media. Either 5% sterile oxalic acid, 3% sterile hydrochloric acid, or 4% sodium hydroxide may be used for this purpose.

The specimen is placed in a 6 inch sterile test tube provided with a cotton stopper. Care should be taken to deliver it to the bottom of the tube to prevent contaminating the sides as much as possible. The sides of the tube are flamed and the cotton plug inserted.

To the specimen, an equal quantity of 3% hydrochloric acid containing brom cresol purple as an indicator is added. (The acid solution is 3% of the concentrated hydrochloric acid and is not 3% hydrogen chloride).

The tube is shaken vigorously with a side to side motion in order to mix the specimen with the reagent.

The tube is flamed well to within an inch of the upper level of the liquid.

A fresh sterile cotton plug is inserted in the tube.

The specimen is left at room temperature for 2 hours.

Using 3% sodium hydroxide, the specimen is made slightly alkaline to the brom cresol purple indicator. This reagent is added aseptically to the tube. (Although the brom cresol purple color change occurs at pH 6.8, it is used rather than brom thymol blue, as it is difficult to determine the pH with brom thymol blue in turbid specimens).

The tube is centrifuged at high speed for 1/2 hour after fastening the cotton stopper so that it will not go to the bottom of the tube.

The supernatant fluid is removed to within 1/2 inch of the top of the sediment. The latter is tested for sterility by inoculating a tube of broth with a loopful.

If growth is observed in 24 hours, the specimen is treated again with 3% hydrochloric acid for 1 hour at room temperature. It is neutralized with sodium hydroxide, centrifuged, and the supernatant fluid drawn off.

The sediment is inoculated onto ordinary culture media to detect the presence of organisms other than M. tuberculosis.

If the specimen shows no growth on ordinary media in 24 hours it may be used for the injection of guinea pigs and for the inoculation of special media for the cultivation of the tubercle bacilli. Smears for acid-fast rods may also be made at this time.

Oxalic acid or sodium hydroxide may be used instead of hydrochloric acid. Four per cent sodium hydroxide may be especially useful with specimens which are not dissolved readily by acids.

Digestion with acid or alkali as above may be used for the concentration of tubercle bacilli not only for cultivation and animal injection but also for smear examination. When the concentrated specimen is to be used for culture or animal injection, the final reaction must be approximately neutral to litmus. When acid digestion is employed neutralization with NaOH may be carried out. When the specimen is digested with 4% NaOH it may be neutralized after digestion with dilute HCl.

If 5% oxalic acid is used to digest the specimen, it is allowed to act for 30 minutes to 1 hour at 37 degrees C. or not more than 5 hours at room temperature.

If 4% NaOH is used to digest the sputum it should be allowed to act for 1/2 hour at 37 degrees C. before neutralization with acid.

CULTURE METHODS FOR M. TUBERCULOSIS: Selected media (See section on media) in slant form are inoculated with approximately 0.25 cc. of the specimen and allowed to dry overnight in a horizontal position. A reduced tension tube is prepared from these tubes the next day by igniting the stoppers, pressing them into the tubes without touching the medium and closing the tube with a sterile rubber cap or stopper. Incubation at 37 degrees C. for 8 weeks is carried out before reporting a negative result.

Screw-cap homeopathic vials with cork discs in their caps may be conveniently used instead of test tubes in the preparation of media for the cultivation of the tubercle bacillus. The cork pad in the screw top insures against the evaporation of the fluid contained in the vial, and if the top is not screwed down too tightly it will insure adequate air exchange and exclude outside contamination during months of incubation.

CONCENTRATION OF SPUTUM FOR THE DETECTION OF ACID-FAST RODS BY SMEAR. Hanks' chemical flocculation (For M. tuberculosis):

Digestive mixture: 4 per cent NaOH
0.2 per cent potassium or sodium alum.
0.002 per cent brom thymol blue.

Equal parts of sputum and digestion mixture are mixed and placed in a 37 degree C. water bath for thirty minutes with occasional shaking. The mixture is neutralized by adding 2.5/N HCl drop by drop until the brom thymol blue begins to change and a precipitate begins to form on shaking. Care should be taken not to add so much acid that an excessive amount of precipitate be formed. The precipitate is collected by centrifugation, smeared and fixed on a slide and stained by the method of Ziehl-Neelsen.

Occasionally flocculation does not occur on neutralization and shaking. By the addition of 0.2 cc. of 1% ferric chloride and again shaking, precipitation may be brought about.

CONCENTRATION OF SPUTUM (Andrus McMahon Method) (For smear demonstration of M. tuberculosis): Place about one ounce of sputum in a clean 6 ounce, wide-mouthed bottle. Add 2 to 4 cc. of a 1% solution of phenol and shake.

Add 1 to 2 volumes (the greater the viscosity of the specimen, the larger the amount of alkali required) of 0.5% sodium hydroxide. Shake vigorously.

Heat in a water bath at 55 degrees C. to 60 degrees C. for 30 minutes or until the mucus is dissolved, shaking vigorously at 5 to 10 minute intervals. Remove from the bath and allow to cool. If insoluble particles settle out, the supernatant fluid should be decanted and the sediment discarded.

Three per cent chloroform is added and after shaking for 20 minutes in a mechanical shaker, the mixture is centrifuged at high speed for 20 minutes in a large centrifuge tube.

The supernatant is removed completely and the sediment smeared on a slide, fixed, and stained. Smears may be made quite thick.

In the concentration procedures described above, it is important that thoroughly clean glassware be used. Distilled water that has been standing for a long time may be the source of acid fast rods and should never be used if there is a scum or precipitate in the container.

GERUNDO MIXTURE FOR THE CONCENTRATION OF SPUTUM (Gerundo, M., J. Lab. & Clin. Med., 1942, 28, 328).

Mixture:

Pepsin	1.0 Gm.
Glycerin	10.0 cc.
HCl (conc.)	15.0 cc.
Sodium fluoride	1.0 Gm.
Dist. H O	1,000 cc.

Mix equal volumes of sputum and solution and leave the mixture for 4 hours in the 37° C. incubator, shaking from time to time. Centrifuge, wash with sterile saline several times to remove the acid and culture the sediment on Petragnani's medium or prepare for microscopic examination.

The method is also useful for the digestion and concentration of clotted joint, pleural fluids, etc.

CONCENTRATION OF PLEURAL FLUID BY THE ACID ALUM METHOD. (McNabb, Diag. Proc. & Reag., 1941, pages 287-288): To the specimen, 2 cc. of 5% potassium alum are added. An equal quantity of 3% hydrochloric acid containing brom cresol purple as an indicator is added. The specimen is allowed to stand at room temperature for 2 hours. Using 3% sodium hydroxide, the specimen is made slightly alkaline to the brom cresol purple indicator. The precipitate is collected by centrifugation, smeared, fixed, and stained for acid-fast rods.

CONCENTRATION OF FECES BY THE ANTIFORMIN METHOD: To 20 cc. of distilled water add 5 cc. of full strength antiformin. Emulsify in this a portion of stool about the size of a pea and allow to stand in a 45-50 degree C. water bath for 1/2 hour. Centrifuge, discard the supernatant and smear the sediment for staining in the usual manner.

CONCENTRATION OF URINE FOR ACID-FAST RODS (Hanks and Feldman, J. Lab. & Clin. Med., 1940, 25, p. 974): Collect a 24 hour specimen of urine in clean containers and place in a large clean cylinder. Add N or 2.5/N NaOH to induce a slight phosphate flocculation and allow the precipitate to settle. Decant the supernatant, centrifuge the sediment and discard the second supernatant. Dissolve this sediment with an equal volume of 12% sulfuric acid (12% by volume). Incubate the mixture at 37 degrees C. for 30 minutes, if for culture, and then adjust the solution with NaOH to a grass green color using brom cresol green as an indicator (pH 4.5). The precipitate that is formed is collected by centrifugation and used for culture, animal injection, or smear for microscopic examination.

CONCENTRATION OF SPINAL FLUID (Hanks & Feldman: J. Lab. & Clin. Med., 1940, 25, p. 886): To each 2 cc. of spinal fluid add 0.5 cc. (4 drops) of chloroform and shake violently for 10 minutes. Centrifuge at high speed for 5 minutes, discard the supernatant fluid, smear the sediment on a slide, fix with heat and stain with Ziehl-Neelsen's stain. This method is recommended for microscopic examination.

Alternate Method: (Hanks and Feldman: J. Lab. & Clin. Med., 1940, 25, p. 886): This method is recommended for cultivation or guinea pig inoculation.

Pipette the specimen forcibly into an autoclaved 1% aqueous solution of potassium alum (0.025 cc. alum solution per 2 cc. of fluid). Shake immediately for 10 minutes. Centrifuge for 5 minutes and discard the supernatant fluid. The sediment may be cultured, injected into a guinea pig or examined microscopically for acid-fast rods.

DEMONSTRATION OF MYCOBACTERIUM LEPRAE: These organisms are demonstrable in nasal and skin lesions of leprosy as acid-fast bacilli occurring in packets.

Swabs or scrapings from nasal lesions are spread onto glass slides, fixed and stained by the method of Ziehl-Neelsen. Skin lesions are incised with a sterile razor blade which is used to scrape the cut lesion from below upward. The deep, rather than the surface skin scraping is desired for the spread.

Typical packet bundles of lepra bacilli, or, in the skin nodules, lepra bacilli packed in "lepra cells" or in endothelial cells, are conclusive of leprosy.

ETHER METHOD FOR THE SEPARATION OF THE POLIO VIRUS FROM BACTERIA IN STOOL SPECIMENS: A thick suspension of the specimen is made in distilled water and shaken in the refrigerator for from one to three hours. The suspension is centrifuged in the angle centrifuge at low speed to bring down the large particles and then filtered through paper. Fifteen per cent ether is added to the supernatant which is then allowed to stand in the refrigerator for 12 to 24 hours. Bacteria are killed while the polio virus survives this treatment. The material may be inoculated into the experimental animal by either the intraperitoneal or intranasal routes or both.

THE DIAGNOSIS OF RABIES INFECTION OF THE ANIMAL BRAIN:

Collection of specimens:

(1) Keep the dog or other animal alive until it definitely becomes sick or dies. Remove the head at the neck immediately after death, and deliver to laboratory within two hours; (2) pack head in ice in a watertight container and deliver to laboratory within one day; (3) remove the brain, pack in a watertight can, seal and pack the can in a larger can of ice and deliver within the melting time of the ice.

If received after hours keep well iced until examined.

Fresh brain tissue is to be preferred. If shipping greater distances, parts of the hippocampus, (Ammon's horn) should be removed and placed in a small bottle of full strength neutral glycerine and mailed at once (for animal inoculation). The remainder, if quite small, should be fixed in Zenker's for 24 hours and changed to 80% alcohol before shipping. If the brain is large, remove and fix in Zenker's, small sections of:

1. Both sides of hippocampus.
2. Cerebral gray matter from near the fissure of Rolando.
3. Cerebellum.

The hippocampus is exposed by opening the lateral ventricles. It is the bulging cylinder lying in the floor of the ventricle. It shows concentric light and dark circles when cut transversely. The dark tissue is sampled.

Fresh smears:

A. Preparation:

1. With a small scissors cut through Ammon's horn (hippocampus) transversely. The cut surface will show light and dark zones. From the cut surface clip a piece no larger than a grain of rice.

2. Transfer this to a clean slide near one end.

3. Press this out flat by means of another slide.

4. Draw the top slide along the length of the bottom one, leaving a thin smear. It must be stained before it dries.

B. Staining:

1. Immerse in Koplin jar of stain and remove at once.
2. Rinse in tap water, dry without blotting and examine.

Stain formula

Basic fuchsin (saturated absolute methyl alcohol solution)	2-4 cc.
Methylene blue (saturated absolute methyl alcohol solution)	15 cc.
Methyl alcohol (absolute, acetone-free)	25 cc.

Mix saturated methylene blue and methyl alcohol in a Koplin jar and add two cc. of basic fuchsin. A trial stain is then made. Macroscopically, the properly stained smear when held up to the light should appear reddish violet in the thinner areas, shading into purplish blue in the thicker ones. If, in the trial stain, the thin parts are blue, add 0.5 cc. more of fuchsin solution and make another trial. Three cc. of fuchsin is nearly always sufficient. The mixed stain improves after 24 hours and keeps indefinitely if tightly corked. It will also work if the alcohol lost by evaporation is replaced by the addition of absolute methyl alcohol.

Staining should be complete (including the rinse) in less than five seconds. Dry slide by air, not by blotting since the smear may easily be wiped off.

Examine the stained smear under the low power of the microscope and look for thin areas showing numerous large nerve cells, well spread out. These are then examined under the oil immersion objective of the microscope.

C. Results:

Negri bodies are stained bright cherry red, in strong relief. They are round or oval bodies, up to 23 microns in diameter, usually containing vacuoles. The basophilic granules in these vacuoles are blue and nearly always clearly visible. There is often one large one surrounded by several small ones. The

cytoplasm of the nerve cells stains purplish blue; nuclei and nucleoli of nerve cells, more deeply blue; stroma, rose pink; nerve fibers, deeper pink. Neural sheaths do not stain. Bacteria, if present, stain an intense blue; muscle fibres, brick red; erythrocytes, a copper color.

Negri bodies are usually more abundant and larger in the nerve cells of the hippocampus. The specimen should not be pronounced negative, however, until at least 50 cells have been searched from each of six areas: the hippocampus, cerebral gray matter and cerebellar gray matter from each side of the brain. It should be borne in mind that Negri bodies will not be seen in about 12 per cent of rabies infected brains.

D. Animal inoculation:

Rabbits, guinea pigs and mice may be employed for animal inoculation in the diagnosis of rabies. For the technique of this procedure see Leach, C. N., Comparative methods of diagnosis of rabies in animals., A.J.P.H., 1938, 28, 162-166; the chapter of Sellers and Carnes, Diag. Proc. & Reagents, A.P.H.A., 1941.

THE MODIFIED SYRINGE METHOD FOR OBTAINING SERUM FROM CHANCRES: After cleaning the chancre and removing the sore and blood, (See section on treatment of specimens - genito-urinary specimens) serum for dark-field examination may be conveniently obtained by applying suction over the chancre. A 5 cc. syringe, the barrel of which has been cut so that the plunger may be inserted from the opposite end serves very well. The smooth open end of the barrel is placed over the chancre and suction is produced by pulling the plunger. The serum may be collected with a capillary tube, a loop, or by touching it with a clean slide or cover-slip.

SPECIAL METHODS FOR THE SELECTIVE SEPARATION OF BACTERIA:

1. The use of potassium or sodium tellurite for the inhibition of bacteria:

Potassium tellurite may be used in media to inhibit the growth of bacteria as follows:

(1) In a dilution of 1:500,000, hemophilic and most coliform organisms are inhibited.

(2) In a dilution of 1:50,000, Ps. aeruginosa and some strains of Proteus are inhibited.

(3) In a dilution of 1:10,000, streptococci, staphylococci and diphtheroids will grow.

The tellurite may be incorporated in the medium before streaking or may be smeared over the surface of an ordinary streaked plate.

In the first method, to a tube of melted agar, 3/4 to 1 cc. of blood and 0.5 cc. of 1:500 sodium or potassium tellurite is added. It is poured into a Petri

dish and when hard, the surface is streaked with a mixed culture. Most gram-negative bacilli grow poorly,if at all,on this medium while the growth of gram-positive organisms is not inhibited. Colonies of streptococci will be small, gray and translucent and will usually show a dark gray or black center. Colonies of staphylococci are larger, opaque and show large, black centers. Since the addition of tellurite to the medium causes hemolysis of the blood,such plates cannot be used to determine the hemolytic characteristics of the gram-positive cocci.

The separation of gram-negative and gram-positive organisms may also be effected by adding 0.25 cc. of 1:500 potassium or sodium tellurite to a tube of blood or serum broth and inoculating this tellurite broth with a small loopful of the bacterial mixture. After 12 to 18 hours of incubation,a culture,in which the gram positive cocci predominate, will usually be obtained.

A simpler method of using tellurite for the isolation of the gram-positive cocci is to inoculate a plate in the usual way and then to spread 2 or 3 drops of 1 in 1,000 tellurite over half of the plate. In this way one half of the plate is an ordinary culture while on the other half the coliforms are generally completely inhibited. In this method, also, blood agar is cleared by the tellurite.

2. The prevention of overgrowth by spreading organisms:

Surface streaked plates may at times be valueless for the separation of organisms due to the presence of spreading organisms. To prevent such spreading, the streaked surface may be covered with melted agar cooled to 45 degrees C. to a depth of 2 or 3 mm. and allowed to set. The spreaders growing between the layers are thus prevented from spreading. Potassium tellurite in a concentration of 1:20,000 to 1:50,000 may be incorporated in the agar which is poured over the surface.

3. The use of pathogenicity for the isolation of pathogenic organisms from grossly contaminated material:

Because the mouse is especially susceptible to the pneumococcus it may be employed for the isolation of this organism especilly from sputum by intraperitoneal injection. (See index).

Such organisms as Pasteurella tularensis and Pasteurella pestis may be isolated from grossly contaminated material by making use of their ability to penetrate the unbroken skin of experimental animals. The specimen is merely rubbed onto the recently shaved and abraded skin of the susceptible animal.

4. See section on media for descriptions of selective media used especially for the separation of pathogens from coliform organisms. (Also crystal-violet medium).

pH DETERMINATION:

Stock solutions of indicators:

Weigh out 0.1 Gm. of the indicator and grind it preferably in an agate mortar, with the volume of N/20 sodium hydroxide given in the table below.

When solution is complete make up to a volume of 25 cc. with water.

For use, dilute the solution as noted in the table below. (Table 43).

(To make N/20 NaOH dissolve 2 Gm. of sodium hydroxide in distilled water and make up the volume to 1 liter).

TABLE 43.

Indicators, their pH range, and the volume of N/20 NaOH necessary to effect the solution of 0.1 Gm.

Indicator	cc. of N/20 NaOH	Test solution: 1.0 cc. indicator plus cc. H_2O*	pH Range
Brom cresol purple	3.7	9	5.2 to 6.8
Brom thymol blue	3.2	9	6.0 to 7.6
Phenol red	5.7	19	6.8 to 8.4
Cresol red	5.3	19	7.2 to 8.8

* Number of cc. of H_2O to be added to 1.0 cc. of the indicator to make the test solution.

The pH of the medium may be determined by adding one of these indicators (10 drops of the test solution) to 10 cc. of a 1:10 dilution of the medium in water.

A series of tubes each of which contains 10 cc. of graded mixtures of KH_2PO_4 and NaOH (See Table 44 below) with indicator added, represents a colorimetric scale against which the media can be standardized.

TABLE 44.

GRADED MIXTURES OF KH_2PO_4 - NaOH

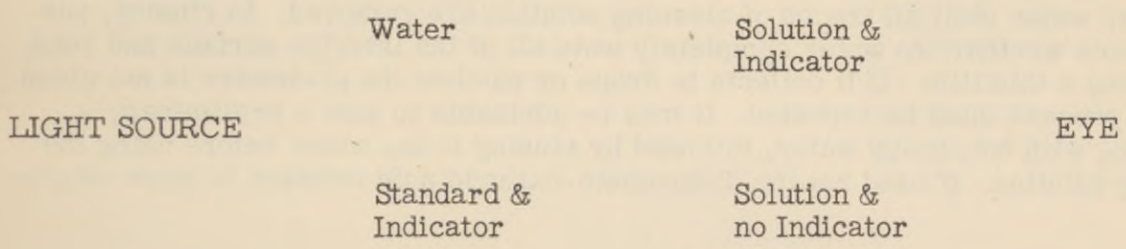
pH								
5.8	50 cc.	M/5	KH_2PO_4	3.72 cc.	M/5	NaOH	Dilute to	200 cc.
6.0	"	"		5.70 "	"		" "	"
6.2	"	"		8.60 "	"		" "	"
6.4	"	"		12.60 "	"		" "	"
6.6	"	"		17.80 "	"		" "	"
6.8	"	"		23.65 "	"		" "	"
7.0	"	"		29.63 "	"		" "	"
7.2	"	"		35.00 "	"		" "	"
7.4	"	"		39.50 "	"		" "	"
7.6	"	"		42.80 "	"		" "	"
7.8	"	"		45.20 "	"		" "	"
8.0	"	"		46.80 "	"		" "	"

In the preparation of the mixtures for use in this colorimetric scale, all glassware must be carefully cleaned and finally thoroughly rinsed in redistilled water. Ten cc. of each of the respective standard mixtures is placed into each test tube and to it 10 drops of the indicator are added. Phenol red will be found most useful since it covers the range from 6.8 to 8.4. If a range from 6 to 7.6 is desired the brom thymol blue solution may be used and when a range just above 8 is desired, cresol red is recommended.

The determination (or adjustment) of the pH of any medium which is not too highly colored and not too turbid may be carried out as follows:

In a clean test tube, 1 cc. of the medium, 9 cc. of water and 10 drops of the indicator are mixed. A color reading is then taken against the scale. If the reaction is too acid, N/20 NaOH is added drop by drop until the color matches that of the standard mixture having the pH that is desired. By calculating from the amount of weak alkali added, the total quantity of medium is then brought to the desired pH with N/1 sodium hydroxide.

In solutions having a certain amount of color or turbidity, a so-called "comparator" may be used in the form of a wooden box with four holes for test tubes and a slit in front and behind, so that it can be looked through against a source of light. The arrangement of this is given in the following diagram:



Arrangement for reading titration

PREPARATION OF GLASSWARE: New glassware is boiled in water to which has been added sufficient white soap or washing soda to provide a good foam. The water is cooled to 45 degrees to 50 degrees C. and the glassware is washed thoroughly using a washrag and brush. It is then rinsed in running tap water and then in distilled water. It is inverted on a drain board or dried in the hot air oven.

Used glassware must be first freed from pathogenic organisms by immersion in a 3 to 5% solution of cresol for several hours or by autoclaving. Glassware smeared with paraffin or petrolatum should be given a preliminary cleansing with xylol.

Glassware that cannot be cleaned by soap and water should be cleaned by soaking overnight in dichromate cleaning solution (See below).

Pipettes, after use, should be placed in tall jars containing 2 to 5% cresol. To wash the inside of a pipette and force out the cotton plug, attach a rubber hose

to the cold water faucet and insert the tip end of the pipette into the hose. Turn on the water and force it through the pipette. Rinse in distilled water and dry. Pipettes that are not perfectly clean should be soaked in dichromate cleaning solution overnight.

The base of each pipette should be packed loosely with cotton (preferably non-absorbent) and sterilized with dry heat after wrapping individually in paper or placing in a pipette can.

Syringes should be dismantled, wrapped in heavy, unbleached muslin and sterilized with dry heat. Small syringes may be sterilized in large cotton-plugged test tubes.

Non-absorbent cotton should be used for the plugging of all glassware.

DICHROMATE CLEANING SOLUTION: Dissolve 300 grams of dichromate (technical) in 2 liters of water with the aid of heat. Add cautiously 1,850 cc. of commercial concentrated sulfuric acid.

Caution: Never pour the aqueous solution into the acid. Handle with care. Avoid contact with flesh and clothing.

Glassware is soaked in this solution overnight and then rinsed repeatedly in hot tap water until all traces of cleaning solution are removed. In rinsing, one should note whether the water completely wets all of the interior surface and runs off leaving a thin film. If it collects in drops or patches the glassware is not clean and the process must be repeated. It may be advisable to give a preliminary scrubbing with hot, soapy water, followed by rinsing in tap water before using the cleaning solution. If used hot the dichromate-sulfuric acid solution is more effective.

STERILIZATION: Glassware wrapped in cloth or paper or plugged with cotton should be sterilized in the hot-air oven by heating for one and one-half hours at 160 to 175 degrees C. Closely packed glassware or glassware in a large container should be heated for a longer period of time to insure penetration of heat and sterilization of the central portion.

When steam under pressure (the autoclave) is employed, routinely 15 pounds steam pressure for 15 minutes is sufficient.* Large packages or media

* In autoclaving sugar fermentation tubes, an exposure of 12 pounds steam pressure for 10 minutes has been recommended (See page 168). This treatment, as a rule, permits sterilization without decomposition of all sugars except maltose. The latter should be sterilized by filtration or autoclaved as a 10 per cent aqueous solution (at 7 to 12 pounds steam pressure for 10 minutes) and then added aseptically to the sterile base medium. Batches of sugars (e.g., xylose, lactose, arabinose) may be frequently encountered which are broken down by the recommended autoclaving. For these, it may be necessary to employ only 7 or 8 pounds steam pressure for 10 minutes. Such media should be incubated to test for sterility.

in bulk will require from 30 minutes to one hour. It is necessary that all air be allowed to escape from the autoclave before closing the exhaust valve and allowing the pressure to rise. A mixture of air and steam at 15 pounds pressure does not have the sterilizing properties of pure steam at this pressure.

When steam without pressure is employed, as for the sterilization of certain media, exposures of 20 to 60 minutes on three successive days are used.

XI. THE MICROSCOPE AND MICROMETRY.

A. STRUCTURE:

The microscope consists of four groups of parts, each group composed of a number of units.

1. Framework:

(a) Base, on which the microscope rests.
(b) Handle, by which it is carried and which supports the magnifying and adjusting systems.
(c) Stage, a perforated horizontal shelf on which the object rests.
(d) Mechanical stage, which moves the object about on the stage.

2. Illumination system:

(a) Mirror, which reflects light upward.
(b) Condenser, placed just beneath hole in stage.
(c) Diaphragm, just beneath condenser, controlled by a small button to open or close it, in controlling the light intensity.

3. Magnification system, through which light passes:

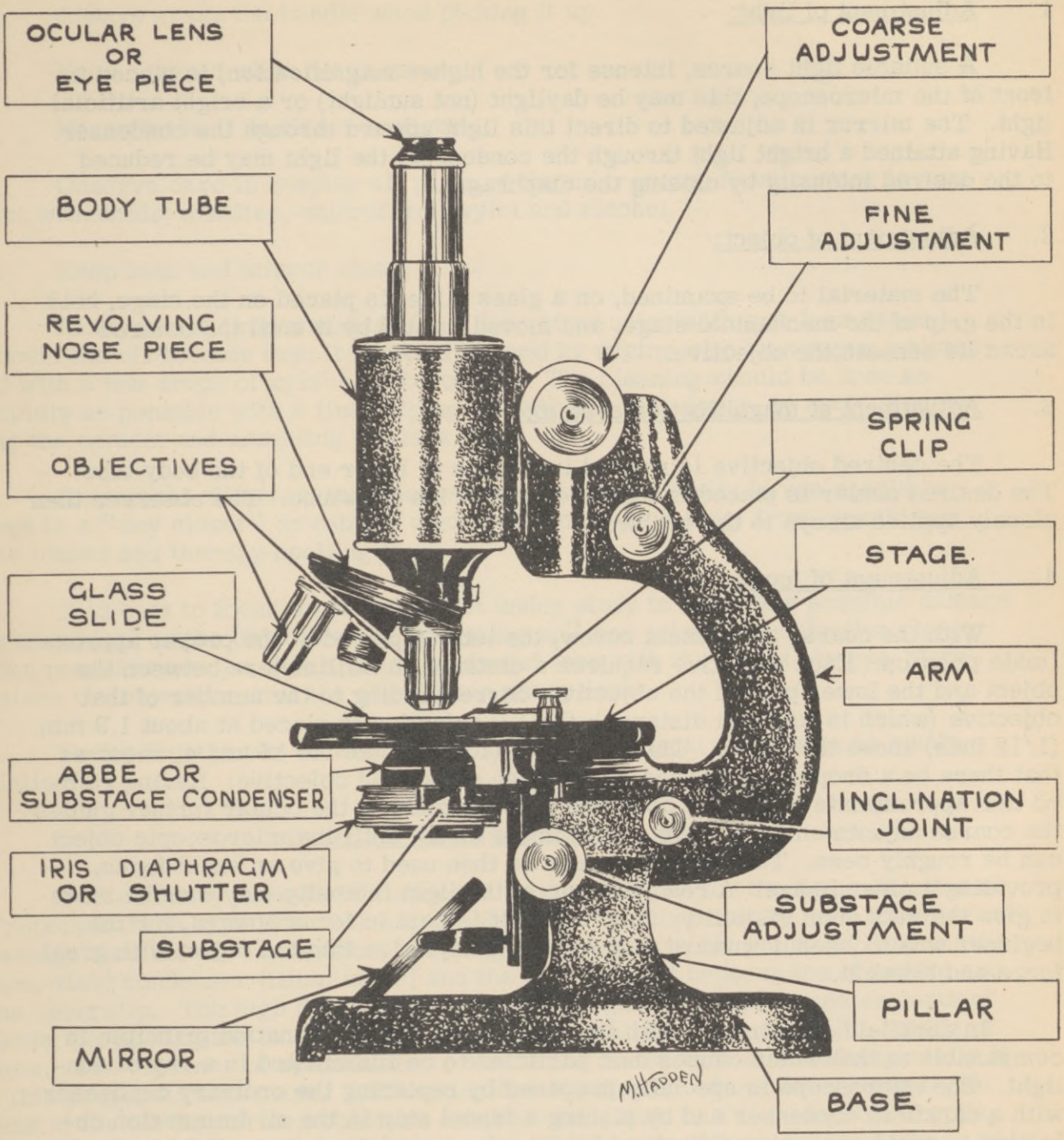
(a) Nose-piece, generally triple, to receive the objectives.
(b) Objectives, generally three, the main magnifying part, designated according to their focal distance as 16, 4 and 1.9 mm., the latter being the highest power and used in most bacterial studies.
(c) Body tube and drawtube, through which the light passes to the ocular.
(d) Ocular, an additional magnifying piece, of which two are generally furnished, 6.4X giving somewhat less magnification than 10X (number indicates times object is magnified).

4. Adjustment system, which moves body tube up or down for focusing of objective to object:

(a) Coarse adjuster gives rapid movement over a wide range and is used to obtain an approximate focus.
(b) Fine adjuster gives a very slow movement over a limited range and is used to obtain an exact focus, after prior coarse adjustment.

The magnification of any combination of objectives and oculars may be obtained by multiplying the magnification of the objective by that of the ocular. The magnification given by different combinations of objectives and oculars is as follows:

Objective	Ocular	
	6.4X	10X
16 mm. (10X).....	X64	X100
4 mm. (43X).....	X275	X430
1.9 mm. (95X).....	X610	X950


OCULAR LENS OR EYE PIECE
BODY TUBE
REVOLVING NOSE PIECE
OBJECTIVES
GLASS SLIDE
ABBE OR SUBSTAGE CONDENSER
IRIS DIAPHRAGM OR SHUTTER
SUBSTAGE
MIRROR
COARSE ADJUSTMENT
FINE ADJUSTMENT
SPRING CLIP
STAGE
ARM
INCLINATION JOINT
SUBSTAGE ADJUSTMENT
PILLAR
BASE
M.H.HADDEN

B. USE:

1. Adjustment of light:

A suitable light source, intense for the higher magnification, is placed in front of the microscope; this may be daylight (not sunlight) or a bright artificial light. The mirror is adjusted to direct this light upward through the condenser. Having attained a bright light through the condenser, the light may be reduced to the desired intensity by closing the diaphragm.

2. Adjustment of object:

The material to be examined, on a glass slide, is placed on the stage, held in the grip of the mechanical stage, and moved around by it until the desired areas lie beneath the objective.

3. Adjustment of magnification system:

The desired objective is rotated into place at lower end of the body tube. The desired ocular is placed in the upper end of the drawtube. The observer then closely applies an eye to the ocular.

4. Adjustment of focus:

With the coarse adjustment screw, the tube is placed at the proper approximate position. Each objective requires a distance in millimeters between the object and the lower part of the objective, corresponding to the number of that objective (which is its focal distance); the 1.9 objective is placed at about 1.9 mm. (1/12 inch) above the object. This objective (1.9), but not the 16 and 4, requires that there be a drop of cedarwood oil between object and objective. Having gained this approximate focus, the observer's eye applied to the ocular further guides the coarse adjustment to a more approximate focus, until the microscopic object can be roughly seen. The fine adjustment is then used to give an exact focus, providing a clear image. A readjustment of the light intensity may then be made to give the maximum visibility. In general, it is best to focus upward, for the beginner who focuses downward may force the objective into the object with great force and break it.

In darkfield examination with the microscope the illumination principle is comparable to that which causes dust particles to be illuminated in a ray of sunlight. The microscope is specially prepared by replacing the ordinary condenser with a darkfield condenser and by placing a funnel stop in the oil immersion objective to avoid excessive diffusion of light. An especially intense light is used. The preparation should be thinly spread, free from bubbles and covered with a thin cover-glass. Immersion oil is placed between the slide and condenser as well as on top of the cover-glass. All highly refractile objects, including bacteria will be seen as bright objects on a dark background.

When the lower power objectives are employed, no oil is placed on top of the slide.

C. CARE:

1. Always grasp the handle when picking it up.

2. Keep covered when not in use.

3. Never leave it in direct sunlight.

4. Observe care in keeping all parts of the microscope from coming in contact with acids, alkalies, chloroform, xylol and alcohol.

5. Keep lens and mirror clean.

6. Always wipe immersion oil from lens after using with soft lens paper. Should the oil become dry, it can be removed by wiping with lens paper moistened with a few drops of xylol or chloroform. The cleaning should be done as rapidly as possible with a final wiping off with dry lens paper, to avoid dissolving the cement and unseating the lens.

7. In tropical climates, the microscope when not actually in use should be kept in a "dry closet" or cabinet desiccator to prevent fungi from growing on the lenses and thereby spoiling them.

8. It is best to focus up on the object under study to eliminate possible damage to the object or the objective. In "focusing up" one brings the objective closer than necessary to the object under study and then with the eye at the eye-piece, raises the objective until the object is in sharp focus.

9. Force must never be used to overcome any unusual resistance to manipulation.

Darkfield Microscopic Technique

For ordinary dark-field diagnostic procedures, as for the demonstration of Treponemata in syphilitic lesions, the high dry objective may be used to better advantage than the oil-immersion. In using this objective, oil is placed between the dark-field condenser (illuminator) and the slide but not between the objective and the coverslip. The high dry objective gives a larger field and a greater depth of focus, permitting the examination of more material and an appreciable saving of time. The tendency of spirochetes to dive out of focus is diminished especially if a thin preparation is made. Difficulties due to adhesion of the coverglass to the lens of the oil-immersion objective are removed, and, to a considerable degree, the mistaking of artefacts for spirochetes. It is, furthermore, unnecessary to reduce the aperture of the high dry objective by inserting a funnel stop. When used in combination with a suitably adjusted strong light and a 10x or 12.5x ocular, the high dry soon becomes the objective of choice. If, at any time, it is desired to study the spirochete under the oil-immersion objective, a drop of oil is placed on the coverglass and an oil-immersion objective of suitable aperture (or one containing a funnel stop) turned into position. This procedure, of course, makes further examination with the high dry objective impossible.

Of the many reasons for failure with the dark-field technique the most common is unsatisfactory lighting. A powerful source of light is essential. Direct sunlight, the arc-light, or a lamp capable of giving a point source of light of high brilliancy should be used. In emergencies, a strong focusing flashlight (three cells or more) may also be employed. Since the special dark-field illuminators (condensers) are designed for parallel beams of light, a parallelizing system should be employed with artificial light and the light then reflected into the condenser with the plane mirror of the microscope. The parallel beams of direct sunlight are also directed into the condenser with the plane mirror. If the sunlight is too brilliant one or more pieces of ground glass may be used between the sun and the mirror. The preparation under examination should be protected from the direct rays of the sun. If a lamp is used without a ground daylight glass or a parallelizing lens, one may get better results with the concave mirror which tends to make the rays from the lamp more perfectly parallel. If the light is not sufficiently strong better results may be obtained by using the concave mirror even though there be some reduction in definition.

The following steps are taken in the performance of a dark-field examination.

1. Remove the Abbe condenser and fasten the clean parabaloid or cardioid illuminator (condenser) in its place. It is necessary that the upper lens surface be clean and that it be possible to bring it into the plane of the upper surface of the stage or a little higher.

2. Direct a strong beam of light (parallel rays if possible) on the plane mirror. Reflect the light into the illuminator.

3. Before placing the preparation to be studied on the stage, focus the low power objective on the upper surface of the illuminator lens. A small circle which is scratched on this surface will be seen. This circle represents the center of the lens and must be brought into the exact center of the field by means of the centering screws on the illuminator.

4. The material for examination is placed on a clean glass slide of correct thickness. The thickness of the slide that may be used is usually indicated on the illuminator mount. If the slide is too thick the object under study cannot be illuminated by the oblique rays coming from the illuminator. If the slide is too thin the illuminator may be racked down to the proper level. Cover the material with a clean coverglass (preferably No. 1) and press with clean paper or gauze to obtain a thin preparation.

5. Place a drop of immersion oil upon the illuminator (condenser) lens and then place the prepared slide in position. Raise the illuminator until fairly intimate contact between its upper surface and the bottom of the slide is obtained making certain that air bubbles are squeezed out of the oil. The thickness of the oil layer may be adjusted later in order to obtain proper illumination. (See #6 below).

6. Examine the preparation first with the low-power objective and then with the high dry objective. The background should be dark and the bodies in the field brilliantly illuminated. By careful adjustment of the position of the mirror and

by racking the illuminator up or down, proper illumination may be obtained. The illumination may be considered adequate when the serum colloids are seen as juggling pin-points of bright light.

7. If use of the oil-immersion objective is desired, a drop of oil is placed on the coverglass and the numerical aperture of the objective cut down by placing a funnel-stop behind the rear lens.

Good results are usually obtained if: (1) Clean slides and coverglasses of correct thickness are used. (2) The special dark-field condenser is carefully centered. (The top of the condenser should be thoroughly cleaned in order to facilitate the finding of the centering circle). (3) The top of the condenser and the bottom of the slide are brought into close proximity and then adjustments made to correct for variation in slide thickness by raising or lowering the illuminator. (4) Air bubbles are removed from the oil between the condenser and the slide. (5) A thin rather than a thick preparation is made. (6) The presence of excessive amounts of large elements such as blood cells is avoided.

MICROMETRY

The unit of length in micrometry is the micron which is designated by u and is 1/1000 of a millimeter. A millimicron is 1/1000 of a micron or 0.000001 millimeters, and is written mu or uu. Measurements may be made with a disc micrometer which is a glass disc with ruled lines which can be placed on the diaphragm of the ordinary ocular with the ruled surface down. The image of the object is formed at the level of this diaphragm so that the lines of the image cut those of the lines on the disc. The micrometer must be calibrated with each objective to determine the value of the spaces. All that is necessary subsequently in measuring is to count the number of lines or spaces which the image of the object fills, and then, knowing the value of each space for that objective, to multiply the number of spaces by the value of a single space. The tube length must always be the same as that used in the standardization.

The ocular micrometer is usually ruled with 50 or 100 lines or spaces, separated by longer lines into groups of 5 and 10. It may be standardized with a regular stage micrometer if one is available. Stage micrometers have ruled lines separated from one another by 1/10 mm. (100u). Some of these 1/10 mm. spaces are again ruled with 10 lines giving spaces which are only 1/100 mm. (10u) apart. If one does not have such a scale, however, a haemocytometer makes a very satisfactory substitute. In any system of ruling of the haemocytometer, whether it be Thoma-Zeiss, Turck or Neubauer type, there are small squares in the central ruling of crossed lines which are used for counting red cells. These are in groups of 16 and each one is 1/20 mm. or 50u square.

Having focused the ruling of the haemocytometer, the number of small squares covered by the 50 or 100 ruled lines of the ocular micrometer is noted and the number of squares so overlaid is multiplied by 50 (since each square

is 50 microns square) which gives the micron value of the entire ocular micrometer ruled space. To obtain the value of each space, divide by 50 or 100 according to the number of lines on the ocular micrometer. To measure a bacterium, for example, the bacterium is brought into focus and the number of spaces covering it noted. This number of spaces is multiplied by the value in micra of the space for the objective used.

The diameter of the microscopic field may be determined by the use of a stage micrometer or an haemocytometer. From this measurement the area covered may be calculated.

XII. DEFINITIONS.

Aerobic, growing in the presence of free oxygen; strictly aerobic, growing only in the presence of free oxygen.

Aerogenic, producing gas.

Agglutinin, an antibody having the power of clumping suspensions of bacteria or other cells.

Alexin, see complement.

Amorphous, without visible differentiation of structure.

Anaerobic, growing in the absence of free oxygen; strictly anaerobic, growing only in the absence of free oxygen; facultative anaerobic, growing both in presence and in absence of oxygen.

Anamnestic reaction, stimulation of further production of antibodies upon injection of an antigenically different substance.

Antibody, a specific substance produced by an animal in response to the introduction of an antigen.

Antiformin, a proprietary preparation of a strongly alkaline solution of sodium hypochlorite. It does not dissolve acid-fast organisms like tubercle bacilli and is used in isolating the latter from feces.

Antigen, a substance which when introduced into an animal body stimulates the animal to produce specific bodies that react or unite with the substance introduced.

Antiseptic, a substance that opposes sepsis, putrefaction or decay by preventing or arresting the growth or action of micro-organisms. This may be accomplished by prevention of growth and reproduction through continued contact between the antiseptic and the organism or through destruction of the organism after a relatively short exposure.

Antitoxin, an antibody having the specific power of uniting with and neutralizing a toxic substance.

Arthropods, fleas, lice, mites, flies, mosquitoes.

Ascospore, one of a set of spores contained in a special sac, or ascus.

Ascus, sporangium or spore case of certain lichens and fungi, consisting of a single terminal cell.

Aseptically, without permitting bacterial contamination.

Autogenous vaccine, prepared from bacteria freshly isolated from the patient who is to be treated with it.

Autolysis, self-disintegration of cells by the action of their own enzymes.

Bacillus, a rod-shaped form of bacterium.

Bactericidal, destructive to bacteria.

Bacteriostatic, preventing bacterial growth, but without killing the bacteria.

Berkefeld filter, a filter made of diatomaceous or fullers' earth.

Bipolar, at both poles or ends of the bacterial cell.

Capsule, a gelatinous envelope surrounding the cell membrane of some kinds of bacteria.

Chromogenesis, the production of color.

Coccus, spherically shaped form of bacterium.

Columella, in molds, the central axis of the spore case, around which the spores are arranged.

Communicable disease, a disease in which the causative agent may readily be transferred from one person to another directly or indirectly; contagious or infectious.

Complement, a normal, thermolabile constituent of serum which reacts together with immune antibodies in the sense that, when the antigen has been specifically sensitized by the antibody, the complex so formed can be acted upon by the complement.

Conidia, asexual spores formed by splitting off from the summit of a conidiophore.

Conidiophore, branch of the mycelium of a fungus which bears conidia.

Cytolysis, a dissolving action on cells.

Disinfectant, an agent that frees from infection by killing bacteria or other micro-organisms.

Endospores, thick-walled spores formed within the bacterial cell; i.e., typical bacterial spores like those of B. anthracis or B. subtilis.

Endotoxin, a toxic substance produced within an organism and not excreted. Feebly antigenic.

Exotoxin, a toxic substance excreted by a micro-organism and hence found outside the cell body. An antigenic poison.

Exudate, a collection or deposit of fluid resulting from an inflammatory process and usually due to an infection. It is usually rich in cells and coagulable materials and is usually alkaline in reaction.

Facultative anaerobe, see anaerobic.

Filtrable virus, etiological agent (of an infectious disease) so small that it will pass through the pores of a Berkefeld or Chamberland filter.

Filtrate, a liquid which has passed through a filter.

Flagellum, a mobile, whip-like process or cilium.

Flocculent growth, particles of small adherent masses of bacteria of various shapes floating in the culture fluid.

Fusiform, spindle-shaped.

Germicide, same as disinfectant.

Hamster, a rat-like rodent with cheek pouches.

Hemolysin, an antibody causing hemolysis.

Hemolysis, a dissolving action on red blood cells resulting in liberation of hemoglobin.

Heterologous, different with respect to type or origin.

Homologous, the same with respect to type or origin.

Hypha, one of the filaments composing the mycelium of a fungus.

Immune serum, an animal fluid containing an antibody.

Intracerebral, into the brain.

Intracutaneous, into the skin.

Intraperitoneal, into the abdominal cavity.

Lysin, an antibody which has the power of causing the dissolution of cells or liberation of their contents.

Monotrichous, having a single flagellum at one pole.

Microaerophile, requiring only a small amount of free oxygen.

Monovalent, as applied to sera, having the ability to combine with only one type of antigen.

Multivalent, (polyvalent) as applied to sera, having the ability to combine with more than one type of antigen.

Mycelium, the vegetative body of a fungus composed of a mass of filaments called hyphae.

Neufeld reaction, used to diagnose type of pneumococci. Upon addition of type-specific immune rabbit serum the capsule swells markedly (Quellung).

Opaque, impervious to light.

Parasitic, deriving its nourishment from some living animal or plant upon which it lives and which acts as a host; not necessarily pathogenic.

Pathogenic, not only parasitic but also causing disease to the host.

Pellicle, bacterial growth forming either a continuous or an interrupted sheet over the culture fluid.

Peptonization, rendering curdled milk soluble by the action of peptonizing enzymes.

Peritrichate, applied to the arrangement of flagella, indicates that they are distributed over the entire surface of an organism.

Per os, through the mouth.

Plasma, animal fluids such as blood or lymph freed only of organized cellular elements. Blood plasma is the fluid portion of unclotted blood.

Pleomorphic, occurring in various distinct forms.

Polyvalent, see multivalent.

Precipitin, an antibody having the power of precipitating soluble antigens.

Prophylaxis, preventive treatment for protection against disease.

Protection test, a procedure in which therapeutic or convalescent sera are evaluated in terms of ability to protect experimental animals against known pathogenic agents.

Proteolytic, capable of splitting proteins into simpler compounds.

Putrefaction, the process of decay accompanied by a disagreeable odor.

Pyogenic, producing pus.

Rennet curd, coagulation of milk due to rennin or rennin-like enzymes, distinguished from acid curd by the absence of acid.

Rugose, much wrinkled.

Saccharolytic, capable of splitting up sugar.

Saprogen, an organism causing putrefaction.

Saprophytic, unable to grow in the absence of organic matter; i.e., not autotrophic but not parasitic, as a living host is not necessary.

Satellite, a smaller colony growing best in the proximity of a larger colony.

Schizomycetes, class of vegetable micro-organisms, the bacteria or fission-fungi.

Septicemia, condition due to the growth of pathogenic bacteria in the blood.

Serum, the fluid portion of clotted blood.

Sinus tract, a suppurating channel or fistula.

Spindled, larger at the middle than at the ends; applied to sporangia; also refers to the forms frequently called clostridia.

Sporangia, cells containing endospores.

Sporangiophore, the thread-like stalk which bears the sporangium of molds at its tip.

Spore, single-celled resistant bodies, capable of developing at once or after a time into an independent organism.

Sterigma, a stalk or support as the specialized cell that forms the immediate support of the conidia in many fungi.

Stock vaccine, a vaccine made from cultures kept on hand in the laboratory.

Stolon, a trailing branch that is disposed to take root.

Subterminal, situated toward the end of the cell but not at the extreme end; i.e., between the positions denoted eccentric and terminal.

Thermophilic, growing best at high temperatures, i.e., 50 degrees C. or over.

Toxoid, detoxified toxin.

Translucent, allowing the passage of some light.

Transudates, collections or deposits of fluid due usually to noninflammatory processes due to disturbances of circulation. They are usually light yellow in appearance and contain less cells than do exudates. Their pH is usually the same as that of blood.

Vaccine, generally any suspension or solution of an infectious organism used as an immunizing agent.

Viscid, sticky or adhesive.

Voges Proskauer reaction, test for the presence of acetyl-methyl-carbinol to distinguish between the colon and the aerogenes groups of bacteria.

Vole, a short-tailed, mouse-like rodent, as the meadow mouse, characterized chiefly by its dentition.

Weil-Felix reaction and Widal test, agglutination tests used in the diagnosis of typhus or typhus-like fevers and typhoid fever respectively.

INDEX

-A-

-B-

-C-

-F-

-G-

-M-

-Q-

-R-

-T-

-U-

-V-

-W-

-X-

-Y-

-Z-

Moore