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# DIAGNOSTIC PROCEDURES AND REAGENTS

TECHNICS FOR THE
LABORATORY DIAGNOSIS AND CONTROL OF THE
COMMUNICABLE DISEASES

THIRD EDITION

The Subcommittee on Diagnostic Procedures and Reagents has prepared this report. It has been reviewed by the Coördinating Committee on Laboratory Methods and recommended for publication. Publication has been authorized by the Committee on Research and Standards of the American Public Health Association.

Publication Office
AMERICAN PUBLIC HEALTH ASSOCIATION, INC.
1790 Broadway
New York 19, N. Y.

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# FOREWORD

THE third edition of *Diagnostic Procedures and Reagents* has been prepared, as were the previous editions, with the primary purpose of being a useful guide to the laboratory worker. This means more than the preparation of a manual, with steps of techniques arranged in consecutive order and with no explanation of the principles involved.

In the preparation of chapters, judgment of the Referee as to the manner of presentation was accepted. The choice of methods to be described in detail was the decision of the individual Referee. Since this volume cannot attempt to standardize methods where many methods are available, and where no satisfactory means of standardization are available, it is felt that this individual manner of presentation enhances the value of the book by giving adequate discussion of the methods with which authors have had the greatest experience. In some chapters it has been necessary to include the essential clinical features of disease in order to guide the laboratory worker in the collection and handling of suitable specimens.

The chapter on the serology of syphilis was prepared as a discussion of basic principles and problems. It was felt that the variety of methods in use and the minute technical details necessary for uniformity, as well as the rapid changes taking place with the introduction of cardiolipin antigen, make a description of techniques impossible in a volume of this size. Complete descriptions of techniques by author-serologists are published by the U. S. Public Health Service.

It is the work of the referees that has made this book possible and it is their expert knowledge and experience that give it value.

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# Culture Media

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*U. S. Army*

I. Introduction
II. Ingredients
III. Sterilization
IV. Table of Culture Media
V. Preparation of Formulas

## I. INTRODUCTION

THE ultimate aim in the preparation of a culture medium is the production of a substrate that will meet the nutritional needs of the organism under study. While most media have been developed as tools for the study of certain phases of bacterial physiology as a means of identification, several important formulas have been derived to supply essential growth factors for the adequate nutrition of fastidious organisms. Although the essential growth requirements have been determined for many organisms, the majority of media in common use are made from empiric formulas of ingredients which experience has proved are adequate sources of energy and structural material for bacterial growth. Many factors other than the supply of proper nutritional needs enter into the preparation of good culture media. Freely available water is essential for all metabolic processes, the hydrogen ion concentration must be carefully controlled and inhibitory substances must be very carefully controlled.

All bacteria except the true autotrophs secure their energy from the oxidative decomposition of organic nitrogenous compounds, carbohydrates, organic acids or salts. A source of nitrogenous food material is required by all bacteria. Most organisms of medical importance require the nitrogenous base as a protein or protein derivative. The use of peptone to supply the required nitrogen was first described by C. von Nägeli[1] in 1872.

Peptones as a general class are crude products resulting from the hydrolytic cleavage of proteins. They consist of mixtures of proteoses,

true peptones, polypeptids and amino acids. The composition of the peptone will vary with the protein substrate digested, the enzyme system used for digestion, and the time period allowed for the digestion process. These protein derivatives are excellent sources of nitrogen because they are soluble and are not coagulated by heat. Peptones are well adapted to the synthetic metabolism of bacteria because of their high amino acid content. Mixtures of amino acids can also be prepared by the strong acid hydrolysis of protein substrates. In these acid hydrolysates, the amino acid tryptophane, an essential metabolite for many pathogens, is destroyed concomitantly with the cleavage of the peptones and peptids and the destruction of several other necessary growth factors. As a result, these substances must subsequently be added to make a complete amino acid mixture.

Meat extract has been used as a growth supplement since its first introduction by C. von Nägeli and W. von Nägeli in 1875. This work was reported in the first comprehensive review of the nutritive requirements of microörganisms in which von Nägeli[2] summarized the historically interesting studies which he and his collaborators had pursued throughout the previous ten years. As the name implies, meat extract is a mixture of the water soluble constituents—nitrogenous compounds, fermentable carbohydrates and minerals, found in muscle tissue. Meat extracts are very useful in empiric formulas because they contain substances which have a markedly stimulating effect on bacterial growth. Many of these substances are not supplied by peptones in adequate quantities.

Amino acids and other protein derivatives used in bacterial media are not only important for their nitrogen supply capacity, but also for other biochemical properties which they possess as a result of their chemical structure. All proteins and protein derivatives are amphoteric and act as buffers tending to stabilize the rapid changes in pH which would ordinarily result from the addition or the production of milli-equivalent amounts of either hydrogen or hydroxyl ions. The metabolic utilization of amino acids by microörganisms generally results in the production of alkaline residues which tend to compensate for the acid residues which ordinarily result from carbohydrate utilization.

Carbohydrates and organic acids or salts are incorporated in culture media as a readily available source of energy for the metabolism of microörganisms. They are also useful in fermentation and utilization tests primarily designed for the identification of bacteria. When an organism elaborates enzymes which are capable of oxidizing carbon com-

pounds, energy is released for the growth processes. Utilization of these compounds can be followed by observation of the cultures for the disappearance of the original compound or the appearance of the end products of the oxidative decomposition. While many of the mechanisms involved in enzymic catalyses remain to be elucidated, stereochemical specificity was noted as long ago as 1858 when Pasteur[3] recorded that d-ammonium tartrate disappeared from an inoculated culture medium, but when l-ammonium tartrate was substituted in the same medium, the organism did not utilize it. Most bacterial enzymes or the ability to produce them are apparently hereditarily transmitted and constant. This fact aids in the identification of bacteria since the presence of certain enzyme systems may be used to establish the pattern of the unknown organism for correlation with previously established patterns.

Very little is known concerning the absolute inorganic requirements for adequate bacterial metabolism, but mineral salts do have an essential role. Salts are important as constituents of the cell protoplasm, as enzyme activators, and as physical agents in the maintenance and regulation of osmotic pressure and the ionic balance between cell and substrate. The metallic ions, iron, copper and manganese, probably assist in the regulation of oxidation reduction potentials by virtue of their reversible valence changes. Sodium, potassium, calcium, and magnesium are important as enzyme activators. Phosphorus in the form of phosphates plays a large part in the release of energy from organic compounds by virtue of its ability to form high energy bonds. It may be presumed that the halides assist in balancing ionic equilibria. Definitive studies of the effect of mineral salts in the metabolism of bacteria are hindered by the fact that many of the common and necessary metallic salts are present in variable amounts in many of the compounds necessary to sustain bacterial growth.

Our knowledge of the influence of growth factors and vitamins on microbial growth has increased markedly in the past two decades. All of this work has shown that the growth requirements of bacteria must be considered individually as to type, species, and strain because there is no pattern of essential ingredients common to all types of bacteria. Many bacteria can synthesize all of their essential amino acids, growth stimulants and vitamins from inorganic sources. In contrast, many bacteria require rather complex mixtures of amino acids, accessory factors and vitamins for adequate growth. Pasteur[3] used yeast extract and muscle juice sterilized by filtration as sources of nitrogen and

commented on the markedly stimulatory effect. Yeast extract is still used empirically and is a very excellent source of growth factors.

An early definitive study of accessory growth factors was made by Davis[4] in 1917 while working with *Hemophilus influenzae*. Davis's work demonstrated that two factors, later termed X and V by Thjotta and Avery,[5] were essential components which had to be added for growth to occur in media of peptone base. In 1937 Lwoff and Lwoff[6] identified the V factor as coenzyme I, diphosphopyridine nucleotide. The X factor had previously been identified as an iron complex. The early studies on the growth factors required by *H. influenzae* as exemplified by the papers cited and by many others of the same period were directed toward the solution of the practical problem of developing media for the isolation and study of an organism. In contrast, the papers subsequently published by the Lwoffs and by many other workers have been primarily concerned with the elucidation of the biochemical nature of the factors involved and study of the probable mechanism of their action.

Comprehensive studies of the nutritional requirements of many other bacteria are available in the literature of bacteriology and biochemistry. Unfortunately, the information derived from the exhaustive study of one genus cannot be utilized in its entirety in the study of a different genus because of the marked discrepancies in the requirements of different genera.

As exemplified by the work with *Neisseria gonorrhoeae*, some of the original studies have laid the groundwork for investigation of strain variation of nutritional requirements within the species. Working with complex media, Lankford, Scott, Cox, and Cooke,[7] and Lankford and Snell[8] demonstrated that many strains of *N. gonorrhoeae* required glutamine for growth on original isolation. Later Lankford and Skaggs[9] reported the isolation of strains which required cocarboxylase. There is some disagreement as to the organic acids essential for growth of *N. gonorrhoeae*. Plack, Stockinger, and Carpenter[10] reported that a medium of nine organic acids, five inorganic salts, and glucose would sustain growth of the gonococcus. Other workers[11] found this medium unsatisfactory. Working with a closely related organism, Frantz[12] found that a medium composed of d-glutamic acid, l-cystine and ammonium chloride supplemented with a mixture of four inorganic salts and glucose was capable of supporting the growth of *Neisseria meningitidis*. It may be that the essential growth requirements of the majority of strains of *N. gonorrhoeae* are relatively as simple and that the main

difficulty to be overcome in the isolation of the gonococcus on media, empiric in formula, is the suppression of inhibitory substances as has been suggested by Mueller and Hinton.[13]

Space does not permit further discussion of specific points in the field of bacterial nutrition. As guides to further study in this interesting field, the excellent reviews of Knight[14, 15] and the well documented chapters of Porter[16] should be utilized for their practical and historical interest.

## II. INGREDIENTS

The quality of the ingredients used in the preparation of culture media is of utmost importance. It is axiomatic that a good workman can do better work with better tools, and culture media are the essential tools used in bacteriology. The quality of a culture medium depends upon the quality of the ingredients of which it is made and on the care with which it is prepared. Uniformity of product can be secured only when all materials used are of uniform quality at all times. In most instances, distilled water should be used in the preparation of bacteriologic media. Chemicals used should conform with the highest standards established by the American Chemical Society unless otherwise specified. Those organic compounds and other chemicals for which standards of purity have not been established, should be procured only from sources of established integrity and reliability.

Bacteriological peptones were studied by a committee of the board for the revision of the *U.S. Pharmacopocia*. As a result of this study, exacting standards and specifications were included in *U.S.P.* XIII for a bacteriological peptone—Pancreatic Digest of Casein. The *U.S.P.* description includes a section listing the cultural characteristics which the peptone must meet in order to be approved. Pancreatic Digest of Casein is available under various trade names from several reliable firms and because the *U.S.P.* standards control the purity as well as the biologic properties, such preparations are strictly comparable. It is highly recommended that Pancreatic Digest of Casein—a bacteriological peptone, *U.S.P.* XIII, be used in the preparation of all basic culture media.

Fermentable compounds—carbohydrates, polyhydric alcohols and the organic acids or their salts, are usually added to culture media to detect specific enzyme systems. It is essential that only substances of highest purity be utilized for this purpose if the results obtained are to be considered reliable. Special precautions are often necessary in

the sterilization of fermentable compounds that are not heat stable, as prolonged or undue exposure to heat will destroy the identity of the substance. Mudge[17] has reported that 4 per cent of added maltose was hydrolyzed to glucose on the first day and "a very great" percentage on the second day when subjected to fractional sterilization in the Arnold. In contrast, less than 1 per cent of the maltose was hydrolyzed by careful autoclaving. These figures indicate that maltose and other compounds similarly hydrolyzable under the influence of heat should be sterilized by filtration unless excellent autoclaves are available.

Adequate growth of most organisms will be sustained on basal media of peptone and meat extract. When it is necessary to supplement such a basal medium, yeast extract, sterilized by filtration, is probably the most economical source of essential metabolites. Commercial liver extracts, malt extracts and extract concentrates are also adequate substitutes in bacterial nutrition. Pure enzymes, vitamins and coenzymes are necessary only in definitive studies for the determination of essential growth factors of bacteria and, in general, their use can be limited to research investigations.

Several infusion base formulas are listed in the compilation of culture media without actual proof that the resultant product will supply the nutritional needs of fastidious organisms any better than a solution of pancreatic digest of casein and meat or yeast extract. Stokes, Gunness, and Foster[18] determined the levels of eight members of the vitamin B-complex supplied by ingredients commonly used in culture media. They were able to classify the culture media ingredients in four groups based upon the abundance of the vitamins of the B-complex. Meat extract, brain heart infusion and heart infusion were of the same order. Brewer[19] has reported that vegetable digests were more satisfactory than meat infusion in supporting adequate growth of several of the more fastidious organisms. Economically, infusion media are very expensive because the high cost of the initial ingredients is coupled with an exorbitant expenditure of time and labor in their preparation. At the present time the cost of the initial ingredients for one liter of extract-peptone solution is approximately 15 cents, and it can be prepared for sterilization in less than 10 minutes. In comparison, the cost of the ingredients for one liter of infusion broth is approximately six to seven times as much and will require from 2 to 5 hours of labor depending on the method of infusion and the type of meat used. Until definitive assays for known and unrecognized growth

factors conclusively prove that infusions are superior to yeast or meat extracts as a source of essential metabolites, it is recommended that infusion formulas be emended by substituting a solution of 0.3 per cent yeast extract and 1.5 per cent pancreatic digest of casein, liter for liter.

Inhibitory substances may be added to culture media when agar containing unknown impurities is incorporated into the medium as a solidifying agent. Levine and Schoenlein [20] cited Dominikiewicz method (1908) for purifying agar to remove inhibitory substances. Ayers, Mudge, and Rupp [21] demonstrated the necessity of washing agar for the elimination of inhibitory substances. These problems can be largely eliminated if agar is procured from reliable firms specializing in the supply of ingredients for culture media preparation. An agar with 1 per cent foreign insolubles and 6.5 per cent ash of unknown mineral composition meets the specifications established in *U.S.P.* XIII. Obviously, such an agar could contain many inhibitory salts and would have to be purified before it could be used in the preparation of culture media.

For economy of time and working space, dehydrated media can be used without sacrificing the quality of the ultimate product. The competition between the producers of dehydrated culture media results in uniformity and quality in the product, not obtainable in small laboratories whose budgets are too small to permit inclusion of a special section for the preparation of media. Constant efforts are being made by all manufacturers to improve production methods. Formulas which contain unstable compounds or unstable compounds formed by mixing two ingredients shortly before use are not adapted to dehydration, although the basal medium, or adequate substitutes, may be available. Many of the formulas listed in the compilation of culture media are available as the dehydrated powder and their use is highly recommended. Economically, it may be advantageous to prepare basic broths and agars from dehydrated ingredients, rather than to purchase the completed medium in dehydrated form. For many of the selective and differential media, the use of dehydrated products is almost mandatory.

## III. STERILIZATION

Autoclave sterilization is the method of choice in the preparation of all culture media except those containing compounds which are destroyed by exposure to heat. It should be the aim in all media prep-

aration to sterilize the product at the lowest temperature and shortest time period which experience has shown essential for sterility. To achieve this aim certain definite principles must be observed:

1. The autoclave must be efficient and should be equipped with a drain line thermometer.
2. The autoclave must not be overloaded.
3. Bulk media should be preheated to avoid undue time lag.
4. Tubes should be packed loosely in baskets and never placed in containers capable of entrapping air.
5. All air must be exhausted from the autoclave to maintain the proper pressure-temperature-time relationships.
6. Flasks and tubes should not be filled to more than two-thirds capacity.
7. The media should be removed from the autoclave as soon as possible.

Adoption of these simple rules will aid in the production of good media. Prolonged sterilization of agar media will result in the production of a precipitate and occasionally in the destruction of the jellifying properties of agar. Excessive heating generally lowers the pH of the medium to a marked degree.

The time-temperature relationships essential for effective sterilization will vary with the quantity of medium in each flask or tube being processed. Toennies and Gallant [22] reported satisfactory sterilization of media for bacterimetry studies by exposing separated tubes at 121° C. (15 lb.) pressure for 2½ minutes. This procedure is not recommended for routine use in sterilization because the wide separation of tubes so definitely limits the capacity of the autoclave. For media in tubes packed *loosely* in baskets and for flasks containing not over 50 ml. of liquid media, exposure at 118° C. (12 lb.) for 10 minutes will insure adequate sterilization. Agar should be dissolved in the medium before sterilization, or, if solidified, the agar should be melted so that the convection currents in the liquefied medium can distribute the heat evenly throughout the flask. Flasks containing 100 ml. to 1,000 ml. can be sterilized by exposure at 121° C. (15 lb.) for 15 minutes, if adequately preheated. Media in quantities greater than one liter must be heated for longer periods depending upon the shape of the container, the efficiency of the autoclave, and the volume of medium being sterilized.

The foregoing discussion is applicable only to liquid, permanently solid and reversibly liquid-solid media. Special additional precautions must be followed to attain successful inspissation and sterilization of

heat coagulable protein media. The autoclave must be equipped with a valve on the exhaust line so that all ports on the autoclave can be completely closed before the steam is turned on. The tubes of media are slanted in racks or baskets not more than three deep. Before placing the racks or baskets in the autoclave, they should be wrapped in cloth or paper and covered with layers of paper to prevent too rapid heat exchange. With all of the ports closed, the steam is turned on and the pressure raised to 15 lb. as rapidly as possible. After 10 minutes, the entrapped air is gradually replaced with steam at a rate that maintains the pressure in the autoclave constant. Timing of the period of sterilization is started when the temperature on the drain line thermometer reaches 120° C. After a 15 minute sterilization period, *all* ports on the autoclave are closed so that the return to atmospheric pressure will be slow and the heat loss gradual. This slow return to atmospheric pressure is very important, and media should not be removed from the autoclave, or the door of the autoclave opened until definitely cooled. The same wrappings that prevented too rapid ingress of the heat during the period of inspissation, tend to retain the heat during the cooling process. The use of screw capped tubes and vials can be definitely recommended for all media requiring inspissation as the increase of the air pressure in the sealed tube assists in the maintenance of a smooth surface.

## IV. TABLE OF CULTURE MEDIA

1. Peptone Solution for Single Carbohydrates
2. Peptone Solution for Indole Production
3. Peptone Broth, Infusion-free and Buffered
4. Nutrient Extract Broth
4a. Nutrient Extract Agar
5. Broth, Meat Infusion, Plain
5a. Agar, Meat Infusion, Plain
6. Broth, Meat Infusion, Double Strength
7. Broth, Liver Infusion
7a. Agar, Liver Infusion
8. Broth, Beef Infusion with Proteose for Streptococci
9. Broth, Beef Infusion with Dextrose and Indicator
10. Broth, 10 Per cent Meat Infusion
11. Broth, Sodium Azide-Crystal Violet Enrichment
12. Broth, Tryptose Phosphate
13. Broth, Tryptose Dextrose Vitamin B
13a. Agar, Tryptose Dextrose Vitamin B
13b. Agar, Tryptose Dextrose

14. Ground Meat Medium for Anaerobes
15. Gelatin, Extract
16. Milk with Indicator
17. Agar, Staphylococcus Medium of Dolman, Wilson and Cockcroft
18. Agar, Russell's Double Sugar, modified
19. Agar, Kligler's Iron, modified
20. Agar, Triple Sugar Iron
20a. Agar, Triple Sugar Iron, modified
21. Agar, Eosin-Methylene Blue, modified
22. Agar, Endo's, Robinson and Rettger
23. Agar, Bismuth Sulfite, Wilson and Blair
24. Agar, Sodium Desoxycholate
24a. Agar, Sodium Desoxycholate Citrate
24b. Agar, Sodium Desoxycholate Citrate, modified
24c. Agar, Sodium Desoxycholate Citrate Lactose Sucrose
25. Agar, Bile Salts Citrate (Difco SS)
26. Agar, Bile Salts, MacConkey, modified
27. Broth, Bile Glycerol Peptone
28. Broth, Tetrathionate Enrichment, modified
29. Broth, Selenite F
30. Broth, Urea, Rustigian and Stuart
31. Agar, Urea, Christensen
32. Agar, Motility, modified
33. Agar, Blood, plain
33a. Agar, Chocolate Blood
33b. Agar, Serum
34. Agar, Blood, Bordet-Gengou, modified
35. Agar, Blood Glucose Cystine, Francis
36. Agar, Blood Cystine Tellurite
36a. Agar, Tellurite for Type Determination
37. Agar, *in vitro* Virulence Test
38. Agar, GC Base with Hemoglobin
39. Agar, McLeod's with Plasma and Hemoglobin
40. Agar, Proteose No. 3 with Plasma and Hemoglobin
41. Agar, Gelatin Blood
42. Agar, Gelatin Egg Albumin
43. Agar, Sabouraud's
44. Agar, Corn Meal
45. Agar, Crystal Violet Oxgall
46. Agar, Potato Dextrose for Fungi
47. Agar, Coccidioides Isolation
48. Agar, Histoplasma Isolation
49. Broth, Synthetic for Histoplasmin and Tuberculin
50. Egg Yolk, Potato Flour Medium, Trudeau Committee
51. Egg, Potato Flour and Mineral Salt Medium, Lowenstein-Jensen
52. Semi-synthetic Tuberculosis Medium, Dubos
53. Egg Yolk, Water Medium, Besredka, modified
54. Egg Media, solidified

55. Blood Serum Mixture, Loeffler's
56. Agar, Semi-fluid
57. Inulin Serum Water, Hiss
58. Broth, Thioglycolate, modified
59. Broth, Endameba Culture
60. Broth, Todd-Hewitt, modified
61. Medium for Gonococci and Meningococci, Mueller's
62. Agar, Serum Tellurite for Diphtheria

## V. PREPARATION AND FORMULAS

### *1. Peptone Solution for Single Carbohydrates*

| | |
|---|---|
| Pancreatic Digest of Casein, U.S.P. XIII * | 10 gm. |
| Sodium chloride | 5 gm. |
| Bromcresol purple (0.04% aqueous solution) † | 10 ml. |
| Water | 1,000 ml. |

* Pancreatic Digest of Casein is specified because it is free of bacteriologically detectable carbohydrate. It is supplied under various trade names by several manufacturers.
† Any other suitable indicator can be substituted.

a. Dissolve the peptone and salt in the water. Add the indicator and adjust to pH 7.4–7.5.
b. Dispense in convenient amounts and sterilize in the autoclave.
c. Aseptically add a sterile aqueous solution of the desired carbohydrate equivalent to 0.5–1.0 gm. of the dry carbohydrate to each 100 ml. of medium.
d. Dispense at least 2 ml. amounts in small sterile tubes and incubate at 37° C. for 24 hours as a sterility check.

or

b. Add 0.5–1.0 gm. of the desired carbohydrates per 100 ml. of medium.
c. Tube, pack *loosely* in baskets and sterilize at 118° C. (12 lb.) for 10 minutes.
d. Remove from the autoclave as soon as possible and incubate.

### *2. Peptone Solution for Indole Production*

| | |
|---|---|
| Pancreatic Digest of Casein, U.S.P. XIII * | 10 gm. |
| Sodium chloride | 5 gm. |
| Water | 1,000 ml. |

* Satisfactory positive results for indole production by bacteriologic test is required by the U.S.P. XIII specifications.

Dissolve the peptone and salt in the water and adjust to pH 7.2. Tube in desired amounts, 3–5 ml., and sterilize in the autoclave at 121° C. (15 lb.) for 15 minutes.

### 3. *Peptone Broth, Infusion-free and Buffered*[23]

| | |
|---|---|
| Peptone * | 20 gm. |
| Sodium chloride | 5 gm. |
| Dipotassium phosphate $K_2HPO_4$ | 1 gm. |
| Monopotassium phosphate $KH_2PO_4$ | 1 gm. |
| Dextrose | 7.5 gm. |
| Tap water to make | 1 kg. |

* Several peptones including Pancreatic Digest of Casein, Nutripeptone (Wilson), Proteose Peptone (Difco), Thiotone (BBL) and Peptone M (Albimi) have been found satisfactory for the growth of hemolytic streptococci in this medium.

a. Dissolve the salts † and peptone in part of the water and make up weight to 1 kg. Autoclave in bulk 40 minutes.
b. Add 7.5 gm. of dextrose and make up weight to 1 kg. Filter through paper. Dispense as desired and autoclave 20 minutes.

### 4. *Nutrient Extract Broth* *

| | |
|---|---|
| Beef extract | 3 gm. |
| Peptone | 10 gm. |
| Water | 1,000 ml. |

* Beef extract contains glycogen and other fermentable carbohydrate derived from muscle which must be destroyed before using this medium as a carbohydrate base. Several lots of yeast extract have been checked with *Escherichia coli* as the test organism and found to be free of fermentable carbohydrate. Referee's note.

a. Dissolve ingredients in water and adjust to pH 7.0.
b. (Only if for carbohydrate base medium) Destroy fermentable carbohydrate by inoculation with *Escherichia coli* or *Escherichia freundii* and incubate at 37° C. for 6 hours. Kill organisms by heating in flowing steam or boiling.
c. Filter to clear. Dispense as desired and autoclave at 121° C. (15 lb.) for 20 minutes. For fermentation media proceed as for CM 1.

### 4a. *Nutrient Extract Agar*

| | |
|---|---|
| Extract broth (CM 4) | 1,000 ml. |
| Agar | 15–20 gm. |

a. Dissolve the agar in the extract broth with heat. The temperature must be raised to the boiling point to obtain satisfactory solution of the agar.

† 2.3 gm. of the anhydrous dipotassium salt will provide an equal amount of phosphate ion and can be substituted for 1.0 gm. of the monopotassium salt and 1.0 gm. of the dipotassium salt. If this is done, less alkali is required to adjust the reaction to pH 7.8. The buffer capacities are equivalent. The difference in buffer capacity when distilled water is substituted for tap water will depend entirely on the buffer capacity of the tap water which will vary widely. Any free $Ca^{++}$ or $Mg^{++}$ ion in the tap water will precipitate some of the phosphate ion and decrease the buffer capacity. The sterilization period is without effect on the buffer capacity, but empirically, the long heat exposure should lower the level of the accessory growth factors so essential to the proper nutrition of the streptococci. It is suggested that a shorter preliminary heating period be tested. Referee's note.

b. Dispense in tubes or flasks as desired and sterilize in the autoclave at 121° C. (15 lb.) for 15 minutes.

### 5. *Meat Infusion Broth, Plain* *

| | |
|---|---|
| Lean beef muscle | 500 gm. |
| Peptone | 20 gm. |
| Sodium chloride | 5 gm. |
| Water | 1,000 ml. |

* In 1930, Levine and Schoenlein[20] tabulated 40 distinct procedures for the preparation of infusion broths using meat of some type as a base. Each of these procedures had been subject to variation by various authors, apparently as ideas occurred to them. There is no exact rule that can be followed for the preparation of an infusion, but the general lines given above can serve as a guide. In the referee's laboratory, a broth of 2 per cent Pancreatic Digest of Casein, 0.5 per cent yeast extract and 0.5 per cent sodium chloride has been substituted for infusion broths in the cultivation of many of the more fastidious organisms. In all cases tried, the growth obtained has been as heavy or heavier than the growth obtained in the infusion media. Limited use has been made of a broth composed of 1 per cent Pancreatic Digest of Casein, 1 per cent of the papaic digest of soy bean meal described by Brewer,[19] and 0.5 per cent yeast extract. This has proved to be a very excellent medium, but cannot be used for the determination of fermentation reactions because fermentable carbohydrate is introduced in the papaic digest.

a. Remove all fascia and fat from the muscle and grind in a food mill.
b. Infuse ground meat overnight in water at 4° to 6° C. in an enameled or stainless steel container.
c. Heat to 45° C. and hold between 45° C. and 50° C. for 1 hour.
d. Boil for ½ hour and allow to cool without stirring. Skim off fat and lift out the firm coagulum.
e. Pour off supernatant fluid into a flask and transfer meat to a meat press to express all juices as completely as possible. Combine expressed juice with the supernate already collected.
f. Filter through glass wool and make up to original volume.
g. Dissolve peptone component and salt and adjust to pH 7.4.
h. Boil for 20 minutes and pour into tall, glass cylinders until suspended particles have settled.
i. Decant clear supernate and dispense as desired. Sterilize in the autoclave at 121° C. (15 lb.) for 15 minutes.

### 5a. *Meat Infusion Agar, Plain*

| | |
|---|---|
| Infusion broth (CM 5) | 1,000 ml. |
| Agar | 15–20 gm. (as desired) |

a. Dissolve the agar in the broth with heat and adjust the reaction to pH 7.2–7.4.
b. Dispense as desired and autoclave at 121° C. (15 lb.) for 15 minutes.

### 6. *Meat Infusion Broth, Double Strength*

Proceed as for Meat Infusion Broth, Plain (CM 5), except that 1 kg. of ground meat is infused with 1,000 ml. of water. Agar in the amount of 15–20 gm. per liter can be added to make Meat Infusion Agar, Double Strength.

### 7. *Liver Infusion Broth*[24]

| | |
|---|---|
| Liver infusion | 500 ml. |
| Peptone (Bacto) | 5 gm. |
| Sodium chloride | 5 gm. |
| Water | 500 ml. |

a. Prepare the liver infusion as in Meat Infusion Broth, Double Strength (CM 6) except that fresh beef liver is substituted for the ground meat. The infusion is not sterilized before incorporation of the other ingredients.
b. Combine all ingredients and heat in flowing steam for 20 minutes. Adjust to pH 7.2.
c. Heat in flowing steam 15 minutes and filter through a coarse paper.
d. Dispense as desired and autoclave at 121° C. (15 lb.) for 30 minutes.* The reaction after sterilization should be pH 6.6–6.8. *Never resterilize liver infusion broth.*

### 7a. *Liver Infusion Agar*

| | |
|---|---|
| Liver infusion | 500 ml. |
| Peptone (Bacto) | 5 gm. |
| Sodium chloride | 5 gm. |
| Washed agar | 20 gm. |
| Water | 500 ml. |

According to Huddleson[24] more heat is required in the preparation of Liver Infusion Agar than is necessary for solution of the agar.
a. Prepare the liver infusion as in Meat Infusion Broth, Double Strength (CM 6) except that fresh beef liver is substituted for the ground meat. The infusion is not sterilized before incorporation of the other ingredients.
b. Combine all ingredients and heat in flowing steam for 1 hour. Cool and adjust to pH 7.2.
c. Heat in flowing steam for ½ hour.

---

* The heating and sterilizing periods prescribed in the preparation of Liver Infusion Broth and Liver Infusion Agar are greater than the periods required to achieve sterility, but essential for the reduction of the reaction to the optimum for the growth of *Brucella*. Such prolonged heating periods will result in partial destruction of essential metabolites. Referee's note.

d. Decant and dispense in sterile flasks or tubes as desired and autoclave at 121° C. (15 lb.) for 30 minutes.

### 8. *Beef Infusion Broth with Proteose for Streptococci*[23]

| | |
|---|---|
| Beef | 450 gm. |
| Peptone (Difco Proteose) | 20 gm. |
| Sodium chloride | 5 gm. |
| Dextrose | 0.5–1.5 gm. |
| Water | 1,000 ml. |

a. Infuse beef as for Meat Infusion Broth, Plain (CM 5).
b. Determine reducing sugar content. If the reducing sugar content is equivalent to from 0.1 per cent to 0.15 per cent dextrose, use 0.5 gm. dextrose per liter; if approximately 0.075 per cent use 1.0 gm.; and if approximately 0.05 per cent or less, use 1.5 gm.
c. Add sodium chloride, peptone and required amount of dextrose and dissolve. Adjust the reaction to pH 8.2.
d. Autoclave in bulk (20–40 kg. amounts) and make up weight to the equivalent of 1,025 gm. for each 1,000 gm. of starting material.
e. Adjust reaction to pH 8.0 and boil 3–5 minutes.
f. Filter through paper, dispense as desired and autoclave at 115°–118° C. (10–12 lb.) for 30 minutes.

### 9. *Beef Infusion Broth with Dextrose and Indicator*[23]

| | |
|---|---|
| Meat infusion broth, plain (CM 5) | 1,000 ml. |
| Dextrose | 10 gm. |
| Phenol red | 0.018 gm. |

Dissolve dextrose and phenol red in broth. Adjust the reaction to pH 7.4–7.8. Dispense as desired and autoclave at 121° C. (15 lb.) for 15 minutes.

### 10. *10% Meat Infusion Broth*[23]

| | |
|---|---|
| Beef or beef heart muscle | 500 gm. |
| Peptone (Difco Proteose) | 20 gm. |
| Sodium chloride | 5 gm. |
| Dipotassium phosphate $K_2HPO_4$ | 1 gm. |
| Monopotassium phosphate $KH_2PO_4$ | 1 gm. |
| Dextrose | 1–2 gm. |
| Calcium chloride $CaCl_2$ 10 per cent solution | 2 ml. |
| Water to make | 1 kg. |

a. Infuse meat with 250 gm. of water as under CM 5 and determine reducing sugar content of the meat juice. If reducing sugar content is the equivalent of from 0.125 per cent to 0.15 per cent dextrose, add 1

gm. of dextrose; if between 0.075 per cent and 0.1 per cent, add 1.5 gm.; if less than 0.05 per cent, add 1.75 to 2.0 gm.

b. Dissolve peptone, salts, and the required amount of dextrose in water, combine with the meat juices and make up weight to 1 kg.

c. Adjust the reaction to pH 8.0–8.2 and add the calcium chloride.*

d. Autoclave in bulk and make up to original weight.

e. Adjust the reaction to pH 7.8–8.0, boil 3 to 5 minutes and filter through paper.

f. Dispense as desired and autoclave at 115°–118° C. (10–12 lb.) for 30 minutes.

* As was noted in the remarks following CM 3, Peptone Broth, Infusion-free and Buffered, adjusting the reaction to pH 8.0 will convert virtually all of the monopotassium salt to the dibasic form. The addition of the calcium chloride solution will precipitate 39 per cent of the added phosphate in the form of insoluble calcium phosphate. Calcium is also a good precipitant for some members of the B-complex. Referee's note.

### 11. *Sodium Azide-Crystal Violet Enrichment Broth*[25]

| | |
|---|---|
| Infusion broth (CM 5) | 500 ml. |
| Rabbit red cells, washed, sterile | 10 ml. |
| Crystal violet, 0.01 per cent aqueous solution | 1 ml. |
| Sodium azide, 0.625 per cent aqueous solution | 5.2 ml. |

a. Sterilize the infusion broth and aseptically add the rabbit cells and previously sterilized crystal violet and sodium azide solutions.

b. While agitating to keep the cells evenly suspended, aseptically dispense in sterile tubes.

c. Store at 4°–6° C. The finished broth is not satisfactory after 7 days' storage.

### 12. *Tryptose Phosphate Broth*

| | |
|---|---|
| Tryptose | 20 gm. |
| Dextrose | 2 gm. |
| Sodium chloride | 5 gm. |
| Disodium phosphate $K_2HPO_4$ | 2.5 gm. |
| Agar | 1–2 gm. |
| Water | 1,000 ml. |

a. Dissolve all ingredients in the water. If the agar is used, the solution must be raised to the boiling point for satisfactory solution of the agar.

b. Dispense in tubes or flasks as desired and autoclave at 121° C. for 15 minutes.

### 13. *Tryptose Dextrose Vitamin B Broth*[26]

| | |
|---|---|
| Tryptose | 20 gm. |
| Dextrose | 1 gm. |
| Sodium chloride | 5 gm. |
| Thiamine hydrochloride | 5 mg. |
| Water | 1,000 ml. |

a. Dissolve all ingredients in the water. Adjust the reaction to pH 7.2.
b. Dispense in tubes or flasks as desired and autoclave at 121° C. (15 lb.) for 15 minutes.

### 13a. *Tryptose Dextrose Vitamin B Agar*[26]

| | |
|---|---|
| Tryptose dextrose vitamin B broth (CM 13) | 1,000 ml. |
| Agar | 15 gm. |

a. Dissolve the agar in the broth by heating to the boiling point. Adjust the reaction to pH 7.2.
b. Dispense in tubes or flasks as desired and autoclave at 121° C. for 15 minutes.

### 13b. *Tryptose Dextrose Agar*

Exactly as CM 13a, Tryptose Dextrose Vitamin B Agar, except that the thiamine hydrochloride is omitted.

### 14. *Ground Meat Medium for Anaerobes*

a. Prepare tubes with infusion, peptone or extract broth with 0.5 per cent sodium chloride and 0.1 per cent dextrose. The reaction of the medium before tubing should be pH 7.5.
b. Add enough ground *lean* beef, beef heart, or veal to occupy half the column of liquid.
c. Autoclave at 121° C. (15 lb.) for 15 minutes.

### 15. *Extract Gelatin*[27]

| | |
|---|---|
| Peptone | 5 gm. |
| Meat extract | 3 gm. |
| Gelatin | 120 gm. |
| Water | 1,000 ml. |

a. Soak the gelatin in the water for 15 minutes before heating. Dissolve peptone and meat extract in the water and gradually raise the temperature to 65° C. to dissolve the gelatin. This procedure should be carried out in a double boiler to avoid burning the gelatin.
b. Make up to original volume and adjust reaction to pH 7.0. If the medium is not clear, heat to the boiling point and filter while very hot.

c. Dispense in tubes as desired and autoclave. Prolonged heating must be avoided or the power of the gelatin to solidify will be lost.

### 16. *Milk with Indicator*

a. Add 1 ml. of 1.6 per cent alcoholic solution of bromcresol purple to 1,000 ml. of skim milk. Litmus, if available, may be substituted.
b. Dispense in tubes as desired and autoclave at 116° C. (11 lb.) for 10 minutes.

### 17. *Staphylococcus Agar*[28]

| | |
|---|---|
| Proteose peptone | 20 gm. |
| Sodium chloride | 5 gm. |
| Dipotassium phosphate $K_2HPO_4$ | 1 gm. |
| Monopotassium phosphate $KH_2PO_4$ | 1 gm. |
| Magnesium sulfate $MgSO_4 \cdot 7\ H_2O$ | 0.2 gm. |
| Calcium chloride $CaCl_2$ | 0.1 gm. |
| Agar | 3 gm. |
| Water | 1,000 ml. |

a. Dissolve all ingredients in water with heat. The solution must be raised to the boiling point in order to secure a complete solution and uniform distribution of the agar.
b. Adjust the reaction to pH 7.4 and dispense as desired.
c. Autoclave at 121° C. (15 lb.) for 15 minutes.

### 18. *Russell's Double Sugar Agar,*[67] *modified*

| | |
|---|---|
| Meat extract | 3 gm. |
| Pancreatic Digest of Casein | 10 gm. |
| Lactose | 10 gm. |
| Dextrose | 0.5 gm. |
| Phenol red, 0.25 per cent solution | 10 ml. |
| Agar | 12–20 gm. |
| Water | 1,000 ml. |

a. Dissolve all ingredients in water with heat and adjust the reaction to pH 7.4.
b. Dispense in tubes and autoclave at 121° C. (15 lb.) for 15 minutes.
c. Cool in a slanted position so that the agar will form a deep butt.

### 19. *Kligler's Iron Agar*[29]

Essentially, Kligler included lead acetate in the double sugar medium of Russell to detect the formation of $H_2S$. This medium has been modified to incorporate more sensitive indicators of $H_2S$ production and acidity.

| | |
|---|---|
| Peptone components | 20 gm. |
| Lactose | 10 gm. |
| Dextrose | 1 gm. |
| Sodium chloride | 5 gm. |
| Agar | 15 gm. |
| Phenol red, 0.25 per cent aqueous solution | 10 ml. |
| Water | 1,000 ml. |

*plus, modification No. 1* [30]

| | |
|---|---|
| Ferrous sulfate | 0.2 gm. |
| Sodium sulfite | 0.4 gm. |
| Sodium thiosulfate | 0.08 gm. |

*or, modification No. 2* [31]

| | |
|---|---|
| Ferric ammonium citrate | 0.5 gm. |
| Sodium thiosulfate | 0.5 gm. |

Prepared and used exactly as Russell's Double Sugar Agar.

20. *Triple Sugar Iron Agar*

Hajna [32] modified the medium of Krumweide [33] by including an indicator of $H_2S$ production and substituting phenol red for litmus.

| | |
|---|---|
| Peptone | 20 gm. |
| Sodium chloride | 5 gm. |
| Lactose | 10 gm. |
| Sucrose | 10 gm. |
| Dextrose | 1 gm. |
| Ferrous ammonium sulfate | 0.2 gm. |
| Sodium thiosulfate | 0.2 gm. |
| Phenol red, 0.25 per cent aqueous solution | 10 ml. |
| Agar | 17 gm. |
| Water | 1,000 ml. |

Prepared and used exactly as Russell's Double Sugar Agar.

20a. *Triple Sugar Iron Agar, modified* [40]

| | |
|---|---|
| Beef extract | 3 gm. |
| Yeast extract | 3 gm. |
| Peptone | 15 gm. |
| Proteose-peptone | 5 gm. |
| Dextrose | 1 gm. |
| Lactose | 10 gm. |
| Sucrose | 10 gm. |
| Ferrous sulfate | 0.2 gm. |
| Sodium thiosulfate | 0.3 gm. |
| Sodium chloride | 5 gm. |
| Phenol red, 0.25 per cent aqueous solution | 10 ml. |
| Agar | 15 gm. |
| Water | 1,000 ml. |

Prepared and used exactly as Russell's Double Sugar Agar. Recommended for cultivation of *Brucella.*

### 21. *Eosin-Methylene Blue Agar, modified*

Several modifications of the original formula proposed by Holt-Harris and Teague[34] have been developed. Levine[35] evaluated the influence of variation of the ratio of eosin Y to methylene blue. He reported that the optimum ratio was eosin 6: methylene blue 1 when based upon actual dye content.

| | |
|---|---|
| Peptone | 10 gm. |
| Dibasic potassium phosphate | 2 gm. |
| Lactose | 10 gm. |
| Eosin Y,* certified, 2 per cent aqueous solution | 20 ml. |
| Methylene Blue,* cert. 0.5 per cent aqueous solution | 13 ml. |
| Agar | 15 gm. |
| Water | 1,000 ml. |

* Certified dyes must be used if the volumes listed in this formula are to be followed. Any variation in dye content must be checked by assay to be certain that the complex formed by the eosin and methylene blue is correctly proportioned. The carbohydrate component of the original Holt-Harris and Teague formula was 0.5 per cent lactose and 0.5 per cent sucrose. This combination can be used if desired for the detection of intestinal pathogens, but should not be used in water bacteriology.

a. Dissolve all ingredients in water with heat and adjust the reaction to pH 7.2.

b. Dispense in flasks as desired and autoclave at 121° C. (15 lb.) for 15 minutes. For use, melt media and pour plates.

### 22. *Endo's Agar, Robinson and Rettger*[36]

| | |
|---|---|
| Extract agar (CM 4a) (agar content 2-2.5%) | 1,000 ml. |
| Sodium carbonate, 10 per cent aqueous solution | 10 ml. |
| Lactose | 10 gm. |
| Sodium bisulfite, 10 per cent w/v aqueous solution | 10 ml. |
| Basic fuchsin (ca. 10%) sat. alc. solution | 0.5–3 ml.* |

* Exact quantity of basic fuchsin must be determined by standardization.

a. Melt agar and add sodium carbonate. The reaction should be between pH 7.6 and 8.0.

b. Steam or heat in boiling water for 10 minutes and add the lactose, basic fuchsin and sodium bisulfite.

c. Dispense as desired and autoclave at 116° C. (11 lb.) for 10 minutes. Cool and pour plates. Porous tops are desirable.

If the sodium bisulfite solution is not freshly prepared, it should be stored under a layer of mineral oil to slow down the loss of the sulfur dioxide by diffusion. Prepared plates of Endo's medium should be stored in the dark and used within 4 days of preparation. Referee's note.

### 23. *Bismuth Sulfite Agar, Wilson and Blair*[37, 68]

Agar Base

| | |
|---|---|
| Peptone | 10 gm. |
| Meat extract | 5 gm. |
| Agar | 20 gm. |
| Water | 1.000 ml. |

a. Dissolve all ingredients in water with heat and adjust reaction to pH 7.2.

b. Dispense in flasks in 100 ml. quantities or other desired volumes and autoclave at 121° C. (15 lb.) for 15 minutes.

Bismuth Sulfite Solution

| | |
|---|---|
| Bismuth ammonium citrate, scales U.S.P. IX | 6 gm. |
| Sodium sulfite, anhydrous | 10-20 gm.* |
| Dextrose | 10 gm. |
| Disodium phosphate, anhydrous | 10 gm. |
| Water | 200 ml. |

* The weight of sodium sulfite may be varied within limits as a means of standardizing other ingredients in the medium.

a. Dissolve the bismuth ammonium citrate in 50 ml. of boiling water, the dextrose in 50 ml. of boiling water and the sodium sulfite in 100 ml. of boiling water. (Caution—Sulfur dioxide is evolved rapidly from hot solutions.)

b. Mix the bismuth ammonium citrate solution and the sodium sulfite solution; boil, and while boiling, add the disodium phosphate.

c. Cool and add the dextrose solution. Restore to 200 ml.

d. Store at room temperature in a well stoppered Pyrex flask in the *dark*.

Iron Citrate-Brilliant Green Solution

| | |
|---|---|
| Ferric citrate | 1 gm. |
| Brilliant green, 1 per cent aqueous solution | 12.5 ml. |
| Water | 100 ml. |

Dissolve the ferric citrate in water and add the brilliant green solution. Store in a well stoppered Pyrex bottle at room temperature in the dark.

*Complete medium*

| | |
|---|---|
| Agar base | 1.000 ml. |
| Bismuth sulfite solution | 200 ml. |
| Iron citrate-brilliant green solution | 45 ml. |

a. Melt the agar base and add the bismuth sulfite and the iron citrate-brilliant green solutions with thorough mixing.

b. Pour in porous top plates at least ¼ inch deep and use within 4 days of preparation. Store in a refrigerator.

### 24. *Sodium Desoxycholate Agar,*[38] *modified*[81]

| | |
|---|---|
| Peptone | 10 gm. |
| Lactose | 10 gm. |
| Dipotassium phosphate | 2 gm. |
| Ferric citrate | 1 gm. |
| Sodium citrate | 1 gm. |
| Sodium desoxycholate | 1 gm. |
| Sodium chloride | 5 gm. |
| Agar | 16 gm. |
| Neutral red, 1 per cent solution | 3 ml. |
| Water | 1,000 ml. |

a. Dissolve all soluble ingredients in water and add agar. Allow to soak at least 10 minutes to wet agar thoroughly.

b. Heat gently with constant agitation and boil for 1 minute to dissolve agar. Do not overheat, as excessive heating increases the inhibitory effect. Adjust reaction to pH 7.2.

c. Pour in porous top plates or dispense in tubes in 20 ml. volumes for later preparation of plates. Larger volumes should not be used, or there will be too much exposure to heat in melting the agar.

### 24a. *Sodium Desoxycholate Citrate Agar,*[38] *modified*[81]

| | |
|---|---|
| Meat infusion (horse, beef, pork) | 1,000 ml. |
| Peptone | 10 gm. |
| Lactose | 10 gm. |
| Sodium citrate | 20 gm. |
| Ferric citrate | 1 gm. |
| Sodium desoxycholate | 5 gm. |
| Agar | 17 gm. |
| Neutral red, 1 per cent solution | 2 ml. |

Prepare and use exactly as Sodium Desoxycholate Agar, CM 24.

Brodie[39] recently investigated the mechanism of inhibition of bile salt media and found that sodium taurocholate could be substituted for sodium desoxycholate if the latter is not available. He demonstrated that the inhibitory effect is the result of the combined action

of the electrolytes and the bile salt. Any variation in the bile salt concentration or the bile salt itself must be controlled by variation of the electrolyte concentration. The exact ratio can only be determined by titration.

24b. *Sodium Desoxycholate Citrate Lactose Agar, modified*[81]

| Ingredient | Amount |
|---|---|
| Meat extract | 3 gm. |
| Peptone | 7 gm. |
| Lactose | 10 gm. |
| Sodium desoxycholate | 2.5 gm. |
| Sodium thiosulfate | 5 gm. |
| Sodium citrate | 10.5 gm. |
| Agar | 15 gm. |
| Neutral red, 1 per cent solution | 3 ml. |
| Water | 1,000 ml. |

Prepare and use exactly as Sodium Desoxycholate Agar, CM 24.

24c. *Sodium Desoxycholate Citrate Lactose Sucrose Agar*[81]

| Ingredient | Amount |
|---|---|
| Meat extract | 3 gm. |
| Peptone | 7 gm. |
| Lactose | 5 gm. |
| Sucrose | 5 gm. |
| Sodium desoxycholate | 2.5 gm. |
| Sodium thiosulfate | 5 gm. |
| Sodium citrate | 10.5 gm. |
| Neutral red, 1 per cent solution | 3 ml. |
| Agar | 15 gm. |
| Water | 1,000 ml. |

Prepare and use exactly as Sodium Desoxycholate Agar, CM 24.

25. *Bile Salts Citrate Agar (Difco SS)*[30, 80]

Agar Base

| Ingredient | Amount |
|---|---|
| Beef extract | 5 gm. |
| Proteose peptone | 5 gm. |
| Bile salts No. 3 | 8.5 gm. |
| Agar | 13.5–17 gm. |
| Water | 1,000 ml. |

a. Dissolve the agar in 500 ml. of the water with heat.
b. Dissolve the other ingredients in the balance of the water, combine, and restore to original volume.
c. Dispense as desired and autoclave at 121° C. (15 lb.) for 15 minutes.

*Complete medium*

| | |
|---|---|
| Agar base | 1,000 ml. |
| Lactose | 10 gm. |
| Sodium citrate $Na_3C_6H_5O_7 \cdot 2H_2O$ | 8.5 gm. |
| Sodium thiosulfate $Na_2S_2O_3 \cdot 5H_2O$ | 8.5 gm. |
| Ferric citrate pearls, U.S.P., 10 per cent solution | 10 ml. |
| Neutral red, 1 per cent solution | 2.5 ml. |
| Brilliant green, cert., 0.1 per cent solution | 0.33 ml. |

a. Melt the agar base and add all the ingredients except the dye solutions.
b. Adjust the reaction to pH 7.0 and add the neutral red and brilliant green.
c. Mix thoroughly and pour deep plates. DO NOT AUTOCLAVE.

### 26. *Bile Salts Agar, MacConkey, modified*[30, 42]

| | |
|---|---|
| Peptone | 20 gm. |
| Lactose | 10 gm. |
| Bile salts No. 3 | 1.5 gm. |
| Sodium chloride | 5 gm. |
| Agar | 13.5-15 gm. |
| Neutral red, 1 per cent solution | 3 ml. |
| Crystal violet, 0.1 per cent solution | 1 ml. |
| Water | 1,000 ml. |

a. Add all dry ingredients to water and dissolve with heat.
b. Adjust the reaction to pH 7.2 and add dye solutions.
c. Dispense in flasks or tubes as desired and autoclave at 121° C. (15 lb.) for 15 minutes. Pour in deep plates and allow surface to dry.

### 27. *Bile Glycerol Peptone Broth*

| | |
|---|---|
| Ox bile, dehydrated | 100 gm. |
| Glycerol | 80 ml. |
| Peptone | 20 gm. |
| Water to make | 1,000 ml. |

a. Dissolve the bile in a small amount of water by rubbing into a paste. Dissolve the peptone in a small amount of water by rubbing to a paste. It is advantageous to combine the dry peptone and the dry bile powder and thoroughly mix before adding the water. Gradually add water to a volume of approximately 500 ml.
b. Add the glycerol and mix thoroughly. Make up volume to 1,000 ml. with more water.
c. Dispense 8 ml. to 15 ml. quantities in wide mouth jars and autoclave at 121° C. (15 lb.) for 15 minutes.

### 28. *Tetrathionate Enrichment Broth,*[43] *modified*[68]

| | |
|---|---|
| Peptone (Proteose No. 3) | 5 gm. |
| Bile salts (Bacto No. 3) | 1 gm. |
| Calcium carbonate Ca $CO_3$ | 10 gm. |
| Sodium thiosulfate, $Na_2S_2O_3 \cdot 5H_2O$ | 30 gm. |
| Brilliant green, cert. 0.1 per cent aqueous sol. | 11 ml. |
| Iodine, 25% aqueous solution | 25 ml. |
| Water | 1,000 ml. |

a. Dissolve the peptone and bile salts in the water and dispense 500 ml. amounts in liter flasks. Add 5 gm. calcium carbonate to each flask and sterilize.

b. As required, add the sodium thiosulfate and brilliant green solution and dispense aseptically in jars or tubes, exactly 12 ml. per container. It is essential to maintain a uniform suspension of the calcium carbonate while dispensing.

c. Immediately prior to inoculation, add 0.3 ml. of the iodine solution to each 12 ml. tube.

*Aqueous iodine solution, 25 per cent*

| | |
|---|---|
| Iodine, resublimed | 25 gm. |
| Potassium iodide KI | 20 gm. |
| Water, to make | 100 ml. |

Dissolve the potassium iodide and iodine in part of the water and make up to volume.

### 29. *Selenite F Broth*[44]

| | |
|---|---|
| Peptone | 5 gm. |
| Lactose | 4 gm. |
| Sodium phosphates, anhydrous * | 10 gm. |
| Sodium acid selenite | 4 gm. |
| Water | 1,000 ml. |

* The ratio of monosodium phosphate to disodium phosphate must be determined with each lot of sodium acid selenite. The total phosphate concentration is 1 per cent, adjusted so that the solution containing the sodium acid selenite is buffered at pH 7.0.

a. Dissolve the dry ingredients in the water with gentle heat.

b. Dispense in tubes and sterilize by exposure to flowing steam for not over 30 minutes. DO NOT AUTOCLAVE.

Alternatively, a proportionate amount of the dry ingredients can be added to a sewage effluent, water sample, or other large specimen and dissolved without heat for the enrichment of suspected enteric pathogens.

### 30. *Urea Broth, Rustigian and Stuart* [78]

| Ingredient | Amount |
|---|---|
| Urea | 20 gm. |
| Monopotassium phosphate | 9.1 gm. |
| Dipotassium phosphate | 9.5 gm. |
| Yeast extract | 0.1 gm. |
| Phenol red, 0.25 per cent solution | 4 ml. |
| Water | 1,000 ml. |

a. Dissolve dry ingredients in the water and add the phenol red solution. DO NOT HEAT.

b. Sterilize by filtration, preferably through a glass or porcelain filter, and tube aseptically in at least 2 ml. amounts.

### 31. *Urea Agar, Christensen* [79]

| Ingredient | Amount |
|---|---|
| Urea | 20 gm. |
| Dextrose | 1 gm. |
| Peptone | 1 gm. |
| Sodium chloride | 5 gm. |
| Monopotassium phosphate | 2 gm. |
| Phenol red, 0.25 per cent solution | 5 ml. |
| Water | 1,000 ml. |
| Agar | 17–20 gm. |

a. Dissolve all ingredients except agar in 100 ml. of water and sterilize by filtration. DO NOT HEAT.

b. Dissolve the agar in 900 ml. of water with heat and autoclave at 121° C. (15 lb.) for 15 minutes.

c. Cool agar to 50° C. and add the filter-sterilized solution of chemicals. Mix thoroughly. Tube aseptically in tubes and slant so that a deep butt results.

### 32. *Motility Agar,* [60] *modified*

| Ingredient | Amount |
|---|---|
| Pancreatic Digest of Casein | 10 gm. |
| Meat extract | 3 gm. |
| Gelatin * | 80 gm. |
| Agar | 3 gm. |
| Water | 1,000 ml. |

* For detection of motility at 22° C., reduce the gelatin concentration to 4 per cent.

a. Dissolve the dry ingredients in the water with gentle heat. The temperature must be raised to the boiling point to insure adequate solution and even distribution of the agar.

b. Adjust the reaction to pH 7.0 and dispense in tubes or flasks as desired.

c. Autoclave at 121° C. (15 lb.) for 15 minutes. In any subsequent reheating, particular care must be taken to see that the agar is liquefied. The medium becomes liquid at about 50° C., but the agar will not melt under 95° C.

### 33. *Blood Agar, Plain*

| | |
|---|---|
| Agar base | 100 ml. |
| Defibrinated blood | 5 ml. |

a. Melt previously sterilized agar base and cool to 45° C. and add 5 per cent of sterile, defibrinated horse, rabbit or sheep blood.

b. Dispense in tubes or plates as desired and incubate to check sterility. Bubbles on the surface of the medium can be destroyed by flaming the surface with a Bunsen burner.

Any agar desired can be used in the preparation of blood agar, provided that the base is isotonic with red cells. The agar concentration should be carefully adjusted so that the surface of the plates and tubes will be of the desired softness. The medium should not contain any compound that has a tendency to increase the fragility of the red cells and the red cells, if old, should be checked for hypersusceptibility to lysis. All plates used for the detection of hemolytic activity should be checked for adequacy by the inoculation of cultures of established hemolytic power.

Blood for use in the preparation of blood agar can be derived from virtually any source, although there are known differences in the hemolytic response of the cells of different mammals to the hemolysins of different bacteria. Brown[45] pointed out the necessity of a standardized technic when the agar is to be used in a critical study of the type of hemolysis produced by streptococci. He advocated the use of horse blood and the observation of deep (subsurface) colonies in double poured plates. For routine recognition of hemolytic strains in primary mixed cultures, surface inoculation is usually satisfactory.

Blood should be defibrinated by the addition of sodium citrate, 5 mg. per ml., the addition of heparin, 0.2 mg. per ml. or by shaking with beads which will collect the fibrin as it is formed. Blood defibrinated with potassium oxalate or sodium fluoride should not be used for the preparation of blood plates because the essential concentration of the anticoagulant may be bacteriostatic for certain genera of bacteria. The concentration of potassium oxalate directly increases the fragility of the red cell by strong shift of the electolyte balance.

### 33a. *Chocolate Blood Agar*

| | |
|---|---|
| Agar base | 100 ml. |
| Defibrinated blood | 5 ml. |

a. Melt previously sterilized agar base. Meat infusion agar (CM 5a) is recommended for *N. Meningitidis.* Cool to 60° C.
b. Add the sterile, defibrinated blood and hold at 60° C. for 15 minutes or until the blood turns chocolate brown. Do not overheat or the blood will coagulate.
c. Dispense in tubes or plates as desired and incubate to check sterility.

### 33b. *Serum Agar*

| | |
|---|---|
| Agar base | 100 ml. |
| Sterile serum | 5 ml. |
| Dextrose, 20% sterile solution | 5 ml. |

a. Melt the agar base (pH 7.4) and cool to 45° C. Infusion agar (CM 5a) is recommended for meningococci.
b. Add the serum and dextrose solution and dispense aseptically in tubes or plates as desired. Incubate to check sterility.

### 34. *Blood Agar, Bordet-Gengou, modified*[46]

Potato infusion

| | |
|---|---|
| Peeled, sliced potatoes | 500 gm. |
| Glycerol | 40 ml. |
| Water | 1,000 ml. |

a. Boil the potatoes in the solution of glycerol and water until very soft. Strain through several layers of gauze and restore volume.
b. Pour infusion into tall cylinders and allow to stand until a clear supernate can be filtered off. If not used immediately, the infusion should be sterilized.

*Base agar*

| | |
|---|---|
| Potato infusion | 500 ml. |
| Sodium chloride | 11.25 gm. |
| Agar | 50 gm. |
| Water | 1,500 ml. |

For vaccine production supplement with 1 per cent of a peptone which cultural tests have shown will support the growth of *Hemophilus pertussis.*
a. Dissolve salt and thoroughly wet the agar with a portion of the water. Add the balance of the water and dissolve the agar with heat.
b. Add the potato infusion and dispense as desired. No adjustment of the reaction is necessary.
c. Autoclave at 121° C. (15 lb.) for 15 minutes.

***Finished medium***

| | |
|---|---|
| Base agar | 100 ml. |
| Defibrinated blood | 20 ml. |

a. Melt the base agar and for each 100 ml. add 20 ml. of blood after the base agar has been cooled to 45° C. The blood may be rabbit, sheep, human, or horse less than 72 hours old. Horse blood should not be used in media for vaccine production.

b. Dispense the medium in plates or tubes aseptically and incubate to check sterility. Each lot of medium should be checked for growth promoting properties, colony appearance and characteristic hemolytic zone with a control culture of *H. pertussis*. Satisfactory plates should have a moist surface and be cherry red in color.

### 35. *Blood Glucose Cystine Agar, Francis* [47]

| | |
|---|---|
| Veal infusion, double strength, CM 6 | 1,000 ml. |
| Rabbit or horse blood | 80 ml. |
| Pancreatic digest of casein, U.S P. XIII | 10 gm. |
| Dextrose | 10 gm. |
| Sodium chloride | 5 gm. |
| Cystine or cystine hydrochloride | 1 gm. |
| Agar | 20 gm. |

a. Dissolve the peptone, sodium chloride and agar in 900 ml. of the veal infusion with heat. Dissolve the cystine or cystine-HCL in a small amount of 0.1 molar sodium hydroxide and add to the base mixture.
b. Dissolve the dextrose in 100 ml. of the veal infusion and sterilize by filtration.
c. Adjust the reaction of the base agar to pH 7.6–7.8 and autoclave at 121° C. (15 lb.) for 20 minutes. Cool to 60° C. and add the blood and dextrose solution.
d. Maintain the complete medium at 60° C. in a water bath for 3 hours with frequent mixing to insure full solution of the cystine.
e. Dispense in tubes and plates as desired and incubate to check sterility.

### 36. *Blood Cystine Tellurite Agar* [48]

| | |
|---|---|
| Heart infusion agar CM 5a | 1,000 ml. |
| Sterile blood | 50 ml. |
| Potassium tellurite, 0.3 per cent aqueous solution * | 150 ml. |
| Dry cystine † | 35–50 mg. |

* Certified material is available from A. H. Thomas.
† The dry cystine need not be sterilized. The exact weight must be determined for each lot by cultural assay with several stock strains of *Corynebacterium diphtheriae*.

a. Melt the previously sterilized beef heart infusion agar and cool to 50° C. Add the blood, sterile potassium tellurite solution and the dry cystine.
b. Mix thoroughly and dispense in pour plates, approximately 15 ml. of the medium in each standard Petri dish.

### 36a. *Tellurite Agar for Type Determination*[69]

| | |
|---|---|
| Meat infusion agar CM 5a | 1,000 ml. |
| Sterile, defibrinated blood | 50 ml. |
| Potassium tellurite, 0.3 per cent aqueous solution | 150 ml. |

a. Melt agar and cool to 90° C.
b. Add sterile blood and hold at 90° C. for 10 minutes.
c. Cool to 42° C. and add potassium tellurite solution. Mix well and pour plates, about 12 ml. per plate.

### 37. *In vitro Virulence Test Agar*[82]

| | |
|---|---|
| Proteose peptone (Difco) | 20.0 gm. |
| Maltose (C. P.) | 3.0 gm. |
| Lactic acid (C. P.) | 0.7 ml. |
| Agar, granulated | 15.0 gm. |
| Sodium chloride (C. P.) | 5.0 gm. |
| Distilled water | 1,000 ml. |

Dissolve in boiling water. Adjust to pH 7.8. Dispense in measured amounts (10 ml.) and autoclave at 121° C. (15 lb.) for 15 minutes. For use, melt the agar, cool to 45° C. and add 2 ml. of rabbit serum * to 10 ml. of agar. Pour into plates and place the strip of sterile filter paper saturated with antitoxin diluted to 500 units per ml. Allow to harden and then to dry 3 or 4 hours in the 37° C. incubator.

### 38. *GC Base Agar with Hemoglobin*[30, 51]

Base Agar

| | |
|---|---|
| Proteose peptone No. 3 | 15 gm. |
| Sodium chloride | 5 gm. |
| Dipotassium phosphate | 4 gm. |
| Monopotassium phosphate | 1 gm. |
| Corn starch | 1 gm. |
| Agar | 10 gm. |
| Water | 500 ml. |

* This must be prepared according to special precautions. See reference.

a. Suspend the dry ingredients in the cold water and heat gently to tl boiling point to dissolve the agar.

b. Dispense 100 ml. amounts in 250 ml. flasks and autoclave at 121° ( (15 lb.) for 15 minutes.

Hemoglobin solution

| | |
|---|---|
| Hemoglobin, Bacto | 10 gm. |
| Water | 500 ml. |

a. Dissolve the hemoglobin completely in cool water with frequen agitation and stirring. This may take 15–20 minutes.

b. After solution of the hemoglobin is accomplished, dispense in 100 m amounts. Autoclave at 121° C. (15 lb.) for 15 minutes.

*Finished Medium*

| | |
|---|---|
| Base agar | 100 ml. |
| Hemoglobin solution | 100 ml. |
| Supplement A or Supplement B, Bacto | 2 ml. |

a. Melt agar base and cool to 50°–60° C. Warm hemoglobin solutior to the same temperature.

b. Mix agar base and hemoglobin solution and add the supplement Mix thoroughly and pour plates.

This medium when completed has an agar concentration of only 1 per cent. This will require extreme care in handling and inoculating the plates, because the surface will be very moist and soft. Supplement A is a fortified yeast concentrate prepared to conserve both thermolabile and thermostable accessory growth factors, to which has been added crystal violet 1:6,000. Supplement B is the fortified concentrate without the crystal violet.

### 39. *McLeod's Agar with Plasma and Hemoglobin*[49, 50, 80]

Base Agar

| | |
|---|---|
| Fresh, ground beef heart muscle | 600 gm. |
| Proteose peptone No. 3 | 10 gm. |
| Disodium phosphate | 2 gm. |
| Agar | 20 gm. |
| Water | 1,000 ml. |

a. Add peptone, ground heart muscle, and disodium phosphate to the water and bring to a boil. The infusion mixture should be stirred

constantly to prevent burning, unless heating is done in a double boiler.

b. Cool to 60° C. and continue the extraction at that temperature for 45 minutes, stirring the mixture frequently.

c. Place the mixture in flowing steam so that the temperature of the medium will be between 80° and 90° C. Continue the extraction for 30 minutes.

d. Cool to 45° C. and filter through two layers of cheese cloth, expressing all the liquid. Then filter through filter paper to clear.

e. Restore to original volume, dissolve agar with heat, and adjust the reaction to pH 7.4.

f. Dispense suitable volumes in sterile flasks and autoclave at 121° C. (15 lb.) for 10 minutes.

*Enrichment solution*

| | |
|---|---|
| Sterile horse plasma | 300 ml. |
| Hemoglobin solution | 500 ml. |
| (25 ml. of settled horse red cells plus 475 ml. water) | |
| Dextrose, 20 per cent sterile, aqueous solution | 10 ml. |
| Nile blue A, 0.04 per cent aqueous solution | 24 ml. |
| Disodium phosphate, 15 per cent aqueous solution | 100 ml. |

All the sterile ingredients are combined aseptically and the mixture then stored in the refrigerator until needed. Do not store for more than 8 weeks.

*Final medium*

Melt agar base and cool to 60° C. For each 100 ml. of the melted agar base, add 31.1 ml. of the stock enrichment solution. Mix thoroughly and pour plates.

### 40. *Proteose No. 3 Agar with Plasma and Hemoglobin*[80]

*Base Agar*

| | |
|---|---|
| Proteose peptone No. 3 | 20 gm. |
| Sodium chloride | 5 gm. |
| Agar | 20 gm. |
| Water | 1,000 ml. |

a. Suspend dry ingredients in water and dissolve the agar with heat. No adjustment of the reaction is necessary.

b. Dispense in flasks in suitable amounts and autoclave at 121° C. (15 lb.) for 20 minutes.

*Enrichment mixture*

| | |
|---|---|
| Horse plasma | 260 ml. |
| Horse hemoglobin, 0.5 per cent solution | 430 ml. |
| (5 ml. settled horse red cells plus 1,000 ml. of water) | |
| Disodium phosphate, 15 per cent aqueous solution | 50 ml. |
| Dextrose, 20 per cent solution | 10 ml. |
| Nile blue A, 0.04 per cent solution | 9 ml. |

Mix all ingredients aseptically and store in the refrigerator. The 15 per cent disodium phosphate ($Na_2HPO_4 \cdot 12H_2O$) can be conveniently sterilized by autoclaving at 121° C. (15 lb.) for 15 minutes; 50 ml. in 4 oz. bottles. The Nile blue A solution can also be heat sterilized, while the dextrose solution should be sterilized by Seitz filtration.

*Final medium*

Melt the agar base and cool to 60° C. For each 100 ml. of the agar base, add 50 ml. of the enrichment mixture. Mix thoroughly with a swirling motion to prevent bubble formation and pour plates.

### 41. *Gelatin Blood Agar*[52]

| | |
|---|---|
| Proteose Peptone, No. 3 | 20 gm. |
| Sodium chloride | 5 gm. |
| Disodium phosphate | 5 gm. |
| Dextrose | 0.5 gm. |
| Gelatin | 40 gm. |
| Blood, sterile, defibrinated | 250 ml. |
| Nile blue A, 0.04 per cent sterile aqueous solution | 7.5 ml. |
| Agar | 20 gm. |
| Water, to make | 1,000 gm. |

a. Suspend the gelatin, salts, peptone, and dextrose in half the water and soak for 20 minutes so that the gelatin will be thoroughly wetted. Dissolve with gentle heat to avoid burning the gelatin.

b. Suspend the agar in the balance of the water and dissolve in the autoclave.

c. Combine the two solutions, make up weight to 1 kg. and adjust the reaction to pH 7.4–7.6.

d. Dispense in flasks in definite volumes and autoclave at 121° C. (15 lb.) for 15 minutes. Store flasks not to be used immediately.

*Final medium*

a. Melt the agar base and for each 1,000 ml. add 250 ml. of blood and 7.5 ml. of the Nile blue A solution. The agar base should be at a temperature of 85°–90° C. when the additions are made.

b. Dispense rapidly as the mixture is thick and hardens quickly. Six ml. volumes of the medium can be dispensed either in 16 x 133 mm. culture tubes stoppered with paraffined corks or in suitable screw cap vials with a phenol-formalin free plastic cap.

### 42. *Gelatin Egg Albumin Agar*[53]

| | |
|---|---|
| Glycerol | 4 ml. |
| Egg albumin, fresh | 15 ml. |
| Ox serum * | 3 ml. |
| Gelatin | 10 gm. |
| Agar | 0.4 gm. |
| Water | 100 ml. |

* 0.25 ml. of Supplement A or B, Difco may be used instead of the ox serum.

a. Soak agar and gelatin in cold water for 20 minutes. Dissolve with heat, taking care not to burn the gelatin. Add the glycerol with thorough mixing.
b. Adjust the reaction to pH 6.5 with 1 per cent potassium dihydrogen phosphate (app. 30 ml.) and autoclave at 121° C. (15 lb.) for 12 minutes.
c. Cool in a water bath adjusted to 45° C. and add the supplements. The temperature must not exceed 45° C. after supplements have been added.

*Preparation of the Serum*

Serum collected as aseptically as possible is sterilized by Seitz filtration. The filter must be preliminarily corrected by filtration of 50 ml. of 1 per cent solution potassium dihydrogen phosphate. After filtration, the serum is transferred to sterile serum bottles and inactivated on two successive days at 56° for 30 minutes and then stored at 4° C. until used.

*Preparation of the Egg Albumin*

Fresh eggs are scrubbed with " Emulsept " and soaked for 1 hour in a solution of " Roccal." Other wetting and disinfecting agents may be used if satisfactory disinfection of the shell can be demonstrated. No egg should be used from which bubbles have arisen during the soaking period as the disinfectant may have penetrated the shell and shell membranes. After disinfection of the egg is completed, the larger (butt) end of the egg is opened aseptically and the albumin removed with a pipette or syringe and transferred to sterile bottles. Care must be taken to avoid rupture of the egg yolk sac, as any admixture of

yolk makes the albumin unsuitable for use. The egg albumin must be used *fresh* as it deteriorates rapidly under storage.

*Final medium*

After addition of the supplements, 3 ml. of the finished medium is rapidly dispensed in vials * closed with a phenol-formalin free plastic screw cap which has a well to hold a cotton swab applicator. The applicator should be long enough to reach the bottom of the vial and, in use, it is virtually embedded in the medium. The vials are filled with $CO_2$, tightly closed to prevent loss of the added $CO_2$ by diffusion and stored at room temperature for use within 8 days of preparation. The vials must be kept upright at time of use to prevent excessive loss of the $CO_2$.

### 43. *Sabouraud's Agar* [54]

| | |
|---|---|
| Peptone | 10 gm. |
| Dextrose or maltose | 40 gm. |
| Agar | 18 gm. |
| Water | 1.000 ml. |

a. Suspend dry ingredients in the water and dissolve the agar by gradually raising the temperature to the boiling point. No adjustment of the reaction is necessary.

b. Dispense in tubes or flasks as desired and autoclave at 121° C. (15 lb.) for 15 minutes.

c. For isolation of *Coccidioides immitis*,[56] add cupric sulfate to 0.05 per cent concentration before pouring plates.

### 44. *Corn Meal Agar* [54]

| | |
|---|---|
| Corn meal | 40 gm. |
| Agar | 20 gm. |
| Water | 1 000 ml. |

a. Suspend the corn meal in water and simmer for 1 hour. While still hot filter through a gauze and cotton pad just thick enough to retain the corn meal.

b. Restore to original volume, add agar and dissolve with heat. No adjustment of the reaction is necessary.

c. Dispense in tubes or flasks as desired and autoclave at 121° C. (15 lb.) for 15 minutes.

* Glasco No. 7350

Some lots of Corn Meal Agar, Bacto will not stimulate chlamydospore formation of *Candida albicans*.[54] All lots of medium prepared should be checked for this property, as that is its primary use.

### 45. *Crystal Violet Oxgall Agar*[55]

| | |
|---|---|
| Peptone | 10 gm. |
| Dextrose | 10 gm. |
| Oxgall, dehydrated * | 15 gm. |
| Crystal violet, 1 per cent solution | 1 ml. |
| Streptomycin | 30,000 units |
| Agar | 20 gm. |
| Water | 1,000 ml. |

* Unless the dehydrated complete medium is used, considerable difficulty may be encountered in preparing a clear medium, free from surface film. Studies are now under way to determine the concentration of individual purified bile acids necessary to obtain the inhibition produced by the dehydrated bile. When this information is available, media of high uniformity can be prepared.[81]

a. Suspend the dry ingredients in the water and dissolve with heat. Add the crystal violet solution.

b. Dispense in flasks in definite quantities and autoclave at 121° C. (15 lb.) for 15 minutes. No adjustment of the reaction is necessary.

c. For use, melt the agar base and when cooled to 45° C. add 30 units of streptomycin per ml. of medium and pour plates.

### 46. *Potato Dextrose Agar for Fungi*[56]

| | |
|---|---|
| Sliced potatoes | 300 gm. |
| Dextrose | 10 gm. |
| Agar | 15 gm. |
| Water | 1,000 ml. |

a. Boil the potatoes in water for 15 minutes, filter through cotton and make up volume to 1,000 ml.

b. Add dry ingredients and dissolve agar with heat. No adjustment of the reaction is necessary.

c. Dispense in tubes or flasks as desired and autoclave at 121° C. (15 lb.) for 15 minutes.

### 47. *Coccidioides Isolation Agar*[57]

| | |
|---|---|
| Ammonium chloride | 10 gm. |
| Sodium acetate | 10 gm. |
| Potassium phosphate, tribasic | 8 gm. |
| Cupric sulfate, 10 per cent solution | 4 ml. |
| Agar | 20 gm. |
| Water | 1,000 ml. |

a. Suspend dry ingredients in water and dissolve with heat. Autoclave at 121° C. (15 lb.) for 15 minutes.

b. Add cupric sulfate solution and pour plates.

### 48. *Histoplasma Isolation Agar*[58]

*Base Agar*

| Ingredient | Amount |
|---|---|
| Calf brain | 200 gm. |
| Beef heart muscle | 250 gm. |
| Peptone | 10 gm. |
| Dextrose | 2 gm. |
| Sodium chloride | 5 gm. |
| Disodium phosphate | 2.5 gm. |
| Agar | 20 gm. |
| Water | 1,000 ml. |

a. Infuse brain and heart separately in 500 ml. volumes of water as directed for Meat Infusion Broth, Plain CM 5.

b. Combine infusions, make up volume to 1,000 ml. and add dry ingredients. Dissolve the agar with heat.

c. Adjust the reaction to pH 7.4 and dispense in flasks as desired. Autoclave at 121° C. (15 lb.) for 15 minutes.

*Final medium*

Melt base agar and cool to 45° C. Supplement with 10 per cent sterile blood, 20 units of penicillin and 40 units of streptomycin per ml. of medium. Pour plates.

Brain heart infusion agar, Difco, is an adequate substitute for the base agar. A medium substituting 2 per cent pancreatic digest of casein and 0.5 per cent yeast extract for the infusion and peptone components has supported excellent growth of *Histoplasma capsulatum* and is easier to prepare. Referee's note.

### 49. *Synthetic Broth for Histoplasmin and Tuberculin*[61, 66]

| Ingredient | Amount |
|---|---|
| Asparagin | 14 gm. |
| Dipotassium phosphate, c.p. Anhyd. | 1.31 gm. |
| Sodium citrate, c.p. $Na_3C_6H_5O_7 \cdot 5\frac{1}{2}H_2O$ | 0.90 gm. |
| Magnesium sulfate, USP · $MgSO_4 \cdot 7H_2O$ | 1.50 gm. |
| Ferric citrate, USP VIII, scales | 0.30 gm. |
| Cerelose, USP X | 10 gm. |
| Glycerol (for tuberculin) | 100 ml. |
| (for histoplasmin) | 25 ml. |
| (for coccidioides) | 25 ml. |
| Water to make | 1,000 ml. |

Dissolve all ingredients in water and make up to a volume of one liter. Dispense in flasks to half volume and autoclave at 121° C. (15 lb.) for 15 minutes.

### 50. *Egg Yolk-Potato Flour Medium, Trudeau Committee*[63]

| | |
|---|---|
| Egg yolk | 500 ml. |
| Potato flour water | 500 ml. |
| Malachite green, 1 per cent solution in 50 per cent alcohol | 20 ml. |

a. Suspend 20 gm. of potato flour in 500 ml. of water to which 10 ml. of glycerol has been added. Heat to boiling with constant stirring. Cool to 60° C.
b. Cleanse the shells of fresh eggs with wet gauze. Rinse the eggs in alcohol and flame. Separate the yolks from the whites as is done in any kitchen. Combine in proportions of 11 egg yolks to one whole egg until a total of 500 ml. of egg yolk-whole egg mixture is secured (approximately 24 eggs).
c. Blend the 500 ml. of egg mixture with 500 ml. of the potato flour-glycerol mixture and add the malachite green solution. All components are thoroughly mixed and dispensed in previously sterilized tubes or screw capped vials.
d. Sterilize and coagulate the medium by a single one hour exposure in an inspissator heated to 90° C. Incubate 48 hours as a check of the sterility. Contaminations are rare if the technic is maintained aseptic throughout.

### 51. *Egg-Potato-Mineral Medium, Lowenstein-Jensen*[62]

*Salt Solution*

| | |
|---|---|
| Monopotassium phosphate | 2.4 gm. |
| Magnesium sulfate | 0.24 gm. |
| Magnesium citrate | 0.6 gm. |
| Asparagin | 3.6 gm. |
| Glycerol | 12 ml. |
| Redistilled water | 600 ml. |

a. Dissolve the dry ingredients and glycerol in the water with heat.
b. Heat in flowing steam in the autoclave for 2 hours and store over night.

*Final Medium*

| | |
|---|---|
| Salt solution | 612 ml. |
| Potato flour | 30 gm. |
| Eggs | 1,000 ml. |
| Malachite green, 2 per cent aqueous solution | 20 ml. |

a. Suspend potato flour in salt solution, being very careful to break up all lumps.
b. Heat in boiling water bath (double boiler) with frequent stirring until the mixture clears. Continue heating for 15 minutes in the bath. Cool to 56° C. and maintain at that temperature for 1 hour.
c. Wash fresh (must be less than 48 hours old) eggs in 5 per cent soda and a soap solution for 30 minutes. (Alternatively, the shells can be sterilized with "Emulsept" and "Roccal" or other combination of disinfecting and wetting agents.) Then place the eggs in running cold water to remove the antiseptic or soap solution.
d. Break eggs into a sterile flask, shake well and filter through sterile gauze. (A Waring blendor or sterilized kitchen egg beater is an excellent tool for homogenizing the whole egg meat.)
e. Combine the egg fluid with the flour-salt solution mixture, add the malachite green solution and mix thoroughly until the dye is evenly distributed throughout the medium.
f. Dispense in previously sterilized tubes or screw capped vials and sterilize as directed in the section on sterilization.

### 52. *Semi-synthetic Tuberculosis Medium, Dubos*[64]

***Basal Medium***

| | |
|---|---|
| Monopotassium phosphate $KH_2PO_4$ | 1 gm. |
| Disodium phosphate, $Na_2HPO_4$ | 6.3 gm. |
| Asparagin | 2 gm. |

Dissolve with heat in 100 ml. of distilled water in a 2 liter flask and add:

| | |
|---|---|
| Distilled water | 850 ml. |
| Enzymatic digest of casein (40 ml. of a 5% autoclaved solution) | 2 gm. |
| Magnesium sulfate $MgSO_4$–$7H_2O$ (1 ml. of a 1% solution) | 0.01 gm. |
| Ferric ammonium citrate (1 ml. of a 5% solution) | 0.05 gm. |
| Calcium chloride $CaCl_2$ (1 ml. of a 0.05% solution) | 0.0005 gm. |
| Zinc sulfate $ZnSO_4$ (1 ml. of a 0.01% solution) | 0.0001 gm. |
| Copper sulfate $CuSO_4$ (1 ml. of a 0.01% solution) | 0.0001 gm. |

Adjust reaction to pH 6.5–6.8 and distribute in flasks for the preparation of Tween-Albumin Medium or Oleic Acid-Albumin Medium.

*Tween-Albumin Medium (for submerged growth)*

Basal medium .......................................... 900 ml.
Tween 80 .......................................... 0.5 ml.
(5 ml. of a 10% solution stored under refrigeration less than 4 weeks)

Autoclave at 121° C. (15 lb.) for 15 minutes. Cool to 45° C. and add:

Albumin (serum fraction V) .......................... 5 gm.
(100 ml. of a 5% solution in 0.85% sodium chloride solution, neutralized with sodium hydroxide solution and sterilized by filtration)
Dextrose .......................................... 5 gm.
(10 ml. of a 50% autoclaved solution)

Dispense aseptically in sterilized tubes, flasks, or screw capped vials, and incubate to check sterility.

Cultures of *Mycobacteria* in this medium are said to be "fully grown" when macroscopic "creeping" of clumps can be seen on the sides of the tube or flask; at this time, each ml. of the medium will contain approximately 0.2 mg. dry weight of organisms.

*Oleic Acid-Albumin Medium*

Basal medium .......................................... 900 ml.
Oleic acid-albumin complex solution .................... 100 ml.

a. Autoclave the basal medium at 121° C. (15 lb.) for 15 minutes and cool below 60° C.
b. Add the oleic acid-albumin complex solution aseptically, mix thoroughly and dispense as desired.

*Oleic Acid-Albumin Complex*

a. Dissolve 0.12 ml. (0.1 gm.) of oleic acid in 10 ml. of N/20 sodium hydroxide by shaking with a rotary motion in a small flask.
b. Add 5 ml. of this solution to 95 ml. of a 5 per cent neutralized 0.85 per cent sodium chloride solution of albumin (serum fraction V).
c. Sterilize by filtration through a glass or porcelain bacteriologic filter.

In order to facilitate isolation of *M. tuberculosis* from material contaminated with other bacteria, an attempt has been made to render the medium as selective as possible by limiting the variety and concentration of the nutrients in the basal medium. Glucose and glycerol are entirely omitted. It may be advisable to reduce the asparagin from 2 gm. to 1 gm., the enzymatic digest of casein from 2 gm. to 1 gm., and the

ferric ammonium citrate from 0.05 gm. to 0.005 gm. Satisfactory growth of *Mycobacteria* is obtained at pH 6.5, a reaction which suppresses the growth of many contaminants. Substitution of the Tween 80 by oleic acid causes the growth of *M. tuberculosis* to be granular instead of diffuse. This may facilitate the detection of large clumps of acid-fast bacteria under the low power objective.

### 53. *Egg Yolk-Water Medium, Besredka,*[65] *modified*[63]

| | |
|---|---|
| Egg yolks (10) | 175 ml. |
| Redistilled water, to make | 3,500 ml. |
| Sodium hydroxide, 1 per cent solution | 60–80 ml. |
| Malachite green, 2 per cent aqueous solution | 17.5 ml. |

a. Add 175 ml. (approximately 10) egg yolks to 500 ml. of water and mix thoroughly. Filter through gauze.
b. Add 1 per cent sodium hydroxide to adjust the reaction to pH 9.0. Make up volume to 3,500 ml.
c. Add the malachite green solution and dispense in tubes or flasks as desired and autoclave at 110° C. (6 lb.) for 20 minutes.

### 54. *Egg Media, Solidified*

*Basic formula*

| | |
|---|---|
| Egg fluid | 750 ml. |
| Water or other diluent | 250 ml. |

Mix thoroughly, dispense in previously sterilized tubes or screw capped vials. Sterilized as directed in the section on sterilization. Use aseptic technic throughout to minimize contamination.

There are many variations of this basic formula, apparently depending upon the availability of various diluents and the desires of the bacteriologist. Coleman[68] has recommended 0.9 per cent sodium chloride solution as the diluent in the preparation of a medium for the preservation of *Vi* antigen. McGuigan and Frobisher[69] suggest the use of either water or physiological salt solution for *Corynebacterium diphtheriae*. As early as 1902, Dorset[70] suggested the use of whole egg without diluent, egg yolk without diluent, and egg yolk with water as a diluent for the cultivation of *Mycobacterium tuberculosis*. Since that time, the variations suggested in the literature have been very numerous. Levine and Schoenlein[20] compiled five modifications labelled Dorset's medium; and all five had been subjected to further variation. Francis[72] used McCoy and Chapin's[71] egg yolk medium in which the egg yolk was diluted with two volumes of physiological salt solution.

Many of these variations followed as a result of poor success with the original formulas. It is safe to say that the primary reason for dissatisfaction with an egg medium is a result of failure to sterilize the medium properly. The important principles to be followed as a guide in the preparation of any egg medium are: (1) The eggs must be fresh; (2) the procedure should be carried out using all possible precautions to avoid any and all unnecessary contamination including preliminary disinfection of the shell, and (3) the exposure of the medium to heat should not be greater than the minimum time period required to secure satisfactory coagulation of the egg protein and effective sterilization. Any egg medium prepared in such a manner that a hard, dry surface results, will undoubtedly be unsatisfactory for good growth of microörganisms. A good medium has a moist, soft, glistening surface.

### 55. *Blood Serum Mixture, Loeffler's*

| | |
|---|---|
| Blood serum | 750 ml. |
| Broth | 250 ml. |

a. Mix the serum with the broth and dispense in tubes or screw capped vials.
b. Sterilize as directed in the section on sterilization.

The formula as listed is the basic formula as proposed by Dr. Loeffler[73] at a session of the Military Medical Society of Berlin in 1887. Many of the subsequent variations introduced have varied as to the broth used as well as the source of the serum. From a summary of the literature, it is apparent that the serum of any animal is satisfactory, and, if preserved with a suitable volatile or otherwise easily separable preservative, need not be fresh. Generally the broth constituent has been supplemented by the addition of 1 per cent of an easily assimilable carbohydrate such as dextrose, as an additional source of energy. Proper and careful sterilization to avoid unnecessary exposure to heat is the primary principle guiding the preparation of a satisfactory medium.

### 56. *Semi-fluid Agar*[59]

| | |
|---|---|
| Meat infusion broth CM 5 | 1,000 ml. |
| Agar | 1–1.5 gm. |
| Dextrose | 1 gm. |

a. Dissolve the agar and dextrose in the meat infusion broth with heat. Adjust the reaction to pH 8.0.

b. Dispense in tubes or flasks as desired and autoclave at 121° C. (15 lb.) for 15 minutes.

*For fermentation tests*

a. Omit dextrose and adjust reaction to pH 7.6.
b. Add 10 ml. of 0.04 per cent solution of bromcresol purple or other desired and suitable indicator.
c. Dispense in flasks and autoclave at 121° C. (15 lb.) for 15 minutes.
d. Add carbohydrate solution, previously sterilized by filtration, sufficient to make the concentration 0.5 per cent.
e. Dispense aseptically in tubes or screw capped vials.

For *Neisseria meningitidis*
Adjust reaction of basic formula to pH 7.3 and add:

| | |
|---|---|
| Potassium chloride | 0.2 gm. |
| Calcium chloride | 0.1 gm. |

Dispense in tubes and autoclave at 121° C. (15 lb.) for 15 minutes.

### 57. *Inulin Serum Water, Hiss*[74]

| | |
|---|---|
| Serum | 100 ml. |
| Water | 200 ml. |
| Inulin | 3 gm. |
| Bromcresol purple 0.04 per cent solution | 3 ml. |

a. Dissolve the inulin and dye in the water with heat and admix the serum.
b. Dispense in tubes or screw capped vials, and sterilize at 100° C. for 10 minutes. It may be necessary to heat at 110° C. (6 lb.) for 10 minutes if the serum has not been handled aseptically, or if experience has demonstrated the presence of resistant spores in the inulin.

### 58. *Thioglycolate Broth,*[76] *modified*[77]

| | |
|---|---|
| Pancreatic digest of casein, U.S.P. XIII | 15 gm. |
| l-cystine | 0.75 gm. |
| Dextrose | 5 gm. |
| Yeast extract | 5 gm. |
| Sodium chloride | 2.5 gm. |
| Sodium thioglycholate | 0.5 gm. |
| Resazurin, 0.1 per cent solution | 1 ml. |
| Agar | 0.75 gm. |

a. Suspend dry ingredients in water and dissolve with heat. Boil for at least 1 minute to insure solution of the agar.

b. Dispense in tubes, filling them to a depth of at least 10 cm. A small amount (0.1 gm.) of dry calcium carbonate may be added to each tube before filling if the medium is intended for the preservation of stock cultures.

c. Sterilize at 121° C. (15 lb.) for 15 minutes and store in the dark at room temperature. DO NOT REFRIGERATE.

d. If oxidation occurs, as will be indicated by the pink color of the resazurin, the tubes can be reheated ONCE in flowing steam or a boiling water bath to lower the potential.

### 59. *Endameba Culture Broth*[75]

| | |
|---|---|
| Heart muscle, dehydrated | 1 gm. |
| or | |
| Heart muscle, minced, fresh | 5 gm |
| Sodium chloride | 8 gm. |
| Calcium chloride | 0.[illegible] gm. |
| Potassium chloride | 0.[illegible] gm. |
| Magnesium chloride | 0.1 gm. |
| Monosodium phosphate | 0.1 gm |
| Sodium bicarbonate | 0.4 gm |
| Whole wheat flour | 25-50 mg. per tube |
| Water | 1,000 ml. |

a. Suspend the heart muscle and dissolve other ingredients in the water with heat. Bring to a boil and extract for 1 hour in a double boiler.

b. Filter through medium paper and tube in 10–15 ml. amounts.

c. Autoclave at 121° C. (15 lb.) for 15 minutes.

Immediately before use add 25–50 mg. of wheat flour to each tube. This can be easily done by filling a dry, sterile 1 ml. pipette to the 0.05 mark and tapping it against the side of the tube. The flour is sterilized in a foil covered tube in the autoclave at 121° C. (15 lb.) for 30 minutes, dried overnight in the hot air oven at 55° C. and then heated in the hot air oven at 180° C. for 30 minutes.

### 60. *Todd-Hewitt Broth,*[83] *modified*[84]

Remove fat and fascia from fresh beef heart or horse meat. Grind fine and infuse one lb. in 1,050 ml. distilled water in the refrigerator overnight. After 18 hours, heat to 85° C. and hold at 85° C. for 30 minutes. Skim off fat and filter through coarse filter paper. To each 1,000 ml. of

infusion add 20 gm. of neopeptone. Adjust reaction to pH 7.0 with N/1 NaOH and add:

| | |
|---|---|
| NaCl | 2 gm. |
| $NaHCO_3$ | 2 gm. |
| $Na_2HPO_4 \cdot 12\ H_2O$ | 1 gm. |
| Dextrose | 1 gm. |

Boil 15 minutes and filter through paper. Tube (40 ml. volumes in 50 ml. centrifuge tubes) and sterilize in the Arnold for one hour on three successive days. (Autoclaving in separated tubes at 115° C. (10 lb.) for 12 minutes has produced very satisfactory media. Referee's note.) The final reaction should be pH 8.0. If a precipitate has formed, it must be removed by filtration, the reaction adjusted to pH 8.0 and the medium resterilized.

### 61. *Mueller's Medium for Gonococci and Meningococci*[85]

a. Starch Paste

(1) Suspend 1.5 gm. of ordinary starch (corn starch or laundry starch, not "soluble starch") in 10 ml. of cold water.

(2) Pour slowly into 90 ml. of boiling water while stirring.

b. Agar Solution

Dissolve 17 gm. of agar in 500 ml. of tap water by autoclaving for 15 minutes at 121° C. (15 lb.).

c. Complete Medium

| | |
|---|---|
| Meat infusion, double strength (C.M. No. 6) | 300 ml. |
| Casamino acids, technical (Difco) | 17.5 gm. |
| Starch paste (a) | 100 ml. |
| Water | 100 ml. |

(1) Adjust pH to 7.4–7.6.

(2) Add to the hot agar solution (b).

(3) Mix and distribute into test tubes or small flasks.

(4) Autoclave *not more than 10 minutes at 115° C. (10 lb.).*

### 62. *Serum Tellurite Agar for Diphtheria*[82]

| | |
|---|---|
| Pancreatic digest of casein, U.S.P. XIII | 20 gm. |
| Sodium chloride | 5 gm. |
| Dextrose | 2 gm. |
| Sterile serum | 50 ml. |
| Potassium tellurite, 1 per cent sterile solution | 10 ml. |
| Agar | 20 gm. |
| Water | 1,000 ml. |

a. Dissolve the dry ingredients in the water with heat and adjust the reaction to pH 7.4–7.6.

b. Autoclave at 121° C. (15 lb.) for 15 minutes. Cool to 50° C. and add the potassium tellurite solution and the serum.

c. Mix thoroughly and dispense aseptically in tubes or plates as desired.

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# The Streptococci

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## I. INTRODUCTION

CLASSIFICATION of the streptococci has long presented a difficult problem in taxonomy.[1-7] The data available for classification have been so confusing, so full of exceptions and irregularities that they have often suggested the query: Is a useful classification of the streptococci possible? Various criteria have been used in the attempts to arrange in more or less well defined groups these Gram-positive spherical or ovoid microörganisms which grow in long or short chains and divide in one plane. Many attempts to do so on the basis of fermentation of carbohydrates have failed to establish differentials which can be relied upon for purposes of grouping and classification; many exceptions are always found. But fermentations and allied biochemical reactions, although unsuitable for the primary groupings of the streptococci, have unquestionable usefulness in the separation of species or types within major or primary categories as determined by antigenic composition[14-16] or action on blood agar media.

Habitats and pathogenicity also fail to satisfy requirements as acceptable differential characters for classification. Exceptions, intermediate characteristics, and poorly defined affinities have nullified attempts to use these criteria, with or without the help that can be obtained from biochemical characters, for the primary groupings of the streptococci. Schottmüller's [8] early observations extended by Brown,[9] on the different kinds of action of streptococci on blood agar media have established themselves in bacteriological thinking and literature and have survived as differentials; these criteria manifest a partial correlation with pathogenesis, but tend to assemble in the same category strains of various origins and habitats, dissimilar biochemical characters and quite different antigenic compositions. Actually, as Andrews [10] pointed out long ago, it is not surprising that among the streptococci there are very numerous species, types, intermediates, and variants. Bacteria which have survived in the struggle for existence are precisely those which have, by a capacity for variability, adapted themselves to inconstant environmental conditions; hence, among the surviving types and species poorly defined groups with transitional forms are common. Those groups, on the other hand, characterized by greater uniformity and less capacity for variation have tended to disappear.

A new era in the taxonomy of the streptococci began with the illuminating demonstrations by Lancefield [11-15] that polysaccharide components, the C-substances revealed by precipitin reactions, indicate boundaries that separate streptococci into groups which, with only a few exceptions, are correlated with the usual habitats and animal pathogenicities. Extending the pioneer observations of Griffith [16] on streptococcal types, Lancefield and others have further subdivided these primary groupings into numerous types, determined in Group A, by protein components, the M-antigens of the streptococcal cell; in Group C the type-specific antigen is also a protein but in other groups the type-specific antigen is a carbohydrate; the M-antigens, like the C-substance, are demonstrated by precipitin reactions. Groups of streptococci defined by the possession of the same group-specific C-substance do not, however, coincide precisely with the grouping which depends on the kind of action on blood agar media. Almost all Group A strains (i.e., the species *Streptococcus pyogenes*) are, to be sure, beta-hemolytic but beta-hemolytic streptococci are not confined to Group A; Group D, for example, although predominantly composed of alpha-hemolytic (viridans) and gamma-hemolytic (non-hemolytic)

strains, includes some strains which are beta-hemolytic; and occasional strains will produce beta-hemolytic colonies when grown anaerobically and alpha-hemolytic colonies when grown in atmospheric air[4]; or again certain strains after repeated subculturing will lose the capacity to cause beta hemolysis, without demonstrable change of any other character.[17] This alteration in one of the important criteria of classification has its counterpart in the disappearance of the type-specific M-antigen after long cultivation on artificial media without animal passage. The M-antigen which under these conditions is no longer demonstrable in the precipitin reaction usually reappears after repeated mouse passage. Even though the C-anti-C and M-anti-M precipitin reactions offer relatively stable and dependable criteria for classification of the streptococci, they do not eliminate all the difficulties and confusions which have for so long bedeviled the efforts to classify the streptococci in well defined categories. Strains which cannot be grouped or typed by either the Lancefield or the Griffith technic are by no means rare[18-20]; correlations with virulence are very incomplete; correlations with origins, host affinities, and pathogenetic capabilities are characterized by exceptions. Thus, among the streptococci, divisions and subdivisions are generally statistical rather than absolute. The classification presented in *Bergey's Manual*[21] is probably the best compromise between the conflicting claims of the major classificatory criteria. Some of the more important differentials are shown in Tables 1, 2, and 3.

TABLE 1

*Group and Type-specific Antigens of Hemolytic Streptococci*
*(Modified from Lancefield,[17] 1941; and Wilson and Miles[1] (Topley and Wilson) 1946)*

| *Group Specificity* | | *Type Specificity* | | |
|---|---|---|---|---|
| | | | *Type-specific Substances* | |
| *Serological Group* | *Group-Specific Carbohydrates ("C" substance)* | *Specific Types Recognized* | *Designation* | *Chemical Nature* |
| A | *Polysaccharides immunologically distinct for each group* | At least 40 | M<br>T | Proteins<br>Probably proteins |
| B | | Several | S | Polysaccharides |
| C | | Several | — | Proteins |
| D *, E, F, G. H, K, L, M, N. | | Several | S | Polysaccharides * |

* In group D the type-specific substances of 3 types appear to be polysaccharides and the group-specific substances are probably proteins; these are the "pneumococcus-like" types; in the remaining type (type 2) the reverse relationship exists: the type-specific substance is a protein and the group-specific substance is a polysaccharide (22).

Since a high percentage of human streptococcal infections are due to Group A strains, numerous attempts have been made to identify individual Group A types with the syndromes of streptococcal infection. It has finally become quite clear that none of the various streptococcal infections is regularly associated with one and only one type of Group

TABLE 2

*Divisions and Some Differential Characters of the Streptococci*

*(After Sherman [1])*

| Division | Group or Species | Lancefield Group | Hemolysis | Growth at 10° C. | Growth at 45° C. | Growth in Presence of 6.5 Per cent NaCl | Growth in Presence of pH 9.6 | Growth in Presence of 0.1 Per cent Methylene Blue | Strong Reduction | Survival 60° C., 30 Minutes | NH₃ from Peptone |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Pyogenic | S. pyogenes | A | + | | | | | | | | + |
| | S. mastitidis | B | ± | — | — | | — | — | — | — | + |
| | S. equi | C | + | — | — | — | — | — | — | — | |
| | " Animal pyogenes " | C | + | — | — | | — | — | — | — | + |
| | The " human C " | C | + | — | — | — | — | — | — | — | + |
| | " Minute hemolytic " | F | + | — | —* | — | — | — | — | — | + |
| | Group G streptococci | G | + | — | —* | — | — | — | — | — | + |
| | Group E streptococci | E | + | — | — | — | — | — | | | + |
| | Group H streptococci | H | ± | — | + | — | — | — | — | ± | ± |
| Viridans † | S. salivarius | | — | — | ± | — | — | — | | — | — |
| | S. mitis | | — | — | —* | — | — | — | | — | —* |
| | S. equinus | | — | — | + | — | — | — | — | ± | — |
| | S. bovis | | — | — | + | — | — | — | — | + | — |
| | Varieties of S. bovis | | — | — | + | — | — | — | — | + | — |
| | S. thermophilus | | — | — | + | — | — | — | — | + | — |
| Lactic | S. lactis | N | — | + | — | — | — | + | + | +* | + |
| | S. cremoris | | — | + | — | — | — | +* | +* | ± | — |
| Enterococcus | S. fecalis | D | — | + | + | + | + | + | +* | + | + |
| | S. liquefaciens | D | — | + | + | + | + | + | +* | + | + |
| | S. zymogenes | D | + | + | +* | + | + | + | +* | + | + |
| | S. durans | D | + | + | +* | + | + | + | — | + | + |

* Indicates occasional variation from type reaction.
† Many other viridans streptococci have been described, but have not as yet acquired (Bergey) specific status. For descriptions of their biological characters, original descriptions, and other bibliographical data, references (2) and (21) should be consulted.

A streptococcus. In scarlet fever or erysipelas, for example, the streptococcus causing the infection may be any one of many Group A types: this holds true for all the numerous syndromes of streptococcal infection. Thus the implications of type specificity do not include disease specificity: hence the inappropriateness of designating streptococci by a name associating it with a single clinical entity; for

TABLE 3

*Additional Characters of the Pyogenic Streptococci (After Sherman [1])*

| Group or Species | Lancefield Group | Active Fibrinolysis | Sodium Hippurate Hydrolyzed | Starch Hydrolyzed | Esculin Split | Growth on 40 Per Cent Bile Blood Agar | Gelatin Liquefied | Milk Curdled | Final pH in Glucose Broth | Acid Produced from: Arabinose | Maltose | Sucrose | Lactose | Trehalose | Raffinose | Inulin | Glycerol | Mannitol | Sorbitol | Salicin |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| S. pyogenes......... | A | +* | — | —? | ± | —* | — | —* | 6.0 4.8 | — | + | + | +* | +* | — | — | —? | ± | —* | ± |
| S. mastitidis....... | B | — | + | — | — | + | — | +* | 4.8 4.2 | — | + | + | +* | + | — | — | — | — | — | ± |
| S. equi............ | C | — | — | — | — | — | — | — | 5.5 4.8 | — | + | + | — | — | — | — | — | — | — | + |
| "Animal pyogenes".. | C | — | — | +* | + | —* | — | —* | 5.0 4.6 | — | ± | + | + | — | — | — | — | — | + | + |
| The "human C"... | C | ± | — | ± | ± | —* | — | ± | 5.4–4.6 | — | + | + | ± | + | — | — | + | — | — | ± |
| "Minute hemolytic" | F | — | — | — | ± | — | — | — | 5.4 4.6 | — | + | + | ± | ± | — | — | — | — | — | ± |
| Group G streptococci | G | ± | —* | ± | ± | ± | — | ± | 6.0 4.6 | —* | + | + | ±* | +* | ± | —* | ± | — | — | +* |
| Group E streptococci | E | — | — | ± | ± | — | — | — | 4.8 4.2 | — | + | ± | + | + | — | — | ± | ± | + | ± |
| Group H streptococci | H | | | | ± | | | | 5.0 4.5 | | + | + | ± | ± | + | — | — | —* | —* | +* |

* Indicates occasional variation from type reaction.

examples, *Streptococcus scarlatinae, Streptococcus erysipelatis, streptococcus s. b. e.* Disease specificity is more in evidence but far from absolute in the Lancefield groups. While Group A accounts for a large majority of human streptococcal infections, rare cases of scarlet fever are associated with Group C strains, and occasional human infections are due to strains which are in Groups B, C, D, F, G, and H.[17] In infants and young children severe and even fatal infections due to Group D streptococci are by no means rare.[23] Subacute bacterial endocarditis, which may be due to any one of several species in the viridans and enterococcus divisions of the streptococci (or even to species in genera other than the genus Streptococcus), and the postpartum and postabortum infections due to anaerobic streptococci are additional examples of rather frequent human streptococcal infections due to strains which do not belong to Group A.

Nevertheless, the technics of grouping and typing streptococci have made possible epidemiological studies which formerly were impossible. Investigations of the inciting agent in the complications of scarlet fever, making use of type differentiation technics, have shown that an important percentage are not due to the same type of streptococcus that caused the primary disease.[24] The patient whose complication is due to a type different from the type associated with the primary disease, probably acquired the second type in most cases, from someone with whom he came in contact possibly before, but more probably, after the onset of the primary disease. There is evidence, however, which seems to indicate that the occurrence in association with a complication or as a carrier strain from a convalescent, of a type not previously present may be due to type transformation within the host rather than to direct or indirect transmission of the second type from a human contact.[25] With the advent of penicillin the identification of carriers of Group A hemolytic streptococci has acquired new importance because it is now frequently possible to terminate the streptococcus carrier state.[26] The more accurate identification of strains of hemolytic streptococci by serologic grouping and typing and the control of infections and the carrier state by penicillin have placed in the hands of the health officer important tools for the analysis and control of outbreaks of streptococcal infections whether milk-borne or otherwise transmitted; and the new serological technics plus the antibiotics now make possible more rational and practicable quarantine regulations for streptococcal infections.

## II. COLLECTION AND PREPARATION OF SPECIMENS

### A. BLOOD

After skin sterilization, with careful aseptic technic, avoiding drafts, droplets, and other sources of contamination:

1. Draw approximately 25 ml. of blood into a dry 20 ml. syringe (18 or 20 gauge needle).

2. To 10 ml. of 2 per cent sodium citrate in a 22 x 150 mm. test tube add blood up to a 20 ml. mark. Swirl gently.

3. Divide the balance of the blood equally among 3 flasks (about 5 ml. in each flask) each containing 50 ml. of beef infusion broth (C.M. No. 5). Bottles serve the purpose as well as flasks. One flask is incubated aerobically, one anaerobically, and one in an atmosphere of approximately 10 per cent $CO_2$.

4. With the citrated blood 5 pour plates are prepared, each receiving 2 ml. (i.e., 1.0 ml. of undiluted blood) (C.M. No. 33). The balance of the citrated blood is distributed equally among 3 flasks of beef infusion broth (C.M. No. 5) each receiving about 3 ml. These pour plates and flasks inoculated with citrated blood should be incubated as follows: *Aerobic*—2 pour plates, 1 flask; *Anaerobic* (McIntosh and Fildes[27] jar or similar equipment for anaerobiosis)—1 pour plate, 1 flask; in $CO_2$ (approximately 10 per cent)—2 pour plates, 1 flask. An abbreviated blood culture procedure which for routine use will yield results only a little less satisfactory than the procedure just described is to prepare 30 ml. of citrated blood (15 ml. of 2 per cent sodium citrate and 15 ml. of blood) and omit the 3 flasks with untreated blood.

5. In case the patient from whom the blood was taken is at the time on penicillin therapy a penicillin inhibitor, "Clarase"[28] or "Penicillinase"[29] should be added to the medium. "Clarase" may be used in 1 per cent solution in 0.85 per cent NaCl sterilized by Seitz filtration; 0.5 ml. for each 5.0 ml. of blood should be added to the medium. "Penicillinase," a penicillin inactivator of bacterial origin is also commercially available. In case the patient is on sulfonamide therapy at the time blood is taken for cultural examination 0.25 ml. of a 1 per cent solution of para-amino-benzoic acid in distilled water should be added to the medium for each 5.0 ml. of blood.

6. Unless there is great clinical urgency (and this is far from infrequent) for confirmation or elimination of a suspicion that bac-

teriemia exists, the flasks should be incubated 48 hours before being opened for the preparation of Gram-stained films and subcultures to blood agar plates (C.M. No. 33). They should be examined for growth on the 2nd, 7th, 10th and 14th days, and should not be discarded as negative before the 14th day.

7. Cultural examination of the blood of infants and young children, because of contraindications to taking as much as 25 ml. of blood or because of technical difficulties. will often have to be done with smaller quantities of blood. Under these circumstances it is generally advisable, with or without the use of sodium citrate, to divide the amount of blood available equally among 3 flasks of beef infusion broth (C.M. No. 5), one of which is incubated aerobically, one anaerobically, and one in an atmosphere of approximately 10 per cent $CO_2$.

8. Clotted blood may be prepared for cultural examination by pipetting off the serum, placing the clot in the barrel of a 20 ml. syringe and with the plunger forcing it through the tip into a flask of beef infusion broth. This procedure requires very careful aseptic technic; contaminations are frequent. Blood agar plates should be inoculated with the serum.

The somewhat complicated procedure for blood cultures described above is known to yield good results in isolating from the blood the various bacterial species, with widely different requirements for growth, which may be present in clinical material. Not infrequently initial isolation is difficult because some strains cultured directly from an infected host are very fastidious and find conditions of growth in laboratory media unsatisfactory. Only after repeated subcultures do they grow promptly and freely. The variety of conditions offered by the blood culture technic outlined insures a high percentage of positive cultures when there is actually a bacteriemia. Less comprehensive technical procedures will, in some cases, fail to reveal a bacteriemia when it is in fact present and would be demonstrated by the more diversified technic.

An alternative procedure for anaerobiosis is available in the thioglycollate medium[30-32] (C.M. No. 58) which has been found satisfactory for the isolation of contaminants of biological products. Whether or not it will satisfactorily meet the diverse and frequently exacting demands of clinical material has not yet been fully demonstrated, but preliminary reports are favorable.[33] If the colonies in the pour plates are numerous, accurate counting is greatly facilitated by use of a Quebec counter.

### B. SWABS FROM NASAL PASSAGES AND THROAT

1. During active infection—When there is an active streptococcal infection in the nasopharynx the isolation of streptococci from throat swabs usually presents little or no difficulty. The swab is swept lightly across one side of a blood agar plate and then with a loop or glass spreader the rest of the plate is seeded in such a way that scattered colonies will be available for subcultures. Gram-stained films prepared from the material on the swab after inoculation of the plates should be examined. Cultures of Gram-positive cocci with streptococcal morphology obtained from the isolated colonies should be examined for purity and identified by serological and biochemical methods.

2. To identify hemolytic streptococcus carriers—The isolation of streptococci from convalescents and carriers is more difficult and calls for a different technique [34] : otherwise, rapidly growing microörganisms (e.g., *N. catarrhalis,* staphylococci and members of the coliform group) which form large colonies may prevent isolation of streptococci if present in small numbers or if their colonies develop slowly.

Nose and throat swabs from convalescents and suspected carriers should be incubated in a selective enrichment medium [34, 35] consisting of beef infusion broth containing 5 per cent rabbit, human, or horse blood, sodium azide 1:16,000 and crystal violet 1:500,000 (C.M. No. 11). Glucose should not exceed 0.02 per cent. Incubate at 37° for 12–18 hours. Blood plates seeded from this culture should be treated as usual for scattering colonies, isolation of streptococci in pure culture, and identification. In view of the convincing demonstration [36] that individuals with positive nose cultures disseminate much larger numbers of beta-hemolytic streptococci, and hence have a greater potential as transmitters of disease, than individuals who have positive throat and negative nose cultures, especial care should be exercised in obtaining and studying nose cultures.

### C. SPUTUM

Specimens of sputum should be received in sterile, widemouth bottles without disinfectant. The specimen need not be copious but should be fresh, should come from the deeper air passages, and should be mucopurulent or purulent with or without an admixture of blood. Patience and persistent efforts on the part of the attendant may be necessary in order to get a satisfactory specimen. Prepare Gram-stained films from a purulent or bloody portion of the specimen and inoculate blood agar

plates with a technic which will give well scattered colonies on at least a portion of one plate.

### D. Lung Juice and Material Obtained by Bronchoscopy

Lung juice, obtained by lung puncture, should be prepared for bacteriological examination in the same way as sputum and in addition a film should be stained by Wright's method to determine the kinds of cells present. Material obtained at bronchoscopic examinations should be prepared for examination in the same way as lung juice. If any of these materials is foul smelling, cultures should be incubated anaerobically.

### E. Spinal Fluid

Spinal fluid may be prepared for isolation of streptococci by centrifugation at approximately 2,000 r.p.m. for ½ hour. After removal of the supernatant, Gram-stained and Wright-stained films should be prepared from the sediment and blood agar plates inoculated.

### F. Purulent Discharges, Catheterized Urine from Infected Urinary Tracts, Peritoneal and Pleural Exudates, etc.

After preliminary centrifugation (may be omitted if the specimen consists of thick pus), prepare Gram-stained films and, with proper attention to obtaining well scattered colonies, inoculate blood agar plates. If the films show Gram-negative microörganisms in addition to Gram-positive cocci and the primary objective is only to determine the presence or absence of streptococci, a preliminary culture in beef infusion broth containing 5 per cent horse, rabbit, or human blood, plus sodium azide 1:16,000 and crystal violet 1:500,000 (C.M. No. 11) should be incubated overnight and from this culture blood agar plates should be inoculated.

### G. Swabs from Cervix Uteri and Vagina

Specimens should be collected on swabs after several hours at least have elapsed since a vaginal douche. With the aid of a speculum the physician can usually obtain a specimen from the cervical os without contamination by vaginal flora. Blood agar plates inoculated with a technic to insure scattered colonies should be incubated aerobically,

anaerobically, and in an atmosphere of approximately 10 per cent $CO_2$. Gram-stained films should also be prepared from the specimen on the swabs. Anaerobiosis is important in the bacteriological examination of specimens from the female genital tract. Puerperal infections are frequently due to strictly anaerobic or microaerophilic streptococci.

### H. MILK

Samples of milk to be examined for hemolytic streptococci should be kept in properly iced or refrigerated containers until they are examined. Bacteriological examinations should be made as soon as possible after collection. Centrifuge the specimen at approximately 2,000 r.p.m. for ½ hour. With a capillary pipette draw off and discard the skim milk. From mixed cream and sediment make films; stain one with Loeffler's alkaline methylene blue and examine for pus cells; stain another for bacteria by Gram's method. Inoculate blood agar plates, preferably pour plates. Incubate aerobically and anaerobically.

## III. BACTERIOLOGICAL EXAMINATION

### A. PLATING SPECIMENS [37]

Three plating technics are available: (1) Streaked plates: A single loopful of the material submitted for culture may be streaked in parallel rows on the surface of a 5 per cent defibrinated horse or human blood agar (C.M. No. 33) plate. If preliminary microscopic examination shows that large numbers of microörganisms are present, dilution of the specimen or inoculation of additional plates with a technic to yield scattered colonies is advisable; (2) Pour plates: Beef infusion agar dispensed in volumes of approximately 12 ml. is melted in boiling water; when it has cooled to 45°, 5 per cent defibrinated horse or human blood is added, and thoroughly mixed with the melted agar by rotating the tube between the palms of the hands. With a pipette 1 ml. of the specimen, diluted if necessary, is placed in a Petri dish and the melted blood agar poured over it. The inoculum may be evenly mixed with the blood agar by carefully swirling the plate before the agar solidifies. (3) Streaked pour plates: These are prepared by inoculating beef infusion agar plates with the material to be examined and then pouring over the inoculated surface about 5 ml. of 5 per cent defibrinated horse or human blood agar.

## B. TYPES OF GROWTH ON OR IN BLOOD AGAR

The sixteen recognized (Bergey)[21] species of the genus Streptococcus (omitting the anaerobic species) are, by established practice, descriptively designated and classified by the characteristics of their colonies when grown aerobically on blood agar; a more precise differentiation of the four categories of streptococci based on the changes produced in blood agar is possible if cultures are grown in pour plates in which horse blood is used.[9, 38] There are three main types and one sub-type of colonial appearance in pour plates of this medium.

(1) The *alpha-type*. The colony is immediately surrounded by a zone, which may be very narrow, of discolored erythrocytes which have a green or brownish-green color. This zone may not be unmistakably visible to the naked eye; to see it clearly it may be necessary to use the low power of the microscope or a dissecting microscope. Outside of the zone of discoloration a zone of hemolysis may be seen; this zone may be either so narrow as to be almost invisible or it may attain considerable width; refrigeration will usually widen this zone. Alpha-type colonies may easily be mistaken for beta-type colonies if the hemolytic zone is wide and the inner zone of greening is narrow; the microscope may be necessary to make the distinction; and the differentiation is usually more definite in the deep than in the surface colonies. Some strains which produce alpha-hemolytic colonies in atmospheric air produce beta-hemolytic colonies when grown under anaerobic conditions.[4] Strains characterized by alpha-hemolytic colonies in blood agar are commonly referred to as the *viridans group*. The *alpha-prime-type*. The colonies are not sharply outlined; they are surrounded by a slightly hazy zone of hemolysis less sharply defined than in beta-hemolysis. By using the microscope one can see that the zone of hemolysis contains a moderate number of unaltered corpuscles which are most numerous in the immediate neighborhood of the colony. No visible discoloration occurs. Refrigeration for 24 hours causes considerable widening of the zone of hemolysis. Some strains which produce alpha-prime hemolysis on horse blood agar produce typical alpha-hemolysis on rabbit blood agar.

(2) The *beta-type*. The colonies of beta-hemolytic streptococci in blood agar plates are surrounded by a clear zone in which no intact erythrocytes are visible. A subdivision of this type is the double zone beta-hemolytic type. This colonial type, after producing a zone of hemolysis like that of other beta-type strains, forms on standing at room

temperature or on refrigeration, a second ring of hemolysis separated from the first ring by a zone of intact erythrocytes. All of the double zone beta-hemolytic strains which have been recorded are members of Group B (Lancefield), but not all Group B strains produce double zones. Alternate zones of hemolysis and unhemolyzed erythrocytes may be produced with these strains by alternating incubation and refrigeration.

(3) The *gamma type.* The third type of effect of streptococci on blood agar is seen in those strains which in blood agar produce colonies with no detectable change in the blood surrounding the colony. These are commonly referred to as *indifferent* or *non-hemolytic* types.

Bacterial species which produce colonies resembling those of beta-hemolytic streptococci, for example, *Cornyebacterium pyogenes, Listeria monocytogenes, Hemophilus hemolyticus,* may usually be differentiated from streptococci by the Gram stain; and pneumococci producing alpha hemolytic or alpha-prime hemolytic colonies may be differentiated from viridans streptococci by inulin fermentation,* bile solubility and the Neufeld Quellung reaction.

## C. COLONIAL MORPHOLOGY

Identification of streptococcus strains is aided by critical consideration of the colonial types. On blood agar plates there are four recognized colonial types of surface growths of *Streptococcus pyogenes.*[17, 39-41] (1) *Mucoid* (pseudo-glossy)—The colony has a sharply circumscribed circular outline; it is high convex to domed; surface, smooth and glistening; consistency, mucoid, sometimes almost watery. (2) *Matt*—The colony usually has a circular outline; it is sharply circumscribed; elevation, domed; surface, coarsely stippled. Matt colonies probably represent a form intermediate between mucoid and glossy (smooth). The colony is tough and can be pushed along the surface of the agar without loss of contour. (3) *Glossy* (smooth)—The colony is grayish white, slightly opaque, smooth, glistening; outline, circular; the colony disintegrates when touched. (4) *Rough*—The colony has an irregular fimbriated outline; elevation, flat; color, grayish. The colony tends to be larger than the mucoid, matt, or glossy colony, and the zone of hemolysis narrower. Usually strains in the mucoid or matt phase contain the M-antigen, those in the glossy or rough phase do not.

Freshly isolated pathogenic strains are usually in the matt or mucoid

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* Many strains of streptococci of serological Groups H and K and occasional strains of Groups E, F, and M ferment inulin.

phase of colonial morphology. Pathogenic strains of groups other than Group A may differ somewhat from Group A strains in colonial appearance. Not infrequently there is considerable difficulty in assigning with certainty the colonies of a culture to one of these categories; the difficulties in deciding which phase of variation a culture is in are often increased by the effects on colonial morphology of drying of the medium. Furthermore the colonies of some strains do not have a uniform appearance on blood of different species of animals.

## D. DIFFERENTIAL CHARACTERS: IDENTIFICATION OF SPECIES, GROUPS, AND TYPES

Having obtained pure cultures of a strain of streptococcus by subculturing from isolated colonies, identification is accomplished by observation of its characters in three catagories: (1) the type of growth on or in blood agar, (2) the biochemical characters, (3) serological reactions in group-specific antisera and, if indicated, in type-specific antisera of Group A.

The differential criteria based on type of growth in or on blood agar have been described above. The biochemical characters which are most useful for species identification are presented in Tables 1, 2, and 3. For serological grouping and typing the prime requisite is group-specific and type-specific antisera of adequate potency, that is, good anti-C and anti-M sera respectively.

Directions for the preparation of antistreptococcus grouping and typing sera may be found in references 42 and 43.

*Determination of serological* (Lancefield) *group.* The extract of the streptococcal cell used in the precipitin test for grouping and typing is prepared as follows[44]: Approximately 40 ml. quantities of Todd-Hewitt[45] broth (modified)[46] (C.M. No. 60) in 50 ml. centrifuge tubes or 125 ml. flasks are inoculated from the pure stock cultures and incubated at least 18 hours or until a heavy growth is obtained. This is checked for purity of growth. The broth culture is centrifuged and the clear supernatant is pipetted off or decanted.

The bacterial sediment is mixed with 0.4 ml. of N/5 HCl. A loopful of the suspension should give an orange-red color with a drop of 0.01 per cent thymol blue, that is, the extractions should be carried out at a pH of 2.0 to 2.4. If necessary, more N/5 HCl is added to obtain this range.

The mixture is transferred to a pointed 15 ml. centrifuge tube and

heated in a boiling water bath, shaken at 3 minute intervals for 10 minutes, cooled, and centrifuged.

The clear supernatant is decanted into a second centrifuge tube and a small drop of 0.01 per cent solution of phenol red is added, which colors the solution a distinct yellow.

Three-tenths to 0.33 ml. of N/5 NaOH is added drop by drop until a faint pink color appears. The first *faint pink* color indicates a pH of 7.0; a good extract may have a pH between 7.0 and 7.8. If too alkaline, the extract is readjusted with N/20 HCl because nonspecific precipitin reactions may occur when the pH of the extract is over 7.8. The slight precipitate formed during neutralization is discarded after centrifugation and the supernatant fluid which should be crystal clear is pipetted or decanted into small test tubes.* This is now ready for testing with antisera, and is used for both grouping and typing. Difficulties in grouping and in typing streptococci are generally traceable to faulty preparation of the extract. It is essential that extraction be carried out at a pH below 2.5. It is also important to keep the final volume small. Cloudy extracts may be caused by contamination, by the use of N/5 NaOH stored in non-Pyrex glass containers and by stirring up the sediment in the bottom of the centrifuge tubes. Contaminated extracts may give false reactions. All glassware must be perfectly clean; and the acid and alkali solutions must be of accurate normality.† Into 8 x 100 mm. tubes pipette 0.1 ml. of undiluted group-specific serum. Layer on the surface of the serum 0.1 ml. of the extract. As controls combine similar volumes of each extract and normal rabbit serum, of each serum and salt solution, and of each serum and the extract of a strain of its homologous group. After 10 minutes' incubation at 35° C. examine for a ring precipitate at the interface of antiserum and extract. Shake the tubes and record the amount of precipitation after 2 hours' incubation at 35° C. and after overnight refrigeration.

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* Larger quantities of extract may of course be prepared by using multiples of the amounts suggested.

† The formamide ($HCO \cdot NH_2$) method[40] of extracting the polysaccharides of hemolytic streptococci is economical and yields potent extracts from which protein substances which may give cross-reactions are largely eliminated: centrifuge 10–15 ml. of a broth culture until the organisms are packed; remove the supernatant as completely as possible and discard; to the sediment add 0.2 ml. of formamide. Shake; and place the tube in an oil bath (automobile or mineral oil) at 150°–180° C. for 15 minutes; cool; and add 0.5 ml. of acid alcohol (1 ml. concentrated HCl with 99 ml. of 95 per cent alcohol); centrifuge. Transfer the supernatant to a clean centrifuge tube; add 1 ml. of acetone and centrifuge lightly; discard the supernatant. Add 2 ml. of normal saline to the sediment; shake and add 1 drop of bromthymol blue indicator; then add sufficient 2 per cent sodium carbonate ($Na_2CO_3 \cdot H_2O$, *not* technical sodium carbonate) to turn the extract blue. Centrifuge before use in the precipitin test.

An alternative technic [47, 48] for the grouping and typing of streptococci by precipitin reactions has been introduced by Swift. It makes possible considerable economy in the use of antisera; instead of 8 x 100 mm. tubes it makes use of capillary pipettes having an external diameter of 1.0 ± 0.02 mm. Details may be found in references 47 and 48.

Mouse-virulent strains of Group A streptococci have the M-antigen, but not all strains with M-antigen are virulent; hence M-antigen is essential for mouse virulence but is not the only determinant.

## IV. REPORTING RESULTS

Results of bacteriological examinations of specimens submitted for isolation and identification of streptococci may be reported as follows:

The laboratory may simply state, "no streptococci isolated," or "no beta-hemolytic streptococci isolated."

If streptococci have been isolated from the specimen a *complete* report should include the following information:

1. Type of action on or in blood agar, i.e., alpha, beta, gamma, or alpha-prime hemolysis.

2. The serological (Lancefield) group to which the strain belongs; if the strain is in Group A (i.e., *Streptococcus pyogenes*), sometimes it is also important to report the serological type; if serological reactions fail to indicate group and type unequivocally the report should include this information.

3. The primary group of the genus Streptococcus in which the strain is classified, i.e., pyogenes, viridans, lactic, or enterococcus group.

4. The species (Bergey) to which the strain is assigned on the basis of biochemical characters.

5. If the strain will not grow in the presence of oxygen the report may state that anaerobic (or microaerophilic) streptococci were isolated and record the kind of action on or in blood agar and the serological group.

6. Reports of positive blood cultures should, whenever possible, include the results of colony counts of the pour plates, stating the estimated number of streptococci per ml.[3]

In routine clinical material there are frequent specimens from infections which are mild, apparently inconsequential, transitory, and without any apparent epidemiological significance; reports of the bacteriological examination of such specimens, if streptococci have been isolated, may reasonably include, in the absence of contrary indications,

fewer data than the requirements of a *complete* report call for. Minimal data should, however, be (1) type of action on blood agar, and (2) serological group by Lancefield precipitin method.

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# The Pneumococcus

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I. Introduction
II. Collection of Specimens and General Procedures
III. Type Identification
IV. General Bacteriological Procedures
V. Interpretation of Results
VI. References.

## I. INTRODUCTION

THE finding and type-differentiation of pneumococci is no longer one of the major activities of the clinical laboratory. Extensive use of antibiotics in therapy makes type determination unnecessary and indeed at times impossible. The basic procedures, however, are useful and used. Even though specific serum therapy has been superseded, type differentiation remains the most satisfactory method for identifying the pneumococcus. Moreover, the finding of certain types of pneumococci by the direct method, as in sputum, is an indication of the etiology of the infection at hand.

This is not the place for a detailed historical review of the typing systems and devices. The present classification into 32 standard types derives almost entirely from the work of Cooper and her associates.[1, 2] Eddy[3] is responsible for a system of nomenclature for 75 types but there is little likelihood that this will be generally adopted. For practical purposes type identification of strains which occur infrequently is of interest only to the epidemiologist. From the standpoint of speed and economy the Neufeld method stands by itself but with this technic, as with any other, a certain amount of skill and patience is required, and the work should not be undertaken unless both attributes are employed. The considerable amount of judgment required in any of these procedures can only be acquired from experience.

## II. COLLECTION OF SPECIMENS AND GENERAL PROCEDURES

The specimens ordinarily employed in the laboratory diagnosis of pneumococcal infections are sputum and pleural exudate in pneumonia, spinal fluid in pneumococcal meningitis, peritoneal exudate in pneumococcal peritonitis, and pus in otitis media and mastoiditis. Blood cultures should be done routinely and frequently in all pneumococcal infections, for the presence or absence of bacteriemia is a guide to prognosis, and the organisms found in blood cultures provide the most satisfactory material for confirming the results of other examinations.

In general the methods employed for the finding and identification of pneumococci are dictated by the time element and by experience as to those procedures which are more likely to be successful. In dealing with materials suspected of containing pneumococci, chances of success are greatly improved if specimens are immediately conveyed to the laboratory.

### A. SPUTUM

The specimen of sputum should be collected and sent to the laboratory in a sterile, wide mouth bottle, containing no disinfectant. Sputum specimens need not be copious.

The greatest care should be taken to obtain sputum as early in the course of pneumonia as possible. The patient may have difficulty in raising sputum and may be inclined to dribble a little saliva into the container, hoping thus to satisfy a perfunctory request that he expectorate. Persistence on the part of the attendant will often result in persuading the patient to raise at least a little material. Early in pneumonia the sputum may be more fluid and watery than it is later, when it is more often muco-purulent and may be blood-streaked or blood-stained (" rusty "). If the specimen is all or mostly saliva, request another specimen, for an examination of salivary specimens is often unsatisfactory.

It is of the greatest importance that sputum (or any other specimens) be obtained before therapy is instituted. With adequate antibiotic administration, as with penicillin, it becomes increasingly difficult to obtain significant results—indeed, within a few hours after the first large amount has been administered it is almost useless to examine sputum. If typing of pneumococci is obtained with such material, however, one may suspect either inadequate therapy or the presence of a " resistant " organism. The converse is definitely not true. Therapy

cannot be regulated on this basis. Essentially the same things are true with sulfa drugs but the time interval is perhaps longer.

If the sample of sputum is of watery consistency and can be handled without undue stringing when tried with the platinum loop no further preparation is necessary. If, however, the specimen is mucoid it is necessary to emulsify in saline since otherwise proper mixture with immune serum cannot be obtained. Draw up 4.5 ml. of sterile saline into sterile syringe, then draw up 0.5 ml. of sputum. Use a sterile Petri dish and work mixture in and out of syringe about 10 times or until completely mixed. Methods employed for the examination of sputum are:

1. *Microscopic Examination*—It is the practice in some laboratories to examine a Gram-stained preparation of sputum. This is not recommended, for it serves no useful purpose.

2. *Culture on Blood Agar*—The sputum is streaked on the surface of a blood agar plate (C.M. No. 33) by the triple streak method. This is a necessary procedure, not for the recovery of pneumococci, but to determine the presence of other significant bacterial forms.

3. *Direct typing*—As hereafter indicated.

4. Should direct typing fail, some method for increasing the number of pneumococci must be employed. The method of choice is that of mouse inoculation. If mice are not available the material may be inoculated into dextrose blood broth ("Avery tube").

## B. THROAT SWABS

Adults with an early pneumonia, and children at any stage of the disease may be truly unable to raise sputum. In such cases, a sterile throat swab should be introduced into the pharynx. If the patient coughs, a fleck of sputum may sometimes be caught on the swab; if not, the pharynx is swabbed.

The swab should be introduced into a tube containing 3 ml. of tryptose phosphate broth (C.M. No. 12) and incubated for 2 hrs. at 37° C. The broth may show sufficient pneumococci for direct typing, but generally best results are obtained by mouse inoculation.

*Saliva and Throat Washings for Determination of Carrier Types*—These materials should be inoculated directly into mice. It is true that this method serves to isolate only mouse virulent forms but other methods are not effective.

### C. Spinal Fluid

This should be collected, with aseptic precautions, in a sterile tube. Microscopic examination should determine whether typing is justified. Typing by the Neufeld method may nearly always be achieved directly with the specimen or with the sediment obtained by centrifuging. If the specimen is old, mouse inoculation may be necessary.

### D. Pleural Fluid, Peritoneal Exudate, Pus, Vomitus, etc.

Microscopic examination should determine whether typing is justified. If the number of organisms is great the material should be diluted. If microscopic examination is negative, mouse inoculation should be undertaken.

### E. Swabbed Specimens from Eyes, Ears, Sinuses, etc.

Swabs are planted in tubes containing 5 ml. of dextrose blood broth and incubated for 3 hrs. at 37° C. Microscopic examination will determine if direct typing is justified. Mouse inoculation should be resorted to in case of doubt.

### F. Postmortem Tissues

Bits of tissue are planted in broth and incubated 3 hrs. at 37° C. Best results are obtained by mouse inoculation. If the tissue is soft the exudate may be used for direct typing. Dilution is necessary.

### G. Blood Cultures

Blood should be collected with a sterile syringe in such amount as is required for whatever cultures are to be made. In many clinics both fluid and solid cultures are used. Any broth of good quality may be used such as C.M. No. 12. Blood agar base Difco is an excellent basis for plate cultures. Care must be taken that the agar has been melted and then properly cooled before venipuncture is undertaken. The amount of blood (usually 1 ml.) is introduced into a sterile Petri dish and the cooled agar quickly introduced. Mixing can be obtained by quick twirling of the plate.

Pneumococci may be typed directly from an original positive fluid culture, but certain precautions are necessary, particularly if the culture has a heavy growth. The culture must be diluted so that there are not more than 50 organisms per microscopic field. If typing fails a mouse should be inoculated. This failing, cultural methods must be employed to determine if the organism is a pneumococcus.

Typing from blood agar pour plates is difficult. The plates serve best in a prognostic sense, the fluid cultures for type determinations.

## III. TYPE IDENTIFICATION

Pneumococcus typing is essentially a two-stage process. The first stage, which may frequently be omitted because of the nature of the specimen, consists in obtaining pneumococci in such numbers that one is warranted in proceeding to the second stage, which consists in deciding to which one of the usual 32 types the particular strain belongs. Although it may be slightly illogical, the typing methods are presented first because all specimens require this; while the isolation and cultural methods are applied to only a portion of the specimens received.

For purposes of type identification only one general method is now used. This system, based upon the Quellung phenomenon of Neufeld[4] is both accurate and rapid. The Quellung (swelling) is a localized immunological reaction in which the capsular polysaccharide of the pneumococcus combines with its type-specific antibody. This combination results in a characteristic enlargement of the capsular structure.

### SPECIFIC TYPING SERA

The performance of a series of tests with sera of each of the 32 types is obviously laborious. In order to facilitate type identification the sera of certain types are pooled. The system of pools is as follows.

A—Types 1, 2, 7
B—Types 3, 4, 5, 6, 8
C—Types 9, 12, 14, 15, 17, 33
D—Types 10, 11, 13, 20, 22, 24, 34
E—Types 16, 18, 19, 21, 28
F—Types 23, 25, 27, 29, 31, 32

When identification of all the recognized types is performed, it is customary to do preliminary tests with pooled sera, it being assumed that lack of reaction with such a pool indicates the absence of pneumococci of any of the types represented in the pool.

Both the monovalent and the pooled sera should be potent, specific, and known to be satisfactory for the Neufeld technic. Most laboratories will find it more economical as well as more satisfactory to obtain them from a commercial source, since these sera conform to the regulations of the National Institutes of Health. The sera should be kept in the refrigerator when not in use. Laboratories doing only an occasional typing should obtain fresh sera perhaps once a year.

## TECHNIC OF NEUFELD PROCEDURE

*Placement of specimen material*—With a small platinum loop pick up and place on a clean slide (or slides) 6 small drops of the material to be examined (one drop for each of the serum pools or individual types within a pool). Large slides (2 x 3 in.) may be used. In placing the drops, separate them sufficiently so that the preparations will not run together when the cover slips are put on.

*Addition of serum*—Add a large loopful of the appropriate serum pool to each drop. Caution must be used in cooling loop before inserting in a serum bottle. In the first series of examinations use the serum pools, A-F. In the second series use individual type sera as indicated from results with the appropriate pools.

*Addition of stain*—Add a small loopful of Loeffler's alkaline methylene blue to each drop and stir until color mixture is complete.

*Covering*—Place a clean cover-glass over each mixture. It is not necessary to use pressure.

*Examination*—Examine with oil immersion lens using a strong light but with the diaphragm partly closed.

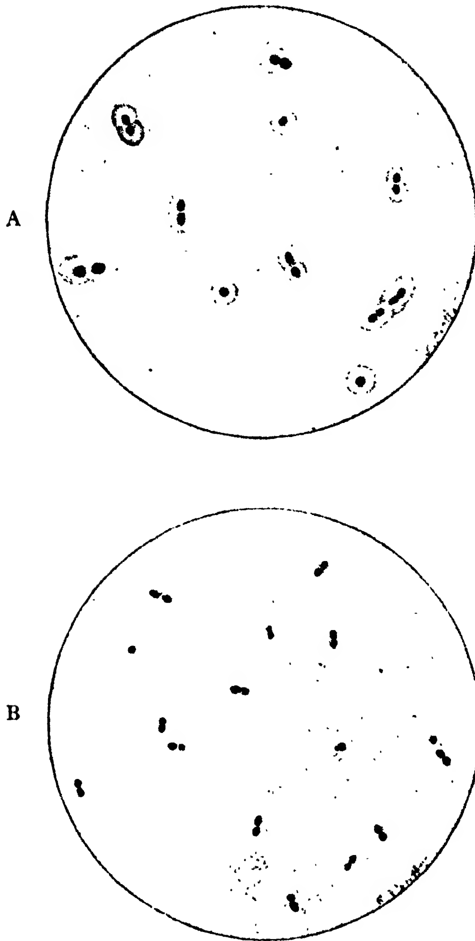
In a positive reaction the body of the pneumococcus, stained blue, is seen surrounded by its swollen capsule (See Plate No. 1). The capsule may be faintly colored but the characteristic feature is its refractile character, sometimes described as a ground-glass appearance. The outer margin is *sharply* defined as a thin dark line. It should be remembered that some pneumococci have small capsules, others large, so that mere capsule *size* must not be mistaken for capsule-*swelling*. Reference can be made to other serum combinations which will show the organisms with capsule but without swelling.

Unfortunately it is usually necessary to traverse many fields in search of positive organisms. Not even the experienced person can give a negative verdict without a considerable effort since sputum often contains micrococci which are surrounded by a halo-like area (usually not a true capsule) and which are easily confused with pneumococci.

If agglutination is observed but no definite capsular swelling is obtained, the set-up must be repeated using less suspected material and a greater proportion of immune serum.

Assuming that a positive has been obtained with one of the serum pools the next step is to repeat the procedure using the sera of the individual types which compose the pool.

PLATE NO. 1




NEUFELD TYPING

A. Appearance of positive reaction showing swollen capsules in preparation **with** serum of homologous type

B. Appearance of negative reaction showing capsules not swollen
Time required: usually five to thirty minutes

From *Pneumonia and Serum Therapy*. Lord and Heffron (2nd ed.).
New York: Commonwealth Fund, 1938.

*Speed of reaction*—Positive reactions are usually evident within a few minutes. If all results are negative it is well to repeat the microscopic examination at the end of one hour. Slow reactions may be due to one or more of several causes, among which are low titer of serum or a disproportionately large number of pneumococci.

*Cross-reactions and reactions to two or more types*—With certain sputa, positive reactions will be obtained with sera of two or more types. This may be due to the presence of two types of pneumococci in the specimen, or to the presence of a single type which reacts with a heterologous as well as the homologous serum. The incidence of cross-reactions is dependent on the sera used and should be largely avoided by the use of properly tested sera. Certain cross-reactions may be due to actual relationships between different types (2 and 5, 3 and 8, etc.), in which case repetition of the test with serial dilutions of the monovalent sera may aid in determining the true type. A finding of two types should be confirmed by repeating the test on the same or another specimen, by mouse inoculation, by blood culture, etc. If other lots of serum of the types involved are available, the test might be repeated with these.

*Mouse Inoculation*—If the material has a poor content of pneumococci and/or if direct typing fails the material should be inoculated into a mouse. Mice weighing 16–24 gm. are suitable. In the case of sputum the material should be emulsified in saline as already described. Inject 0.5 ml. intraperitoneally. At 4–5 hours after inoculation the peritoneal fluid of the mouse will usually show abundant pneumococci. The peritoneal cavity is tapped with the use of a sterile capillary pipette. A rapid insertion is made and the capillary slowly withdrawn. At one point in the withdrawal a fair amount of fluid will enter. This fluid may be mixed with 4 volumes of saline if necessary to obtain sufficient quantity: Type directly.

Should the mouse remain apparently well and show no organisms on peritoneal puncture, it should be killed at the end of 48–96 hours and cultures made from peritoneal cavity and heart's blood.

The use of mouse inoculation requires critical interpretation in the case of sputum since all sputum samples are contaminated with material from the mouth and throat. If typing is obtained within 6 hours after inoculation the organism was probably derived from the sputum. If obtained at a later time it must be considered possible that the type obtained represents the normal carrier type. At least 40 per cent of all individuals are pneumococcus carriers.

## IV. GENERAL BACTERIOLOGICAL PROCEDURES

Gram-positive cocci in pairs or short chains which give characteristic serological results in a typing procedure are so generally accepted as pneumococci that the determination of cultural characteristics is usually considered unnecessary. Organisms which morphologically resemble pneumococci but which fail to react with standard typing sera may be examined as follows:

### 1. *Isolation of pure culture*

Streak blood agar plates (C.M. No. 33) and after 24 hours' incubation pick to blood broth from colonies which are small, translucent, with well defined edges and which are surrounded by a small zone of greenish discoloration. The subcultures should be examined as to morphology, for the pneumococcus not infrequently is involved in a "mixed" colony, especially with *H. influenzae,* and the separation is sometimes troublesome.

### 2. *Typing*

From typical subcultures prepare a dilution of 1–1,000 in broth and inoculate mice. Attempt typing. It should be pointed out that stock cultures in blood broth frequently fail to give significant capsular swelling and that mouse inoculation serves to give suitable material for typing.

### 3. *Bile solubility*

For best results with this test, cultures should be grown in a buffered nutrient medium such as tryptose-phosphate broth (C.M. No. 12). An unbuffered medium containing considerable amounts of fermentable sugar is completely unsuited for this procedure since clearing is not obtained except in the general range of neutrality. Media containing undenatured protein are also undesirable.

Set up two small tubes, one with 0.1 ml. of 10 per cent sodium desoxycholate (or whole sterile ox bile) and one with 0.1 ml. of saline. To each add 0.4 ml. of suitable culture. Incubate at 37° C. and observe after one hour. With a pure culture of pneumococci, the organisms will have gone into solution and the fluid will be completely cleared.

Various modifications of this procedure have been proposed, particularly in the use of microscopic technics, but the standard method remains the most suitable.

### 4. *Fermentation reactions*

It was at one time required by definition that pneumococci ferment inulin as well as dextrose, lactose, and sucrose, but these tests are rather too troublesome for routine purposes. If fermentation is to be attempted the effort should be confined to inulin.

The most satisfactory medium is Hiss serum water containing 0.5 per cent inulin and a suitable indicator (C.M. No. 57). Pneumococci usually give fermentation of inulin with the production of acid (and consequent coagulation). If the results are negative it is necessary to prove by microscopic examination that growth has taken place for failure of multiplication is the most common source of error.

### 5. *Stock cultures*

If it is desired to maintain a given strain for any purpose a considerable amount of attention must be devoted to the problem of preventing variation. Virulence can usually be kept high by transferring in rabbit blood broth at 48 hour intervals, the cultures being kept at 37° C. at all times. For lyophilization a satisfactory result can be obtained by resuspending centrifuged organisms in a sterile 10 per cent solution of amigen (or other casein digest) in distilled water. The dried material has fair stability at room temperature but for best preservation, storage at 4° C. is preferable. An older and still satisfactory method involves the drying of infected mouse spleens over phosphorous pentoxide.

## V. INTERPRETATION OF RESULTS

Although theoretically a laboratory might well confine its efforts to the making of examinations, and leave the interpretation of its findings to the attending physician, this is not always possible. No particular difficulty arises in interpreting the results of examinations of blood, spinal fluid, or pleural fluid, for pneumococci are or are not found; and when found, may be accepted as the infecting agent.

Any of the different pneumococcus types may be found in the sputa of normal persons, or in the sputa of patients with pneumonia due to other bacteria, filterable viruses, rickettsiae, or parasites. Atypical clinical manifestations or positive evidence of other types of acute pulmonary infection are of primary importance for determining the lack of significance of pneumococci in sputa in such cases. Most lobar pneumonias involve pneumococci, however, and the following sugges-

tions are made as to the interpretation of the finding of pneumococci in sputum from typical pneumonia cases and where interpretation is sometimes most difficult.

### 1. *No pneumococci found*

If the patient's condition suggests a pneumococcal infection, another specimen should be examined, and a blood culture taken. The possibility of pneumonia of streptococcal or other etiology should also be kept in mind, but should not preclude further search for pneumococci. It is also necessary to consider the fact that after the use of antibiotics (or sulfa drugs) it may become impossible to demonstrate pneumococci. The time element is referable to the intensity of the therapeutic measures.

### 2. *Pneumococci found, but types not determinable*

With satisfactory antisera this should not occur often. The (suspected) pneumococcus should be isolated in pure culture for determination of bile solubility and for further study (see Section IV, 1 Methods for Cultural Identification). Do not report an organism as pneumococcus unless it is bile-soluble. Another specimen should be examined on the chance that a recognizable type has been missed.

### 3. *Pneumococci found, belonging to a single type*

These can usually be accepted as the etiologic agent, particularly if the type is one of those commonly found in pneumonia and if the demonstration has been direct or from mouse material obtained within 6 hours of inoculation. It should be remembered that pneumococci are at times oral saprophytes, and that in persons who have a pneumonia, due, say, to Type 1 organisms while carrying such a saprophyte, it is possible to find the saprophyte and overlook the pathogen. Therefore, repeated examinations are desirable in all cases and especially so whenever the less common types are found or when Type 3 is the only type recognized.

### 4. *Pneumococci of two or more types found*

It is in this situation that interpretation is most difficult. Pending the results of blood culture examination, a decision as to the most likely agent or agents should be based on the points made in the preceding section and possibly upon the relative numbers of each type

found in the direct typing of the sputum by the Neufeld method. No hard and fast rules can be laid down at present, but some examples may indicate the line of reasoning to follow. If Type 1, which is rarely found except in pneumonia patients or those closely associated with them, and Type 3, which is not uncommon in the normal mouth, are both recognized in the same sputum, the disease is more likely due to Type 1 organism. If any combination of Types 1, 2, 5, and 7 is found, it is probably impossible to estimate, on the basis of sputum examination alone the relative importance of the types found. Purely on the basis of chance, Types 1, 2, 4, 5, 6, 7, 8 (and, in children, 14) are more likely disease producers than Types 25-32. Types 3, 6, and 23 are common mouth contaminants but they both can and do cause pneumonia.

Repeated bacteriological examinations should again be stressed as of the utmost value in arriving at an accurate estimate of the relative importance of the types found.

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# The Meningococcus

## (*Neisseria meningitidis* *)

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* In the new sixth edition of *Bergey's Manual* the name of this organism is given as *Neisseria meningitidis.*

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# I. INTRODUCTION

## A. DEFINITION AND DIAGNOSTIC CHARACTERISTICS

The laboratory diagnosis of meningococcus infection is based chiefly upon the isolation and identification of the meningococcus *(Neisseria meningitidis)*. Therefore, it is important that the laboratory worker have a clear definition of this microörganism, know the conditions under which its characteristic properties may be demonstrated, and when they may be masked. Such a definition may be given briefly as follows:

*" The meningococcus is a Gram-negative coccus, usually occurring in pairs with flattened adjacent sides, which ferments dextrose and maltose with the production of acid."*

The cocci may occur singly or in groups, and the individual cells of a pair often vary greatly in size and in intensity of staining, though they are always Gram-negative when properly stained. Strains that fail to ferment maltose or dextrose, or both of these sugars, when they are first isolated, may be encountered. Such strains usually acquire this property sooner or later, though it may be lacking at a time when it is desired to make a diagnosis of meningococcus infection.

Four other characteristics may be included in the definition in order that they may be discussed: *Characteristic colony formation, lack of pigment production, growth only at body temperatures, and agglutinability by polyvalent antimeningococcus serum.*

As a rule the smooth translucent colonies are quite characteristic, but occasional strains of true meningococci are found that immediately after isolation may have colonies as large as a dime, very convex, or even opaque.

There are four recognized species of the genus *Neisseria* that produce a golden yellow pigment. The meningococcus never produces such a yellow color.

That meningococci grow only at body temperature is, as a rule, a safe generalization to make. Strains are encountered, but rarely, that grow well at room temperature.

Many strains seem to be inagglutinable when they are first recovered from patients. Practically all of these can be coaxed to agglutinate later, though they do not do so at the time when agglutination is useful as a criterion in diagnosis. The significance of this seeming inagglutinability will be discussed later. Many experienced workers do not consider agglutination necessary for identification of the meningococcus.

A more complete definition of the meningococcus may be given thus: "*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 body temperatures, and is, as a rule, at some time agglutinable by polyvalent antimeningococcus serum.*"

Microörganisms having all of the above characteristics are certainly meningococci, but not all meningococci have each and every one of these characteristics.

## B. Distribution and Significance

The normal habitat of the meningococcus is the nasopharynx. In meningococcus infection it may be found in the cerebrospinal fluid either intracellularly or extracellularly, in the blood, or in the petechiae of the skin; its recovery therefrom can be taken as definite evidence of meningococcus infection; but its recovery from the nasopharynx may have a very different, or no significance. Many persons harbor the meningococcus in the nasopharynx, or possibly in the accessory sinuses, for periods varying from a few days to a number of years; sometimes few microörganisms are present, at other times they are predominant. A series of cultures from a given individual may show several in succession that seem completely negative, followed by others that yield the meningococcus in almost pure culture. Carrier surveys, in which a single nasopharyngeal culture is made from each individual, give little information as to the identity of actual carriers in the groups studied. If an inexperienced worker is taking the cultures and the culture medium is not exactly as it should be, many carriers will be missed.

## C. Classification *

The meningococcus, identified in 1887 by Weichselbaum [1] as the cause of epidemic meningitis, was considered a homogeneous species

* A revision of the classification of the meningococcus by the Neisseria Sub-committee of the Nomenclature Committee of the International Association of Microbiologists is now in progress.

until Dopter,[2] in 1909, isolated a strain which differed serologically from the original. Dopter called this the parameningococcus. Intensive work done during World War I indicated that there were two main groups of meningococci and led the French to speak of Group A and Group B,[3] the British equivalents being I and II.[4] Gordon and Murray,[5] using the agglutinin absorption technic, divided the two groups into four types, Types I and III from Group I, and Types II and IV from Group II. The studies made during the years since that time have emphasized the close relationship between Types I and III and have justified their being put together as Group I. But, in the United States at least, the evidence available does not suggest a similar combining of Types II and IV. Strains of Type IV have not been encountered here since 1928, and those studied at that time seemed unrelated to Type II. There are serological as well as other differences among strains classed as Type II, as has been pointed out by Rake,[6] so that the designation Group II seems entirely applicable. In this report the term Group I is used to include Types I and III, and Group II to include all other strains that are agglutinated by a serum representing Type II.

Recently a number of strains that seemed related to Group II by agglutination, have been found on further study to show constant differences.[7] Immunologically they are an independent and homogeneous lot. They produce sera that protect mice well against infections by meningococci of their own group, but there is no cross-protection with sera representing other groups. Because of the agglutinogenic relation to Group II meningococci it has been suggested that, for the present, they be designated as Group II *alpha*[8] following the precedent set by Dopter in his studies of the "parameningococcus" since designated as Group II. Further study may indicate this designation undesirable since the relation to Group II is very superficial. The true position of these newly recognized II *alpha* strains needs clarification as does also that of the American "Type" IV. Some nasopharyngeal strains do not seem to fall into any of the known groups.

When cultures of meningococci are "typed," confusion need not result if the above relationships are kept in mind. Specific strains representing the Gordon-Murray types are available, and type specific sera can be prepared. It must be remembered, however, that most "typing" sera are really "grouping" sera. The same specific carbohydrate is found in Types I and III, and it is difficult to prepare sera which will not "cross." For practical purposes "grouping" is adequate

since Types I and III (Group I) have the same clinical and epidemiological behavior, and this is often different from that of the strains of Group II. More accurate typing is sometimes desirable from an epidemiological point of view.

The importance of serological classification of cultures from patients and from carriers is frequently underestimated. More than 90 per cent of cases of meningococcus infection during epidemics are due to Group I,[9] whereas Group II is more often responsible for sporadic cases. By far the greater number of carrier strains recovered during non-epidemic times are of Group II. Chronic carriers are especially apt to harbor Group II. It is not to be forgotten that while such grouping as that noted above is a practical necessity, the epidemiologist may have occasion to demand finer differentiation. In his search for the origin of an outbreak the results of close type separation may give him a clue of definitive value. This point is well illustrated in the identification of differences among typhoid bacilli, and their epidemiological associations, as reported by Craigie.[10]

### D. Quarantine and Release of Cases and Carriers

In considering the question of quarantine and release of contact and convalescent carriers or patients, the facts just discussed are fundamental; unless the conditions under which the carrier examination is made are ideally controlled the results are certain to be misleading.

During recent years it has been shown that carriers may usually be cleared by the administration of a course of sulfadiazine.[11] Quarantine and release of cases and carriers has been much simplified by this development.

## II. SPECIMENS TO BE EXAMINED AND THEIR TRANSPORTATION

### A. Collection of Specimens

Material submitted for laboratory examination usually consists of spinal fluid, blood, and swabs from the nasopharynx. Occasionally material from petechiae in the skin is examined. Since the disease usually begins as a blood stream infection, blood for cultures should be taken early, just as soon as a meningococcus infection seems to be a possibility, and without waiting for meningeal symptoms to appear. It is usually drawn from the basilic vein. Citrate, in final dilution of 1 per cent, is added only when cultures cannot be made at the bedside.

With the development of meningeal symptoms spinal fluid should

be drawn without delay, though the wisdom of taking such a sample prior to the appearance of symptoms is questioned. The time for tapping the spinal canal must be decided by the clinician who will remember that success in treatment depends largely upon early diagnosis.

Spinal fluid is usually obtained by lumbar puncture, the special needle for this purpose being inserted in the 3rd or 4th lumbar space. The size of the needle used depends to a certain extent upon the size of the patient, though sometimes a very thick and viscid fluid will make a large needle necessary in a child. An 18 gauge needle, 3 or 4 inches long, is the size in common use. In cases where the spinal canal is blocked, cisternal puncture may be resorted to, and special needles are made for this purpose. This is not often necessary for early diagnostic puncture. Ventricular puncture may be done if the fontanelle is still open.

The spinal fluid should be collected directly into at least three sterile tubes. This is imperative when the first fluid is blood tinged, for the presence of blood interferes with some of the examinations to be made. The third tube is to be used for cultures, and the second for cell counts, glucose determinations, and other chemical tests. Some spinal fluids contain enough fibrin to clot quickly. If convenient, it is wise to draw some into a tube containing a crystal of citrate or oxalate for total and differential cell counts. The amount of fluid withdrawn, whether or not it is under increased pressure, and its appearance, *i.e.*, whether blood tinged or yellow, the degree of cloudiness, etc., should be recorded; the fluid, kept constantly warm, should be sent immediately to a laboratory for examination. Bedside culturing is desirable when feasible. In some hospitals a few drops of the spinal fluid are allowed to drip directly through the needle into tubes of culture media.

In cases of meningococcus infection where petechiae are abundant and diagnosis is doubtful, material taken directly from these spots with a fine Pasteur pipette or with a hypodermic needle may be examined microscopically and often successfully cultured. Post-mortem examination of such areas, both bacteriologically and histologically, has proved at times to be the only successful means of accurate diagnosis.

Nasopharyngeal cultures are usually made for the purpose of detecting carriers, and are seldom routinely made from patients with active infection; though it is likely that through such omission interesting information may be missed. Collection of nasopharyngeal material is described in detail in IV, D.

### B. TRANSPORTATION OF MATERIAL

As a rule blood cultures are made at the bedside. Where this is impossible the sample of blood, citrated or otherwise, is transported as quickly as possible to the laboratory. Spinal fluid cultures are also often made at the bedside. In other instances the spinal fluid is carried to the laboratory at once also, and both blood and spinal fluid should be kept as near body temperature as possible in transit. Sometimes these materials will have to be shipped; the meningococcus will occasionally be recovered from them even after long journeys; but the greater the time elapsed and the more variable the temperature, the less likely it is that positive results will be obtained. For cultures from shipped fluids, more than 24 hours old, large inocula are used.

Nasopharyngeal swabs should be cultured immediately from the individual who is being examined. If this is impossible the swab may be placed in a tube containing 1 ml. of defibrinated horse blood or a small amount of semi-solid agar medium [12] until the laboratory can be reached.

## III. PREPARATION OF REAGENTS

### A. CULTURE MEDIA

For isolation of the meningococcus from spinal fluid, the blood agar * plate (C.M. No. 33) and the tube of semi-solid agar (C.M. No. 56), with or without enrichment, are the most practical media; for nasopharyngeal swabs the freshly poured blood agar plate is the medium of choice; and for blood cultures, a meat infusion broth (C.M. No. 5). For fermentation reactions the ordinary semi-solid agar medium with carbohydrate and indicator added (C.M. No. 56) is satisfactory. Should a strain fail to ferment a sugar when the semi-solid medium is used, recourse may be had to a solid medium, *i.e.*, meat infusion agar slants (C.M. No. 5a) with carbohydrate and indicator. It will be remembered that feeble acid producing strains may have this function completely masked in buffered media. For maintenance of stock cultures, semi-solid agar, transplanted every 3 to 4 weeks, or serum dextrose agar (C.M. No. 33b) slants transplanted 2 to 3 times weekly are used. For maintenance of virulence the latter medium is much

* All agar media used for cultivating the meningococcus should have a meat infusion base.

better. Egg slants (C.M. No. 54) are also useful for stock cultures as meningococci may remain viable for a year upon them. Cultures for preparing suspensions for agglutination tests are grown on agar (C.M. No. 5a) with 0.5 per cent dextrose or blood slants (C.M. No. 33).

The best way to preserve cultures is to dry them from a frozen state, as in the "lyophile process" of Flosdorf and Mudd.[13] For this process sterile skimmed milk is an excellent medium in which to suspend the bacteria.

## B. STAINS

1. A smear of the centrifuged spinal fluid sediment stained by Gram's method is an important factor in early diagnosis.
2. A differential count of spinal fluid cells is a special aid when fluids are only slightly turbid. Wright's stain used as for blood smears, or May-Grünwald followed by Giemsa may be employed according to convenience. These may be purchased ready for use.

Various lots of Wright's stain may differ considerably, so that the time intervals for each step should be worked out for each individual lot and adhered to.

Directions for making and using these stains are as follows:

*Gram's Stain, Hucker's Modification*

a. Ammonium oxalate crystal violet solution.

| | |
|---|---|
| Crystal violet (85% dye content, certified) | 4 gm. |
| Ethyl alcohol (95%) | 20 ml. |

Dissolve the crystal violet in the alcohol.

| | |
|---|---|
| Ammonium oxalate | 0.8 gm. |
| Water | 80.0 ml. |

Dissolve the ammonium oxalate in the water.

b. Dilute the crystal violet solution 1:10 with distilled water.

c. Mix one part of the diluted crystal violet solution with 4 parts of ammonium oxalate solution.

*Gram's iodine solution*

| | |
|---|---|
| Iodine | 1 gm. |
| Potassium iodide | 2 gm. |
| Water | 300 ml. |

a. Dissolve the iodine and potassium iodide in the water.

b. This solution deteriorates on standing and should be prepared freshly at least every 2 weeks.

*Counterstain*

| | |
|---|---|
| Safranin (2.5% solution in 95% alcohol) | 10 ml. |
| Water | 100 ml. |

*Staining Procedure*

a. Stain 1 minute with the crystal violet solution.
b. Wash in water.
c. Apply iodine solution for 1 minute.
d. Wash in water.
e. Decolorize in 95 per cent alcohol for 30 seconds with gentle agitation or until violet dye fails to appear in the alcohol.
f. Apply counterstain for 10 seconds.
g. Wash in water; dry without blotting.

*Wright's Stain*

(Many workers prefer to make their own polychrome stains. The ready prepared dry stains and the solutions of them are generally satisfactory.)

| | |
|---|---|
| Methylene blue hydrochloride (certified) | 0.9 gm. |
| Sodium bicarbonate, 0.5 per cent aqueous solution | 100.0 ml. |

a. Heat in a steam sterilizer at 100° C. for 1 hour, in a flask large enough so that the layer of fluid is not over 2 inches deep.
b. Cool and filter.
c. To the filtrate add:

| | |
|---|---|
| Eosin Y (certified) | 1.0 gm. |
| Distilled water | 500.0 ml. |

d. Mix and filter.
e. Collect the precipitate; dry thoroughly at 37° C.
f. Dissolve the dried precipitate in these proportions:

| | |
|---|---|
| Wright's stain (dried precipitate) | 0.1 gm. |
| Methyl alcohol, absolute, neutral, acetone free | 60.0 ml. |

g. Filter before using.

*Staining Procedure*

a. Cover the dried preparation and fix for 1 minute with the methyl alcohol solution of the stain.
b. Dilute and stain by adding an equal quantity of Sörensen's phosphate buffer pH 6.5; metallic film forms on the surface.
c. Stain for 3–5 minutes.
d. Wash by flooding the slide with distilled water or better with phosphate buffer solution.
e. Dry by blotting carefully or dry in the air.

*Technic for using May-Grünwald and Giemsa stains*

A method of using May-Grünwald and Giemsa stains which gives constant results is as follows:

a. Flood the slide with the stain, leave it on for 3 minutes and then pour it off. The slide may be washed with distilled water, but it is unnecessary. Do not blot.
b. Pour on the diluted Giemsa (for 1–10 Giemsa 10 minutes is average; for 1–15, 20 minutes is best). The methyl alcohol used for the Giemsa *must* be acetone free.
c. Wash thoroughly with distilled water from a wash bottle until the preparation has a pink color.

## C. REAGENTS FOR SUGAR DETERMINATIONS[14]

### 1. *Alkaline Copper Tartrate Solution*

a. Dissolve 40 gm. of pure anhydrous sodium carbonate in about 400 ml. of distilled water in a liter flask.

b. Add 7.5 gm. of tartaric acid.

c. When the tartaric acid is dissolved add 4.5 gm. of crystallized copper sulphate.

d. Mix and make up to a volume of 1 liter.

e. Test for the absence of cuprous copper by transferring 2 ml. to a test tube and adding 2 ml. of the molybdate-phosphate solution; the deep blue of the copper should almost vanish.

f. If the chemicals used are not pure, a sediment of cuprous oxide may form in the course of 1 or 2 weeks. If this happens, remove the clear supernatant reagent or filter it through a good quality filter paper. Test for copper. This reagent keeps indefinitely.

### 2. *Molybdate-Phosphate Solution*

a. Transfer 70 gm. of molybdic acid (C.P., Baker's analyzed "special") to a liter beaker.

b. Add 10 gm. of sodium tungstate, 400 ml. of 10 per cent sodium hydroxide, and 400 ml. of distilled water.

c. Boil vigorously for 20 to 40 minutes (to remove ammonia present in molybdic acid).

d. Cool and dilute to about 700 ml.

e. Add 250 ml. of concentrated (85 per cent) phosphoric acid.

f. Make up to a volume of 1 liter.

The solution should be water clear. If it has a yellow tinge the chemicals used are not pure and there will be an error in the determination.

### 3. *Benzoic Acid*

Dissolve 2.5 gm. benzoic acid in 1 liter of hot water and cool. Transfer to a bottle; the solution will keep indefinitely. Filter as necessary.

### 4. *Standard Sugar Solutions*

a. *Stock*—Weigh 1 gm. of pure dextrose (d-glucose) on an analytical balance and dissolve in 100 ml. of the benzoic acid solution. This 1 per cent standard stock solution keeps indefinitely.

b. *Standard Containing 10 mg. Dextrose per 100 ml.*—Pipette 5 ml. of stock solution into a 500 ml. volumetric flask and dilute to the mark with benzoic acid solution.

c. *Standard Containing 20 mg. Dextrose per 100 ml.*—Pipette 10 ml. of stock solution into a 500 ml. volumetric flask and dilute to the mark with benzoic acid solution.

Diluted standards 2 and 3 should be made fresh every month.

## IV. EXAMINATION OF SPECIMENS

### A. CEREBROSPINAL FLUID

#### 1. *Collection*

This has been collected in 3 sterile tubes as described in II, A. The last tube is used for cultures, and the second for cell counts and glucose determinations.

#### 2. *Microscopic examination*

a. Cell counts on fresh unstained fluid—Cell counts must be made immediately if they are to be of value. The sample of fluid should be free from blood, and the cells should be evenly distributed. Usually the fluid may be placed directly, without dilution, in a counting chamber, 9 large squares counted, and the result multiplied by 10/9 to obtain the number of cells in 1 cu. mm. If the cells are too numerous to count by this method, the fluid may be diluted in a "white counting" pipette, as for a regular leucocyte count. The value of the cell count is considered doubtful by some.

b. Preparation, fixation, and staining of films for differential count—A differential count of the spinal fluid cells should also be made at this time. It is a special aid to diagnosis with fluids that are only slightly turbid. The predominance of polymorphonuclear leucocytes will aid in ruling out tuberculous meningitis, poliomyelitis, encephalitis, and lymphocytic choriomeningitis. With very cloudy fluids smears for this purpose can be made without centrifugation; if the fluid is only slightly cloudy it may be centrifuged first and the sediment used. Wright's stain, used as for blood smears, or May-Grünwald followed by Giemsa may be employed.

c. Preparation, fixation, and staining of films for examination of bacteria—A smear of the centrifuged sediment stained by Gram's method is an important factor in early diagnosis. The flattened Gram-negative diplococci may be abundant, both within and without the leucocytes; on the other hand, it may require careful search to detect them, and sometimes they cannot be found. A half-hour is none too long to look for them. This stained smear should be made and examined as soon as possible. A second smear may be stained with methylene blue, as this dye is especially valuable in revealing morphology.

d. Preliminary identification and typing by capsule swelling—Typing of meningococci directly in the spinal fluid by means of demonstrating capsular swelling with homologous antisera was suggested by Clapp [15] and further reported by Beckler.[16] This technique is now used routinely in many laboratories. For its success there must be a sufficient number of meningococci in the fluid to be easily seen, and best results are obtained if the sample of fluid is drawn before the patient has had either sulfonamide or serum therapy. Capsules can be demonstrated for meningococci of Group I and Group II *alpha,* but none has yet been reported for Group II strains.

To perform this test a loopful each of serum, spinal fluid, and methylene blue are mixed together on a cover slip which is inverted on a slide, incubated in a moist atmosphere for 30 minutes at 37° C., and examined microscopically.

e. Evaluation of findings and reporting of results—It is never safe to jump to the conclusion that any Gram-negative bacteria seen are meningococci, for the Pfeiffer bacillus *(Hemophilus influenzae)* is very pleomorphic in spinal fluid and some Gram-positive cocci may seem Gram-negative in such smears. If Gram-negative diplococci are found they should be reported at once as such, and not called meningococci without further identification except in those cases where capsule swelling has been observed. Even in these cases the identity of the organism should be confirmed by cultural and by further serological means.

Further identification consists in cultivating the organism on a suitable medium, and examining its morphology in pure culture, its cultural characteristics, and its action upon dextrose and maltose. The identity of the microörganism may be reported at this point. Corroboratory evidence can be obtained by agglutination with polyvalent serum, and this is especially valuable in the case of strains in which fermentative powers are not well developed. It must be remembered that some freshly isolated strains are inagglutinable in some polyvalent sera. It is not absolutely necessary that the strain should agglutinate with polyvalent serum in order to be called meningococcus.

### 3. *Cultural examination*

a. Preparation of cultures—A generous inoculum should be used; *i.e.,* from 0.5 to 1.0 ml., depending on the nature of the fluid, on a blood agar (C. M. No. 33) plate and into a tube of semi-solid agar (C. M. No. 56), with or without enrichment. In the field, or wherever blood

is unobtainable, Mueller's medium[18] may be used (C.M. No. 61) for isolation and transportation. Centrifuged sediment may be used, or uncentrifuged spinal fluid when the meningococci seem to be especially abundant in the stained smear. The spinal fluid itself as a medium should not be ignored. Incubating it at 35° to 37° C. overnight often results in a multiplication of the meningococci so that their detection in smears and their cultivation are made much easier. The spinal fluids must be kept warm while they await examination. Ideally spinal fluid should be collected before the patient has been given sulfonamides. Actually many samples come from those in whom such therapy is already established. In these cases para amino benzoic acid should be added to the culture medium, enough to give a final concentration of 5 mg. per 100 ml.[17] In some laboratories this para amino benzoic acid is added routinely to all culture media when it is first prepared.

b. Examination of cultures—Colonies of meningococci on blood agar plates are usually characteristic in appearance, being smooth and translucent, and often much larger than textbook description would lead the reader to believe. Growth may be confluent. Colonies may be picked to semi-solid medium (C. M. No. 56), or to blood agar (C. M. No. 33) or serum dextrose (C. M. No. 33b) agar slants or to slants of Mueller's medium (C. M. No. 61).

On semi-solid agar the meningococci grow as a pellicle at the surface of the medium.

c. At this point the microörganisms may be reported as Gram-negative diplococci of typical Neisserian morphology, but they should not be reported as meningococci until identification of the pure culture is complete.

4. *Chemical tests*

Quantitative determinations of sugar in spinal fluid are done routinely in many laboratories and are considered important, not only as an aid in diagnosis, but in prognosis and in following the course of the infection. The amount of sugar is decreased in various forms of purulent meningitis, whereas it remains normal or is increased in poliomyelitis, epidemic encephalitis, or lymphocytic choriomeningitis. The normal amount is 40 to 60 mg. per 100 ml. Persistent low sugar content of spinal fluid in meningococcus meningitis is considered to be indicative of a poor prognosis, and an increase toward the normal is thought to be an encouraging sign. There are several points to remember in the

interpretation of the sugar findings. One is that when spinal fluids are allowed to stand, almost any bacteria will use up the sugar present. Another is that there may be reducing substances other than sugar present. A third is that there is a relation between the sugar content of the spinal fluid and that of the blood.

a. Quantitative estimation of sugar in cerebrospinal fluid—It must be remembered that sugar determinations cannot be made in a blood-tinged fluid. If the cerebrospinal fluid contains a little blood, centrifuge it and use the clear supernatant fluid. The following adaptation of the method of Folin and Wu [19] to cerebrospinal fluids is taken from the laboratory manual of Kolmer and Boerner.[14]

*Principle*

Proteins present are precipitated by tungstic acid and determination is carried out by the Folin-Wu method, using 1–5 dilution of filtrate.

*Procedure*

(1) Pipettes and test tubes must be absolutely clean and dry or the error will be large.

(2) With a 1 ml. pipette transfer 1 ml. of spinal fluid to a *clean, dry* test tube.

(3) Add, with a pipette, 3 ml. of distilled water.

(4) Using a graduated 1 ml. pipette, add 0.5 ml. of 10 per cent sodium tungstate.

(5) Add 0.5 ml. of ⅔ normal $H_2SO_4$.

(6) Mix well and let stand 5 to 10 minutes.

(7) Filter.

(8) Pipette 2 ml. of the clear filtrate into a Folin-Wu sugar tube and proceed as follows:

(9) To a similar tube, add 2 ml. of standard sugar solution 2 containing 10 mg. dextrose per 100 ml. and to a third add 2 ml. of standard sugar solution 3 containing 20 mg. dextrose per 100 ml.

(10) To each tube add 2 ml. of alkaline copper tartrate solution.

(11) Transfer the tubes to a boiling water bath and heat for 6 minutes.

(12) Cool for 2 to 3 minutes in a cold water bath without shaking.

(13) Add to each tube 2 ml. of molybdate-phosphate solution.

(14) Let stand for 3 minutes; dilute the resulting solution to the 25 ml. mark.

(15) Insert a rubber stopper and mix.

(16) Compare in a colorimeter with the nearest matching standard set at 20. The 2 standard is adequate for practically all cases.

(17) Calculation:

Using standard 2 which contains 10 mg. dextrose per 100 ml.

$\frac{20 \times 50}{R}$ = mg. dextrose per 100 ml. fluid. R = Reading.

Using standard 3 which contains 20 mg. dextrose per 100 ml.

$\frac{20 \times 100}{R}$ = mg. dextrose per 100 ml. fluid.

### 5. *Serological test for syphilis*

A routine Wassermann test should be performed on the first specimen of cerebrospinal fluid taken from a patient suspected of having meningococcus meningitis. The results of this test when correlated with the blood test, history, and physical findings may make clear the diagnosis in an otherwise puzzling case. The serological test performed on the cerebrospinal fluid after serum has been administered is not reliable due to the frequency of false-positive reactions.

## B. BLOOD

It is becoming more and more common to make blood cultures as an aid to diagnosis in meningococcus infections. Especially are these of value in very early cases, and in those without meningeal symptoms. These should be made as soon after the blood is drawn as possible, preferably at the bedside. About 5 to 10 ml. of blood, drawn into a sterile syringe from a vein, is added to 100 ml. of warmed infusion broth (C.M. No. 5) in a 250 ml. flask. This gives a relatively large surface, which is desirable, as the meningococcus tends to grow at the surface. Semi-solid agar (C.M. No. 56) gives good results also, as meningococci often grow more easily in it than in liquid media. Numerical results may be obtained by adding the blood to infusion agar (C.M. No. 5a) melted and cooled to about 45° C., mixed, and poured into Petri dishes. Addition of 0.1 to 0.2 per cent dextrose to these media may enhance the growth. When initial cultures are obtained, identification of the organism proceeds as usual.

## C. PETECHIAE

Although meningococci are present in the petechiae, isolation from them is seldom attempted. Material may be withdrawn with a fine

Pasteur pipette or a hypodermic needle, stained smears prepared, and cultures made in semi-solid agar. Occasionally, after death, examination of petechiae allows a diagnosis when other methods are unsuccessful, especially in fulminating cases which have died without showing meningeal symptoms and from which no blood cultures were made. The microörganisms may be seen in properly stained sections (Gram), and they may often be cultivated if the tiny bit of skin is placed in semi-solid media.

### D. NASOPHARYNX

Few hospital laboratories make nasopharyngeal cultures from cases of meningitis routinely. Therefore, we do not know how constantly and for how long they harbor the meningococcus in this region. It can be said, however, that those who have looked for it here during the course of the disease have usually found it. Nasopharyngeal cultures have more frequently been made for the purpose of detecting carriers. In either case, the technic is the same, and is described in great detail in many manuals. Carrier studies should not be undertaken unless they can be made under proper conditions and followed through intensively and completely.

#### 1. *Collecting the material*

*Equipment*

Wooden tongue depressors
Cotton swabs applied to wooden applicators *
  These should be of 2 sizes: small ones for the nasal passages, and larger ones for the nasopharynx
A head mirror or lamp
A cellophane face mask (as suggested by Lombard and Pittman)
Plates of freshly poured blood agar (C.M. No. 33)

It is important that the medium be firm but moist. Meningococci require abundant moisture for their growth. As noted below, the plates should be incubated inside museum jars with wet cotton at the bottom and with the lid fitted on closely.

*Procedure*

When a carrier survey is to be made the demand that the work be expedited results in constant temptation to take short-cuts. Any yielding in such direction is fatal. No procedure requires more strict

* Many prefer a wire swab with the cotton covered end bent at an angle.

attention to every detail. When the work cannot be done thoroughly it should never be attempted, for the results will, without any qualification, be misleading.

Those who are to be examined come before the bacteriologist in single file in a quiet and orderly manner. They need not be seated, but stand in front of the operator who requests that the mouth be opened wide. The dorsum of the tongue is held down, the applicator put inside the mouth, and the subject asked to phonate vigorously. This raises the uvula and permits the swab to be passed under and beyond it. Now, by lifting the uvula more and at the same time lowering the hand, sometimes by using the lower teeth as a fulcrum, the swab is easily pushed up against the posterior nasopharyngeal wall—the region where the nidus of meningococci seems most likely to be. Now, while the swab is pressing gently against the mucous membrane, it is given a slight twirling motion; this tends to wind up some of the local mucus on the swab. Then the swab is withdrawn quickly without its tip coming into contact with anything. The entire procedure requires only two or three seconds.

For specimens to be collected through the nostril, the procedure is simple. The subject is told to look straight ahead and not to throw his head back. The tip of the nose is raised slightly with one hand and with the other the swab is passed *along the floor* of the nasal passage until it meets the posterior wall of the nasopharynx. A gentle twirling motion seems to cause the swab to wind up on itself the mucus and exudate required. If at any time during this procedure the subject begins to move his head around, merely drop or let go of the applicator; the promise to take it out for him without hurting him generally induces him to coöperate.

As to which—throat or nasal cultures—are likely to give better results, there is no definite answer. Sometimes one will be positive and the other negative. It is probable that two throat cultures or two nasal cultures would give about the same averages. When such thoroughness is possible both a throat and a nasal culture should be made.

### 2. *Plating out material*

The blood agar plate (C.M. No. 33) is the medium of choice for the isolation of the meningococcus from the nasopharynx. Where blood is unobtainable Mueller's medium (C.M. No. 61) can be used. Inoculation of the plate from the swab is done variously by different workers. The technic used by Rake[6] in his carrier studies is recommended. The

tip of the swab is brought gently into contact and smeared on the blood agar over a very small area at one edge. Then a fresh, clean, sterile swab is taken and with it the inoculum is spread over one-half of the plate, the excursions back and forth across the plate being made very close together or overlapping; a second fresh swab now spreads the material at a right angle for another one-fourth of the plate; and a final swab is used at a right angle to finish covering the surface of the plate with the progressively decreasing inoculum. Platinum loops may take the place of the swabs after the plate has received the initial inoculum. These plates are incubated for 24 hours, after being placed inside museum jars with wet cotton beneath them. When such jars are not available some other method should be improvised which will insure a very humid atmosphere.

3. *Fishing of colonies for further study*

On the usual blood agar plate (C.M. No. 33) the translucent meningococcus colonies are easily distinguished from those of the commoner inhabitants of the nasopharynx, except for the other members of the genus *Neisseria,* which are often confused with those of the meningococcus. The yellow pigment of the chromogenic species is often slow in appearing. It must not be forgotten that occasionally meningococcus colonies may be very large.

Differentiation of the meningococcus from other *Neisseria* often requires careful cultural, biochemical, and serological studies. This makes carrier detection a laborious procedure.

Sometimes the proportion of meningococci to other bacteria in the nasopharynx will be very small; at other times it may be found in practically pure culture.

The suspected colonies are ringed, transferred to blood agar or serum dextrose agar slants (C.M. No. 33b), and incubated at 35°–37° C. The next day the growth is stained by Gram's method, and if it shows typical Neisserian morphology, cultures are made to test its fermentation reactions; it is transferred to semi-solid agar to keep it alive for future use, and the remainder of the 18–24 hour growth is suspended in salt solution to use for agglutination with both polyvalent and type or group sera. The most specific results of agglutination are obtained with 5–6 hour cultures. If slide agglutination is used at this stage, it should be confirmed later by the test tube method. The "oxidase reaction" may be of some value with nasopharyngeal cultures as it facilitates the recognition of the genus *Neisseria*. It is discussed later.

If the virulence of the culture is to be studied it should be kept on serum glucose agar slants (C.M. No. 33b) and transferred twice a week, or on blood agar slants (C.M. No. 33), and transferred every other day.

## V. IDENTIFICATION OF PURE CULTURES

### A. BACTERIOLOGICAL

When the Gram-negative diplococci of typical morphology are obtained in pure culture, it is necessary to carry their identification through to completion, for all members of the genus *Neisseria* have a similar morphology, and species other than *meningitidis* are occasionally encountered in meningitis.

Colonies of the meningococcus are smooth, round, and translucent with a tendency to confluence when they are abundant. Usually they are 2–3 mm. in diameter, but they may be much larger, even 1 cm.

The only other member of the genus *Neisseria* that produces such translucent colonies is the gonococcus *(Neisseria gonorrhoeae)*. These are usually smaller, growth is less luxuriant, and there is less tendency to confluence.

Colonies of *Neisseria catarrhalis* are more opaque and white, whereas those of *Neisseria sicca* are so dry and adherent that they are either taken up entirely by the inoculating needle, or can be pushed about.

Colonies of the pigmented members; *i.e., Neisseria flava, Neisseria subflava, Neisseria perflava,* and *Neisseria flavescens,* may resemble the meningococcus on the first day, but they gradually become more opaque and develop a yellow color.

Fermentation reactions are useful in identifying members of this group of bacteria. The meningococcus produces acid in dextrose and maltose. Individual strains vary greatly in both the actual and relative amounts of acid produced from these two sugars, and sometimes, soon after isolation, the reaction will be so transient that the strain in question may seem to produce no acid in one of these sugars, especially if a buffered medium is used. Fermentation nearly always becomes more typical as time goes on.

The gonococcus produces acid from dextrose only, and *Neisseria catarrhalis* ferments none at all.

The four pigmented members of the genus are separated from each other chiefly by their fermentation reactions, though *Neisseria flavescens* seems to be more homogenous serologically than the others.

For these fermentation tests, growth from a pure culture is inoculated

into special semi-fluid agar (C.M. No. 56). An acid reaction may be transient; for this reason the cultures should be examined often during several days' incubation.

## B. SEROLOGICAL REACTIONS

### 1. *Identification by agglutination*

The identity of the meningococcus should be confirmed by agglutination with polyvalent serum. Growth not more than 24 hours old, and preferably from 6 to 12, from solid media, is used. The favorite media for this purpose are 0.5 per cent dextrose agar or 5.0 per cent rabbit's blood agar. Growth is suspended in buffered 0.85 per cent sodium chloride solution, and the suspension diluted to a density equivalent to 500 p.p.m. of the silica standards described in *Standard Methods for the Examination of Water and Sewage.*[20] Such a suspension contains, on the average, about one billion organisms per ml. Any other method of estimating turbidity that gives similar results is satisfactory.

It is important to know the pH of the salt solution used, for a culture that may be inagglutinable at pH 7.8 may agglutinate nicely at pH 6.8. Serum dilutions of 1:25 to 1:800 or higher should be made, making the final dilutions after the suspension is added 1:50 to 1:1,600. The total amount in an ordinary sized agglutination tube is 1 ml. Normal horse serum in a dilution of 1:25 and 1:50 and a saline control should also be used. This whole test is incubated at 37° C. for 2 hours and read after storage in icebox overnight.

Some polyvalent sera are made from "rough" stock strains and do not contain agglutinins for the "smooth" specific strains. Such "smooth" strains will seem to be inagglutinable. Later, when the "smooth" strains have lost their specific capsular substances and have become "rough," they may be well agglutinated by that same polyvalent serum.

Some laboratories use microscopic slide agglutination. The advantage of this procedure is that individual colonies from a primary culture may be used and a correct diagnosis is arrived at quickly. The usual method for performing these is as follows[21, 22]:

Polyvalent antimeningococcic horse serum and normal horse serum are diluted 1:10 with 0.85 per cent sodium chloride solution, and a loopful of each placed on a slide. Some of the suspected colony is rubbed up in each, or, some of the colony is mixed with the normal serum and a portion of this suspension transferred to a drop of diluted immune serum.

Agglutination may be observed macroscopically and microscopically. Microscopic agglutination should be confirmed by the macroscopic tube method. Organisms of characteristic morphology and staining, which are agglutinated only by the immune serum, may be tentatively considered meningococci.

Another agglutination technic that is popular in the field is the "rapid" or "short" method described by Noble.[23] The suspension of microörganisms contains 5 times as many bacteria and the serum concentrations are five times as great as for the regular tube agglutination test. Only 0.1 ml. of a serum dilution is placed in each small tube, and 0.1 ml. of suspension added. The rack is inclined to an angle of about 90° and is shaken slowly for 2 minutes in such a way that the mixture of serum and suspension flows up the tube about 1 inch. Then 0.8 ml. of 0.85 per cent sodium chloride solution is added to each tube and the agglutination read. The usual control tubes should be included. This technique has the advantage of giving results quickly.

### 2. *"Grouping" and "typing"*

Determination of serological group of meningococci is necessary from an epidemiological standpoint, and is also an aid from a therapeutic angle. The relative incidence of the principal groups in active cases, convalescents, contact carriers, and healthy chronic carriers may vary widely.

Accurate determination of the type within the group is epidemiologically of value, as there are definite trends to certain types within the groups; *i.e.*, during the years 1930–1936, Type I became increasingly more common than Type III. Mere determination of groups would not have revealed this trend, though for routine purposes there is no practical advantage in spending time and effort in separating Types I and III from each other. Some strains are easily recognized as Type I or Type III, but usually there is considerable "crossing" unless the sera used are highly type-specific. In most routine diagnostic laboratories it will usually be found more satisfactory to refer to these two types together as Group I.

The recovered meningococci may be grouped or typed later if there is no opportunity at the actual time of isolation. However, group determination is quickly made if good sera are available, and usually there will be no need for delay. A much better idea of their antigenic pattern can be obtained immediately after isolation since some strains have a tendency to lose serological specificity on prolonged laboratory

maintenance so that it is more difficult later to determine their type accurately or their relation to the other strains in a given epidemic. In case storage is necessary, as in extensive carrier surveys, such changes are reduced to a minimum if the strains are preserved by drying them from a frozen state.[13]

For "grouping," the sera used should be prepared with "smooth" strains that have abundant specific capsular substance, for it is to this that group specificity seems to be due. The relative amount of this specific substance can be determined by the "halo" reaction described below. Suspensions of the meningococci to be studied are prepared just as described above for agglutination with polyvalent serum. Serum dilutions, however, are lower, and are usually made from 1:10 to 1:320. The pH of the sodium chloride solution used for both suspensions and serum dilutions should be known; generally pH 6.8 gives good results, but if there is a tendency to spontaneous agglutination, the alkalinity should be greater, perhaps up to pH 7.8. Incubation in a water bath at 37° C. for 2 hours, followed by icebox storage overnight, gives the most clear-cut results, as the group specific agglutination occurs at these temperatures. A higher titer may be obtained by overnight incubation at 56° C., but cross-agglutination is more apt to occur, since the nonspecific agglutinins react also under these conditions. The rapid technic described by Noble[23] is often found useful, especially in the field where water baths are not available.

If typing within the groups is to be done, it is essential that the sera be type-specific. The strains of meningococci with which the rabbits are to be immunized must be chosen with great care since the serological types within the groups are not clear-cut and almost every degree of overlapping can be found. Those chosen should correspond as nearly as possible to standard type strains, and they should be "smooth." Preservation in a dried state is the simplest way of keeping biological characteristics of such cultures unchanged. Information about availability of standard type strains can, at the present time, be obtained from the National Institutes of Health, Bethesda, Md.

For typing within a group, serum dilutions are usually made from 1:10 to 1:640, and incubation is at 37° C. for 2 hours, as described above.

A satisfactory method of immunizing rabbits with Group I is by 3 courses of intravenous injections of freshly made suspensions of 5–10 hour cultures of meningococci on 3 successive days, these series being a week apart. A week after the third series, a trial bleeding is made, and if the serum is satisfactory the animal is bled from the

heart. For Group II a longer course of injections may be necessary. If the immunization extends over too long a period the titer of the serum may be increased, but this is apt to be at the expense of specificity, and more "cross-agglutination" is apt to result. Better results are often obtained when the first injection is made intracutaneously.

Recently Phair[24] and his coworkers have reported the preparation of typing serum in chickens. Since chickens are not susceptible to infection with the meningococcus it is possible to give them large intravenous doses and to produce a good agglutinating titer in a very short time. If chicken serum is used for typing it is best to employ the rapid agglutination method. The use of chickens is especially advantageous in preparing serum with Group II strains, since these are often poor antigens.

In "grouping" or "typing" meningococci the strains with which the sera were made should be included in the test; a polyvalent horse serum of good agglutinating titer should be used also for control purposes.

### 3. *Identification by capsule swelling*

Determination of the serological group of meningococci directly in the spinal fluid has been described in Section IV, A, 2. This technique can, of course, be used with pure cultures also, and even with single colonies. A loopful of a 5 hour culture suspension (about 100,000,000 cells per ml.) is substituted for the spinal fluid. A 10 per cent serum broth is an especially good medium for such cultures. This technique is rapid and economical and has become very popular.[10]

## C. REPORTING

When Gram-negative diplococci are seen in a stained smear, they should be reported immediately as such, but not called meningococci until further information is obtained. A strain may be reported as a meningococcus if it is not agglutinated by normal horse serum, does not form pigment, and ferments dextrose and maltose only. However, if it fails to ferment one or both of these sugars, as some strains seem to do immediately after isolation, confirmation of its serological identity must be obtained before reporting it as certainly a meningococcus. This report should be made as promptly as possible without waiting for the typing to be finished. The type or group is reported as soon as it is known.

# VI. SEROLOGICAL EXAMINATIONS, SPINAL FLUID AND PATIENT'S SERUM

## A. SPINAL FLUID

### 1. *Direct precipitation tests upon the cerebrospinal fluid*

Potentially these are of value in arriving at early specific diagnosis. However for the test to be successful the serum used must be rich in precipitins and the spinal fluid should be obtained before therapy is begun.

Rake[25] has described such a test in which he used monovalent rabbit serum especially high in specific group precipitins. He emphasizes the fact that negative results are apt to be obtained with spinal fluids in which the meningococci are few, thus causing very little specific substance to be present. His technic, which is also suitable for use with polyvalent sera, is essentially as follows:

One-tenth ml. of serum is placed in a precipitation tube, and 0.1 ml. of centrifuged spinal fluid is layered over it. Inspection is made at once for a ring at the point of junction; if negative, the tube is incubated at 37° C. A second reading is made after 1 hour, and in the absence of reaction the tube is thoroughly shaken and replaced in the water bath. A third reading for precipitate is made at 2 hours, and if still negative the tube placed in the icebox overnight; final reading is made the following morning.

A positive result with such a test can give a provisional diagnosis very early. A negative test may merely mean that the number of microörganisms or the amount of the specific soluble substance of the meningococcus is too small to be demonstrable, or that the serum used is not sufficiently rich in precipitins. The conditions necessary for the successful performance of this technic limit its usefulness.

### 2. *Capsule swelling*

Typing of meningococci directly in the spinal fluid by means of demonstrating capsular swelling with homologous antisera has been already described in section IV, A, 2.

Capsules can be demonstrated for Group I and Group II *alpha* meningococci but thus far none have been reported for Group II strains.

## B. PATIENT'S SERUM

Several workers have made a study of the agglutinin titers in the serum of patients with meningococcus meningitis during the course of

the disease. Most cases show agglutinins at some time during the infection, usually after the first few days, though the titer may be low. Examination of only one sample will frequently give a negative result. Study of a series of samples can be useful in detecting the presence of meningococcus infection when organisms cannot be found in blood or spinal fluid.

Such tests are generally set up as usual, with serum dilutions of 1:8 to 1:512 or more. All reagents must be accurately checked and controlled since titers may not be high. After setting up the tests they may be centrifuged at high speed for 15 to 20 minutes and then incubated for 2 hours at 37° C. and stored in an icebox overnight [26] or else they may be centrifuged and stored in an icebox without incubation.[27] The former method seems to give higher titers.

## VII. OTHER METHODS NOT IN COMMON USE

### A. Oxidase Test

The method of applying the direct oxidase reaction to the differentiation of bacterial colonies was described by Gordon and McLeod, 1928.[28] Modifications of their technic have been reported by Ellingworth, McLeod, and Gordon,[29] by McLeod, *et al.*,[30] and by Leahy and Carpenter.[31] All of the *Neisseria* produce this reaction, as well as other bacteria which produce an active oxidase. Its practical value in aiding the detection of such bacteria in mixtures is obvious. Though it has chiefly been used for detecting the gonoccocus in plates made from urethral and cervical discharges, it promises to be a useful aid also in the isolation of meningococci and other *Neisseria* from nasopharyngeal cultures.

The technic as applicable to nasopharyngeal swab cultures, is, in general, as follows: "chocolate" agar (C.M. No. 33a) plates are inoculated with the nasopharyngeal swabs, as described in IV, D, 2, and incubated at 37° C. for 24 hours. The plate is then flooded with 1 ml. of a 1 per cent solution of dimethyl-paraphenylenediamine hydrochloride, which is then poured off. It may be considered advantageous to apply the reagent to a single colony or to a small area; for this purpose a platinum loop is highly satisfactory. The *Neisseria* colonies become pink, then purple, and then black, the time required for these color changes being about 6 to 10 minutes. Since upon prolonged contact the dye is apt to be toxic for the bacterial cells, subcultures should be made while the colonies are still pink;

when the colonies become black the cells are likely to be dead. Ellingworth, McLeod, and Gordon[29] prefer the tetramethyl compound to the dimethyl on account of its lower toxicity for bacterial cells. The tetramethyl compound is about ten times as expensive, however, as the dimethyl. A fresh solution of the dye should be prepared each week as it deteriorates on standing.

### B. THE "HALO" REACTION

The development of halos around colonies of meningococci on agar plates containing immune serum has several useful applications in the laboratory. The reaction is group specific, as was first noted by Petrie,[32] and Group II strains can be distinguished from those of Groups I and of II *alpha*. In localities where accurate typing by agglutination is impracticable, this method can be extremely useful for preliminary grouping. The technic described for preliminary evaluation of therapeutic antimeningococcic serum[33] can be modified for this purpose. In general, the following technic is suggested:

1. Melt the agar in tubes, each tube containing approximately 15 to 16 ml.

2. Cool the agar to 50° C., add 0.8 ml. of serum, and pour the mixture into sterile Petri dishes. This gives approximately 5 per cent of the serum.

3. Collect a mass of growth, about 2 mm. in diameter, from an 18 hour culture on glucose serum agar, or 5 hour culture on blood agar.

4. Place this mass upon the surface of the specific serum agar plate without spreading; 6 to 10 different cultures may be tested on one Petri dish.

5. Incubate the plate at 37° C., and examine it after 48 and 72 hours in a strong light against a dark background. The specific precipitins in the serum and the soluble specific antigens of the meningococci will form a visible precipitate in the form of a halo around the bacterial growth. The intensity of the halo is recorded as 1 plus to 4 plus.

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# The Gonococcus

## (*Neisseria gonorrhoeae*)

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## I. INTRODUCTION

The diagnosis of gonococcal infection still constitutes one of the major problems in the public health laboratory. In spite of the extraordinary therapeutic effectiveness of penicillin in gonorrhea many health departments are annually reporting more cases of the disease. The recent development of more dependable cultural technics has aided materially in differentiating gonococcal from non-gonococcal urethritis in the male and facilitated the diagnosis of the disease in the female. In many venereal disease clinics the improved cultural method is likewise used as a test for cure, particularly in the female.

The bacteriologic procedures described herein are those observed to give the most uniform and reliable results after many comparative studies of methods for staining the gonococcus and of media, atmospheric requirements, and temperatures of incubation for growing the gonococcus. Although the complement-fixation test is not, in its present stage of development, a reliable means of diagnosis a method is outlined because the test is employed in a few laboratories.

The film method has in its favor the element of simplicity. It is relatively inexpensive because it requires little equipment, and where laboratory service is not available, it can be performed by the physician in his office. A diagnosis can be made within a few minutes, or, on the other hand, examination of films can be postponed indefinitely without affecting the results. The disadvantages, however, are serious enough to warrant the use of a more reliable method. Films are often negative in chronic cases of gonococcal infection when the number of gonococci has been markedly reduced, with or without secondary infection, and especially after chemotherapy. Rarely, films are positive when infection is due to species of *Neisseria* other than *Neisseria gonorrhoeae*. Furthermore, the direct microscopic examination of slides does not distinguish viable from non-viable gonococci. Results vary with the degree of application, the skill of the technician, and the time devoted to the examination of films.

The excellent results cited in various reports comparing the film and cultural methods indicate that the cultural method is not only a useful adjuvant to the film method, but a necessary procedure for establishing accurate diagnosis. In chronic cases in both sexes and in the detection of carriers, the cultural method is far more dependable. It is pre-eminent, however, in the diagnosis of the disease in females, where it has proved to be as much as 200 per cent superior to the film method.[1] False-positive results are avoided, and since the isolation of *N. gonorrhoeae* in culture constitutes an unquestionable diagnosis, the test is acceptable as legal evidence. The cultural method, in its present stage of development, is limited to use in venereal disease clinics, hospital laboratories, and communities where specimens can be delivered promptly to a public health laboratory equipped to carry out the procedure. If media cannot be inoculated at the time the specimen is collected from the patient, cultures must be made shortly thereafter for the best results. The special media and equipment needed for the test and the greater amount of time expended in performing it increase the cost, although it is no higher than that for cultural tests used in the diagnosis of other infectious diseases. It is, as yet, primarily a procedure for a trained laboratory technician, and for this reason cannot be utilized effectively in the office of the general practitioner.

In view of the limitations of both the film and cultural methods their simultaneous use is strongly recommended to secure a maximum of positive results. The film, however, is most valuable in the diagnosis of acute untreated urethritis in the male. The use of cultures is unnecessary in this type of case unless the information obtained is to be used for research or for medicolegal purposes. The examination of films prepared from prostatic secretion, on the other hand, does not yield dependable evidence. Because of the consistency of the secretion such films are more difficult to stain satisfactorily with Gram's method.

The complement-fixation test, as now performed, cannot be recommended as a sole means of diagnosis. Even the best technic sometimes gives negative results when the patient shows typical symptoms of gonococcal infection and films and cultures are positive. Furthermore, the majority of patients treated with penicillin are cured before antibodies develop. On the other hand, positive results are occasionally obtained in cases with no history of gonococcal infection and negative bacteriologic tests. If used in conjunction with the film and cultural methods, however, it can be of some aid to the physician who is acquainted with its limitations. The most practical application at the

present time is in the diagnosis of gonococcal arthritis, pelvic inflammatory disease, and chronic prostatitis. Further research on the biochemistry of the gonococcus and on related aspects of the problem may produce a complement-fixation test as reliable as that for the diagnosis of syphilis. A dependable serologic test could be not only of practical value to the physician but of invaluable aid to the epidemiologist and in the research laboratory. Although the test is rarely employed in the diagnostic laboratory, the description of the technic is retained for those who may have occasion to use it.

## II. COLLECTION OF SPECIMENS

### A. Outfits

Cotton-tipped applicators, kept sterile in a stoppered test tube, and glass microscopic slides comprise the necessary materials for preparing films. When specimens are to be sent or delivered to the laboratory, a wooden slide holder accommodating two slides, and a mailing container or heavy manila envelope addressed to the laboratory should be included. The swabs are made by tightly winding a small amount of absorbent cotton around the terminal ¾ inch of a 6 inch wooden applicator. The diameter of the swab should not exceed 3/16 inch, and a diameter of ⅛ inch is preferable. The slides must be clean and free from grease; otherwise, the film of exudate will not adhere to the glass. Slides with etched ends are very satisfactory and are especially recommended if the slide is to be used more than once. If etched slides are not used, a diamond-point pencil should be available in the laboratory for marking the slides inasmuch as gummed labels or wax pencils are unsatisfactory.

When cultures are to be taken, at least two sterile swabs are included in the outfit. A tightly stoppered tube or vial containing either 1 ml. of a nutrient broth or the transportation medium of choice is also necessary. The standard Wassermann outfit is satisfactory for the collection of blood for the complement-fixation test.

### B. History Slips

A suitable history form calling for pertinent information is essential for accurate laboratory records. It should provide space for the patient's name, address, age, sex, the source of the specimen, the date taken, and the type of examination desired. The duration of the disease

and whether acute or chronic is also useful information. For cultural tests, the hour the specimen was taken should be recorded. For statistical purposes, it is well to include occupation, color, and marital status. Space, of course, is provided for the name and address of the physician. A properly arranged history form may be used for both the film and cultural examinations although a separate form is advisable for the complement-fixation test. The laboratory report of the bacteriologic examination should provide for the date of examination, the name or initials of the examiner, and the signature of the person responsible for the results. The two items of information to be included in the report on films are (1) the presence or absence of Gram-negative diplococci, and (2) the relative number of pus cells. The report of the cultural examination should state that the gonococcus was (or was not) isolated in culture.

### C. GENERAL DIRECTIONS

In acute cases of gonococcal infection, specimens of exudate for examination by either the film or cultural method are generally taken from the male urethra or cervix. In chronic cases in the male, prostatic secretions and urine may also be submitted. In vulvovaginitis, specimens are obtained from the vagina. Other sources of infectious material are the conjunctiva, abscesses of Bartholin's glands, the Fallopian tubes, pelvic lesions, and rectal discharges, the last especially from females. Cultures of blood and of joint and spinal fluids occasionally reveal the gonococcus. The cultural examination of spinal fluid from atypical cases of meningitis should not be overlooked. Not infrequently the gonococcus can be recovered from the mucous membranes of the genitourinary tract, from prostatic secretions, or from urine sediment of patients who are symptom-free after treatment.[2] Specimens to be examined as a test for cure should not be obtained for at least 48 hours after the termination of therapy, because minimal concentrations of sulfonamides or antibiotics in the inoculum inhibit the growth of the gonococcus.

In taking specimens an important point is to avoid accidental infection of the conjunctiva which is especially vulnerable to invasion by the gonococcus. The use of rubber gloves is recommended, because the patient may be suffering from syphilis as well as from gonococcal infection.

The usual cotton-tipped applicator is suitable for collecting most specimens. Separate swabs, however, should be used for obtaining

exudates for the culture and the film. It is advisable to take first the specimen for culture because more exudate is required for this examination. Aseptic technic is unnecessary except when specimens are obtained from the spinal canal, from joints, or from abscesses. Spinal fluid is taken as ordinarily collected for examination for evidence of meningococcal infection. The technic recommended for procuring joint fluid is as follows: (1) by means of a piece of sterile gauze or a sterile swab, apply tincture of iodine to the skin over the joints; (2) with a 2 ml. sterile syringe and a hypodermic needle, inject 1 per cent procain under the skin and into the subcutaneous tissues at the point of greatest fluctuation; (3) when satisfactory anesthesia has occurred, withdraw the joint fluid by inserting into this area a 20 gauge needle of suitable length, using a 10 or 20 ml. sterile syringe.

To insure the greatest number of positive results the medium should be inoculated immediately after the specimen has been collected from the patient. If possible, the specimen should be inoculated directly onto the surface of the agar plate to be employed for isolating the gonococcus. If the specimen cannot be cultured immediately, the swab with the exudate should be placed at once in a test tube containing 1 ml. of meat infusion broth or in any other suitable menstruum not injurious to the gonococcus. A 2 per cent solution of Proteose No. 3 Broth * to which is added sodium chloride to yield a final concentration of 0.5 per cent is very satisfactory. Such broths will maintain the viability of the gonococcus satisfactorily for only 4 or 5 hours, depending upon the amount of inoculum, number of gonococci in specimen, the temperature, and the number and type of concomitant bacteria in the specimen.

Although specimens of exudate may be inoculated directly onto the culture medium, there are several advantages in first suspending the material in a small amount of broth. It permits delayed inoculation when laboratory facilities are not immediately available; it dilutes the inoculum, thereby reducing overgrowth of the gonococcus with commensal organisms; it provides an increased amount of moisture to the surface of the solid medium.

When the laboratory is distant from the patient and the specimen cannot be cultured for from 18 to 24 hours after collection, a so-called "transportation" medium is essential. As yet, an entirely satisfactory medium for this purpose has not been developed. Comparative tests

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* Prepared by Difco Laboratories, Inc., Detroit, Mich.

for viability of the gonococcus on several media recommended for this use have demonstrated that as the number of hours increases between collection of the specimen and the inoculation of the medium at the laboratory, the number of strains isolated decreases. Nevertheless, under certain circumstances the shipment of specimens to a laboratory from considerable distance is warranted. Two semi-solid media are suggested for this purpose; namely, gelatin blood agar and gelatin egg albumin agar.[3, 4] (C.M. Nos. 41 and 42.)

## D. Male

Aseptic technic is, as a rule, unnecessary in obtaining specimens for culture from the male urethra. When such precautions seem necessary, the foreskin is retracted and the glans penis is cleansed with soap and water; a mild antiseptic, such as 70 per cent alcohol, may be applied. Care must be taken that a minute amount of the antiseptic does not remain in the meatus to be absorbed on the cotton swab when the urethral exudate is collected, thereby inhibiting the growth of the gonococcus. Pus at the meatus is removed with a sterile cotton swab. In the absence of visible exudate, the penile urethra is stripped with the thumb and forefinger and any resulting mucopurulent or mucoid exudate is cultured. Inasmuch as the gonococcus not infrequently can be isolated from the urine sediment the first 10 to 15 ml. of voided urine serves as a useful specimen, especially when no urethral exudate is present.[2]

In chronic cases, prostatic fluid should also be cultured. The penile urethra is compressed with the thumb and finger to prevent loss of fluid while prostatic massage is carried out in the usual manner. Pressure is then released and the prostatic fluid in the urethra is permitted to flow directly onto a chocolate agar plate or into a test tube containing 1 ml. of sterile broth for subsequent culturing. If the quantity is scant, the exudate is collected from the meatus by means of a swab which is then placed in a tube of broth, but preferably on the chocolate agar plate. As a test for cure, a single combined specimen of prostatic fluid and urine may be used, inasmuch as it permits detection of infection in the prostate as well as in the posterior and anterior urethra. From 10 to 15 ml. of urine are collected in a sterile tube immediately after prostatic massage. Recent comparative tests have shown that very seldom is the gonococcus isolated from the prostatic secretion when it is not present in the anterior urethra.

## E. FEMALE

Sterile preparation of the vulva and douching of the vagina are seldom necessary. If the urethral meatus appears normal and no exudate is present, films and cultures from this source are not indicated. The gonococcus is so rarely isolated from the urethra when it cannot be recovered from the cervix that duplicate films and cultures from both sources are unnecessary. This observation is borne out by a

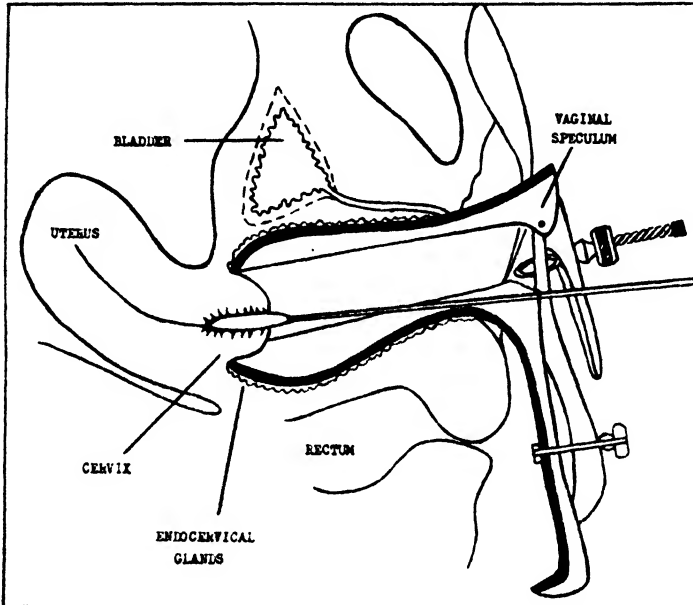


FIGURE 1—Sterile cotton-tipped applicator has been inserted through bivalve speculum into cervical canal (see text).

comparison of hundreds of cultures from the urethra and cervix. Therefore, in most venereal disease clinics cervical films and cultures only are examined, which is a saving in time and expense. If it is deemed necessary to culture the urethra, the following technic is employed: The urethral meatus is cleansed by a sterile cotton pledget. Digital pressure is applied to the urethra with a gloved finger and to Skene's glands. Exudate is then collected on a sterile swab and immersed in a tube containing 1 ml. of suitable broth, or cultured directly on chocolate agar.

Specimens collected for culture from the cervix are most important

and require care and experience to be satisfactory. A bivalve vaginal speculum should always be used, without lubricant other than water or physiologic salt solution (Figure 1). The cervix is cleansed with a cotton pledget on dressing forceps. The cervical plug, if present, is removed with a cotton-tipped applicator. The cervix is then gently compressed with the blades of the speculum to express secretions from the endocervical glands. Specimens are then collected by inserting a small sterile swab into the cervix for from 0.5 to 1 cm. A sufficient quantity of exudate should be obtained to insure successful results. During pregnancy the material should be collected from the cervical os. The swab is cultured as heretofore described.

The collection of specimens from children and infants with vulvovaginitis may be carried out with a cotton-tipped applicator. The use of a female glass catheter, however, is preferable because it causes no trauma and permits collection of more material. The catheter, containing a small amount of physiologic saline solution is introduced into the vagina, and moved about to permit approximately 0.3 to 0.5 ml. of vaginal secretion to flow into the catheter. A film is prepared from one drop and the remainder is suspended in 1 ml. of broth for culture, or is inoculated directly onto a suitable solid medium.

### F. MISCELLANEOUS

In taking specimens from the anorectal region, aseptic technic is important. The area is cleansed with soap and water, an antiseptic applied, the anal sphincter dilated by means of an anoscope, and the specimen collected from the mucosa adjacent to the terminal portion of the anal canal.

Pus from the conjunctiva and from abscesses may be inoculated directly onto the culture medium, or suspended in infusion broth. Joint and spinal fluids and urine are collected in sterile tubes without broth. Blood for cultures is obtained by venipuncture. One ml. and 4 ml. amounts are added to 100 ml. of glucose ascitic fluid broth; 5 ml. are added to from 2 to 4 ml. of 2.5 per cent sodium citrate to prevent coagulation for use in blood agar plates.

### G. TRANSPORTATION OF SPECIMENS

No special precautions are necessary in transmitting films to the laboratory since they are unaffected by time, temperature, or atmospheric conditions. Specimens of urine, blood, exudate in broth, etc.,

on the other hand, should be delivered promptly and cultures made as soon as possible. Not more than 6 hours should elapse between the time the specimen is collected and the time it is cultured. When it is impossible to make cultures directly from the patient, the specimen should be kept in a refrigerator, if possible, otherwise at room temperature. Under no circumstances should it be incubated, because exposure to temperatures as high as body temperature favors the multiplication of contaminants which soon overgrow the gonococcus and make isolation difficult.

## III. EXAMINATION OF SPECIMENS

### A. General Directions

As soon as the outfit is opened in the laboratory, the specimen should be given an accession number and the information checked with the accompanying history form. Care should be taken at all times to preserve the identity of the specimen, and errors in labeling, etc., guarded against.

### B. Films

#### 1. *Preparation*

To prepare a suitable film the exudate which has been obtained on a sterile swab is "rolled out" over the surface of two glass slides by rotating the cotton-tipped applicator between the thumb and index finger. The "rolling" procedure is superior to rubbing the swab over the surface because the pus cells remain intact and more gonococci retain their intracellular position. The film should be placed near one end of the slide so that space remains available at the opposite—etched—end for labeling. The success of the examination depends to a great extent upon the care with which the film is made. Thick films are unsatisfactory and can be avoided by collecting only a small amount of exudate and by rolling the swab over the surface but once. A well made preparation should be not more than one cell thick. When the film has been air dried or fixed by gentle heating of the reverse side of the slide, it should be marked plainly with the patient's name, the source of the specimen, and the date, or with a code number.

#### 2. *Staining*

The procedure recommended is Hucker's[5] modification of Gram's stain. (See page 87.)

The films should be stained individually for the best results, although when large numbers are to be examined, they may be stained in groups by the use of slide holders. If it is necessary to do them collectively, slides with thick films should be removed and stained separately, as they require more decolorization than slides with thin films.

To control the staining reaction, one loopful of a young broth culture of *Staphylococcus aureus* and one of *Escherichia coli* are placed on the slide adjacent to the film before staining. Organisms from solid media may be used if properly diluted with distilled water. While it may not be necessary to control the Gram stain in this manner in laboratories where numerous examinations are made daily, the provision is essential for laboratories in which only an occasional film is examined.

### 3. *Microscopic examination*

A compound miscoscope equipped with a mechanical stage and an oil immersion lens is essential for making a satisfactory examination. When correctly stained, the nuclei of the pus cells should retain some of the violet dye, while the cytoplasm should be pink. The examiner notes first the staining reaction of the staphylococci and colon bacilli placed on the slide for control. (Staphylococci are Gram-positive, i.e., retain the initial violet dye; colon bacilli are Gram-negative, i.e., do not retain the initial dye, but are decolorized and appear pink from the effects of the counterstain.) If the control films are not properly stained, the preparation is unsatisfactory for diagnosis.

The gonococcus is typically Gram-negative when correctly stained and appears as a pink or orange-red coccus, usually arranged in pairs. The approximating surfaces are flattened, producing the well known biscuit or coffee-bean shape. The organisms may be inside or outside of the pus cells. The intracellular location is typical and has considerable diagnostic significance. Extracellular gonococci, as a rule, are found frequently in films prepared from the cervix.

The entire film should be examined for gonococci if necessary. For an experienced observer the period of examination should be at least 3 minutes, preferably 5. Difficult slides may require more time. Special care is required in the examination of vaginal and cervical films from chronic cases in order to avoid missing the occasional gonococcus. All slides should be filed and kept for at least 6 months. While making the examination, the absence or presence of pus cells and their fre-

quency should be noted. If spermatozoa are observed, this fact may also be recorded.

### 4. *Reporting results*

The responsibility for interpreting the results of a microscopic examination is solely that of the physician in charge of the case. The laboratory examiner should report only what is observed in the film, i.e., *Intracellular Gram-negative diplococci resembling gonococci were found*, or . . . *were not found*. If extracellular Gram-negative cocci resembling the gonococcus are observed, usually a continued search will reveal intracellular groups as well. If, however, only extracellular forms are found, the reports should read, *Extracellular Gram-negative diplococci resembling gonococci were found*, and a request for a second film should be made. If the film is too thick for satisfactory staining or no exudate can be detected on the slide, this information should be reported and a second film requested.

The report should include a statement concerning the number of pus cells present, *Many* . . . *Few* . . . or *No pus cells present*.

## C. CULTURES

### 1. *Precautions in handling exudates before culturing*

An important precaution is to prevent drying of the exudate before suspension in broth. Drying destroys the gonococcus rapidly.

Contrary to the general belief that specimens to be cultured for the gonococcus should be kept at body temperature prior to inoculation, better results are obtained when they are held at temperatures of from 4° to 10° C. If facilities for refrigeration are not available, cultures should be kept as cool as possible to avoid overgrowth of the gonococcus with commensal organisms. *It is emphasized that best results are obtained when cultures are made immediately after the specimen is collected.* The findings, however, are usually dependable if inoculation is not delayed for longer than 6 hours.

### 2. *Media*

A comparative study under controlled conditions of 12 media for the isolation of the gonococcus revealed that the greatest number of isolations were made on the three following media[6] (1) Modified McLeod's agar with Nile Blue A and enriched with horse plasma and hemoglobin[7] (C.M. No. 39); (2) Proteose No. 3 Agar with Nile Blue A and enriched with horse plasma and hemoglobin[8] (C.M. No. 40); and

(3) Bacto-GC Medium Base, with Bacto-Hemoglobin and either Supplement A or B[9] (C.M. No. 38).* Although these three media were equally as effective from the standpoint of growing the gonococcus, the "chocolate" agar prepared from the GC Medium Base and Bacto-Hemoglobin was more uniform in quality and more simply prepared than the other two media. The addition of either Bacto-Supplement A or B to the chocolate agar is essential. In the comparative study,[6] Supplement B proved to be slightly superior to Supplement A. These supplements are an especially prepared thermolabile yeast concentrate containing adequate amounts of growth-accessory factors, particularly glutamine, cocarboxylase, and coenzyme, required by the more fastidious strains of the gonococcus. Supplement A differs from Supplement B only in that it contains sufficient crystal violet to yield a final concentration of 1:714,000 in the medium.* The supplements are essential because numerous strains of the gonococcus fail to grow unless the medium is augmented with glutamine.[10]

The dehydrated *GC Medium Base,* with Bacto-Hemoglobin and either Supplement A or B possesses several advantages over the other media described for the isolation of the gonococcus. It is always immediately available and can be readily prepared in either large or small quantities, insuring a supply of fresh moist medium essential for dependable results. Furthermore, the cultures may be examined after incubation for 24 hours instead of 48 hours, which is required by the other media.

The addition of tyrothricin to chocolate agar is another useful aid which may be employed. A final concentration of 1:15,000 inhibits the growth of Gram-positive organisms such as streptococci, lactobacilli, and diphtheroids, thereby facilitating the isolation of the gonococcus.[11]

Many strains of the gonococcus may be isolated on other media prepared from an infusion agar base enriched with either blood, serum, or ascitic fluid. The purpose of the cultural method, however, is to isolate the maximum number of strains and, therefore, the best special media must be employed if the procedure is to be effective.

### 3. *Inoculation of Media*

As previously directed, specimens from the urethra and cervix should, if possible, be inoculated directly onto the surface of the agar

* Prepared by Difco Laboratories, Inc., Detroit, Mich.

plate. Inoculation of the plate is an important procedure and requires skill and experience to obtain suitable cultures because the amount of inoculum collected on a swab varies. Care should be taken to insure that the inoculum is well distributed in order to utilize the maximum surface of the agar and yet avoid overgrowth with commensal bacteria. From one-fourth to one-third of the surface of the agar plate should be streaked back and forth without rotation of the swab. A platinum or chrome wire loop or needle may then be employed to spread further the inoculum at right angles to the originally inoculated area, thereby covering the remainder of the surface.

If the exudate has been suspended in a liquid medium, from 0.05 to 0.1 ml. of inoculum, depending on the turbidity of the specimen, may be pipetted onto the agar and then spread over the surface of the plate with a sterile glass "L-shaped" rod. If the swab employed to collect the exudate is submitted in the tube of broth the applicator should be rotated and compressed against the inner wall of the tube to suspend as much of the exudate as possible. From 0.05 to 0.1 ml. of the suspension is then pipetted to the agar plate and inoculated onto the surface by means of a glass rod or wire loop. If exudate is scant, the suspension should be centrifugalized and the sediment cultured. Joint and spinal fluids and urine are centrifugalized, and the sediment streaked on chocolate agar plates. In the case of blood cultures, citrated blood from the patient is made into blood agar plates by the following procedure: Infusion, hormone, or Douglas's agar is melted and then cooled to about 42 to 45° C., at which temperature 10 per cent of ascitic fluid and 1 per cent of glucose are added; to 20 ml. of this medium, 3 ml. of citrated blood is added and a plate poured; to another 20 ml., 2 ml. of citrated blood is added and a second plate poured.

The inoculated chocolate agar plates and the blood plates are inverted and stacked in jars for incubation in an atmosphere reinforced with carbon dioxide. Dehydrating or museum jars fitted with covers may be used, and can be made airtight with vaseline or plasticene. Any container that has a tightly fitting cover and a mouth wide enough to permit the introduction of the Petri dishes, may be substituted. (Figure 2 shows a 1 gallon wide-mouth glass jar fitted with a metal screw top.)

The atmosphere is reinforced with carbon dioxide in one of several ways. (1) A suitable stopcock, screwed into the cover of the jar, permits rubber hose connections to be made to a cylinder of compressed

carbon dioxide and to a vacuum pump. A mercury, open U-tube type manometer is included to measure the pressure within the jar. Air is evacuated until the pressure within the jar has been reduced by 9 cm. of mercury (removal of approximately 12 per cent of the air), then slightly less carbon dioxide than removed air is released into the jar. This is accomplished by closing the stopcock when the pressure is from 0.5 to 1 cm. of mercury below atmospheric pressure (Figure 3). (2) A less costly and simpler method, but an equally satisfactory one,

FIGURE 2—A—Inexpensive wide-mouthed glass jar, one gallon size, showing method of reducing oxygen tension by use of lighted candle. B—Cover for jar fitted with stopcock for use with equipment as illustrated in Figure 3.

is to place a lighted, smokeless candle approximately 1.5 to 2″ long and 1″ in diameter, on the plates in the jar and then replace the lid. When the flame is extinguished by the depletion of the oxygen, the concentration of carbon dioxide is adequate for the growth of the gonococcus. (3) The atmosphere may be enriched with carbon dioxide by the use of sodium bicarbonate and sulfuric acid, if this method is preferred. It is important that the cultures be incubated in a moist atmosphere which may be insured by placing a thin layer of moist cotton in the bottom of the jar.

The jars containing the cultures are placed in an incubator at 35°

or 36° C. for either 24 or 48 hours, depending upon the medium employed. The flasks containing the blood in glucose ascitic fluid broth are likewise placed in an atmosphere reinforced with carbon dioxide and incubated at the same temperature. An incubator set at 37° C. may be used; however, some strains of gonococci cannot be isolated at this temperature. The cultures may be inspected after 24 hours of incubation, if necessary, at which time there is often sufficient growth for examination. Some strains of gonococci require 48 hours of incubation. A negative report, therefore, should never be made at the end of 24 hours unless the GC agar base, experimental, with Bacto-Hemoglobin and Supplement A or B is employed.

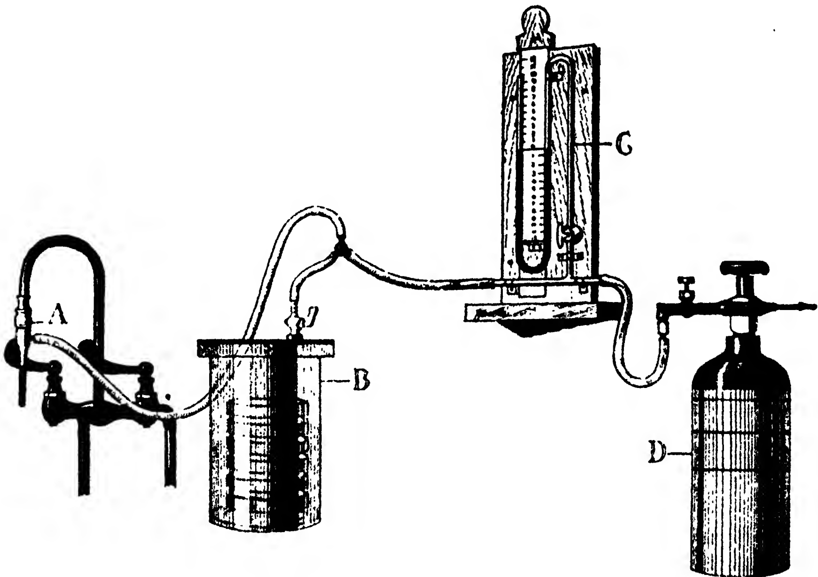


FIGURE 3—Diagram of equipment as used for increasing the carbon dioxide content of the atmosphere surrounding the cultures made for the isolation of the gonococcus. A—Faucet suction pump; B—museum jar containing Petri dishes; C—mercury manometer; and D—tank of compressed carbon dioxide.

## 4. *Examination of Cultures*

### *a. Direct inspection*

After 24 or 48 hours of incubation, the plates are inspected for the presence of colonies of the gonococcus. On a suboptimal medium, only minute pinpoint colonies are observed. On chocolate agar the colonies

are convex, transparent, from 1 to 3 mm. in diameter, with undulate margins (Figure 4). By their transparency and the character of their margins, they can usually be differentiated from young colonies of streptococci and diphtheroids, which they simulate. From the colonies selected films are prepared, stained, and examined.

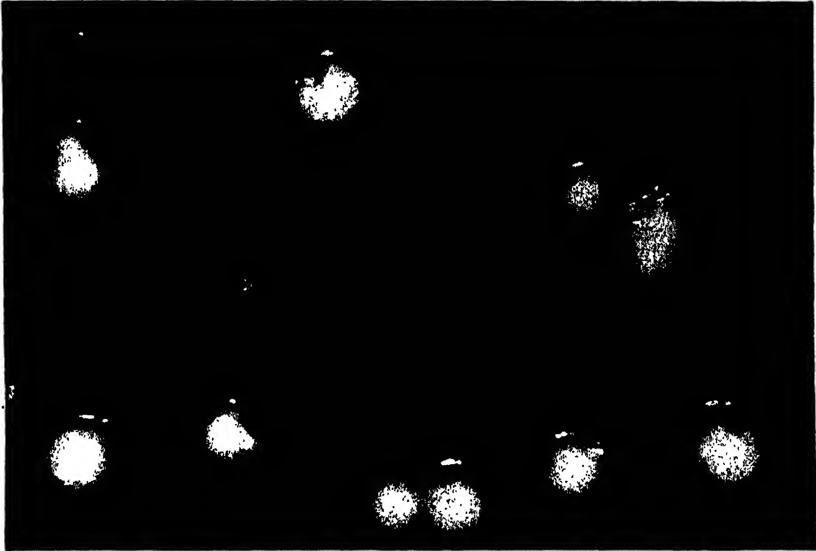

FIGURE 4—Surface colonies of *Neisseria gonorrhoeae* and *Neisseria catarrhalis* on "chocolate" agar, 48 hours.
The gonococcus colonies are gray with undulate margins

*b. The "oxidase test"*

When no colonies, typical of the gonococcus, can be detected by direct inspection, the culture is subjected to the "oxidase test," which is of especial value in detecting colonies of *Neisseria* in mixed cultures (Figure 5). The test is based upon the presence of an enzyme, oxidase, produced by organisms belonging to the genus *Neisseria*. The enzyme is detected by the use of either the oxalate salt [12] or monohydrochloride of the dye component, para-aminodimethylaniline, which, in the presence of oxidase, produces a characteristic series of color changes: first pink, and on further oxidation maroon, and finally black. The oxalate salt of the compound possesses advantages over its monohydrochloride. It is less toxic for the gonococcus and during storage does not deteriorate as rapidly as the monohydrochloride. Likewise, the oxalate salt does

not form the marked black precipitate on chocolate agar sometimes observed from use of the monohydrochloride, especially when a fresh solution is not employed. The oxalate salt is somewhat less soluble than the monohydrochloride, and gentle heating is required in the preparation of a solution. The direct inspection of an agar plate culture containing but a few gonococcus colonies obscured by a luxuriant growth of other microörganisms is almost valueless. The

FIGURE 5—Culture from cervix on surface of "chocolate" agar plate: A—Before oxidase test.

microscopic examination of films from many colonies is impractical. It is in such instances, therefore, that the value of the "oxidase test" is greatest.

A 1 per cent aqueous solution is prepared from the desired compound. Best results are obtained when a fresh preparation is made daily. A solution will, however, continue to be suitable for several days when stored in the refrigerator. An experienced bacteriologist may desire to pick typical colonies of the gonococcus for pure culture studies before the reagent is applied. If there is uncertainty of the

colony type a few drops of the reagent may then be applied to the growth over a small area of the plate. If oxidase-positive colonies appear, subcultures can be made from similar uncontaminated colonies in an adjacent section of the plate. If no oxidase-positive colonies appear from the application of the reagent to a small area, from 1 to 2 ml. of the solution are dropped on the agar plate culture by means of a pipette and the plate tilted so that the entire surface is moistened.

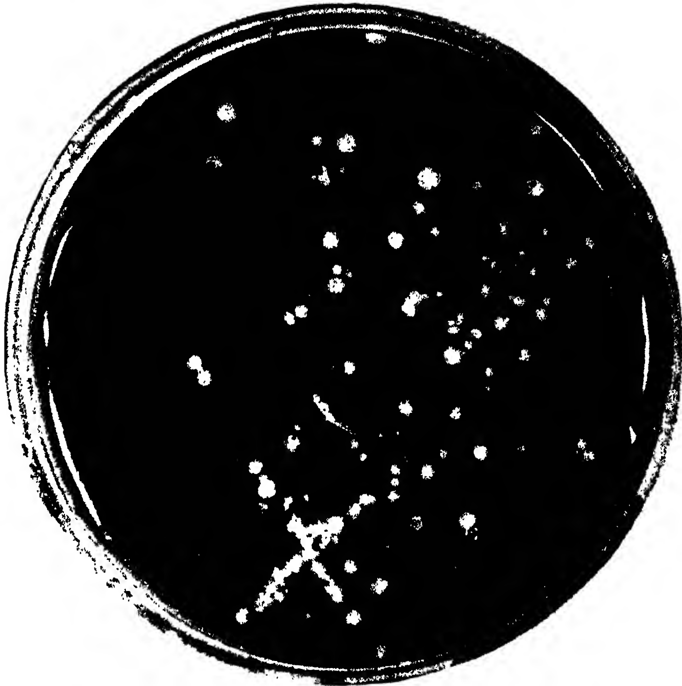

FIGURE 5—Culture from cervix on surface of "chocolate" agar plate:
B—After oxidase test. The gonococcus colonies, which are oxidase-positive, now appear black.

If a large series is to be examined, a "nasal" atomizer provides a simple and economical way to apply the reagent. The plate is observed for a period of from 5 to 8 minutes for evidence of change in the color of the colonies. This usually occurs in less than 2 minutes, but a freshly prepared solution may delay the reaction slightly. The series of color reactions, i.e., pink, maroon, and black, readily identifies the colonies of *Neisseria*. Films are made from "oxidase-positive" colonies, stained, and examined microscopically. 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 organisms. 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.

### 5. *Interpretation of Results*

A Gram-negative diplococcus, isolated from the genital canal, particularly from the male, and showing the typical morphologic characteristics of *Neisseria gonorrhoeae,* may be presumed to be a gonococcus when picked from an "oxidase-positive" colony. Further identification on carbohydrate media is necessary in all medicolegal cases and in doubtful cases, when the history and clinical status of the patient are inconsistent with the bacteriologic findings. Special care should be taken to differentiate the gonococcus from the meningococcus and from *Neisseria catarrhalis* in cultures from the conjunctiva. Cultures from the lower birth canal and from prostatic secretions occasionally show "oxidase-positive" colonies other than those of the *Neisseria,* but difficulty seldom arises in differentiating them, because the majority are either Gram-positive or Gram-negative bacilli. Streptococci and diphtheroids, which are the most difficult organisms to differentiate from *Neisseria gonorrhoeae* by direct inspection of colonies, do not form oxidase.

### 6. *Identification by carbohydrate fermentation*

For the final identification of the gonococcus and differentiation from the other *Neisseria,* it is necessary to inoculate media containing the following carbohydrates: glucose, lactose, sucrose, maltose, levulose, and mannitol. The use of glucose and maltose only will differentiate the gonococcus from the meningococcus. Many basic media for incorporating carbohydrates have been described, but ascitic fluid agar is the best. The ascitic fluid must be bile-free, with a pH range between 7.2 and 8.0. Ascitic fluid of high alkalinity inhibits the growth of the gonococcus; therefore, the pH should be determined before use and the fluid neutralized, if necessary. Even though all the above specifications are met, some lots of ascitic fluid are unsatisfactory, and thus it is advisable to compare the growth-stimulating factor of each new supply with fluid known to be suitable. In laboratories where a supply of ascitic fluid is not available serum may be substituted. Rabbit serum is the most suitable. Human serum may be used. ***The maltose content of beef, sheep, and horse sera make them unsuitable in carbohydrate medium as a growth-promoting substance.***

Typical colonies are selected from the original plate and subcultured on a suitable medium, such as chocolate agar. When growth occurs, transfers are made to a series of slants of ascitic fluid agar containing Andrade's indicator, and 1 per cent of each carbohydrate. Rubber stoppers are inserted in the tubes to limit the oxygen supply; and as growth develops, the carbon dioxide content within the tube is increased. The use of rubber stoppers insures moist, fresh media by preventing the evaporation of condensation water. The cultures are incubated for 48 hours at 36° C. and then inspected.

The fermentation of glucose only, which is indicated by the agar slant turning pink, differentiates *Neisseria gonorrhoeae* from other members of the genus.

Another satisfactory medium is Bacto-phenol red carbohydrate broth containing 0.5 per cent of the desired carbohydrate. A semi-solid

TABLE 1

*Fermentation of carbohydrates by the Neisseria*

| *Microörganisms* | *Glucose* | *Lactose* | *Sucrose* | *Maltose* | *Levulose* | *Mannitol* |
|---|---|---|---|---|---|---|
| *Neisseria gonorrhoeae* | + | — | — | — | — | — |
| *Neisseria meningitidis* * | + | — | — | + | — | — |
| *Neisseria catarrhalis* † | — | — | — | — | — | — |
| *Neisseria sicca* | + | — | + | + | + | — |
| *Neisseria flava* | + | — | — | + | + | — |
| *Neisseria perflava* | + | — | + | + | + | + |
| *Neisseria subflava* | + | — | — | + | — | — |
| *Neisseria flavescens* | — | | — | — | — | — |

* *N. subflava* produces yellow pigment on agar which differentiates it from *N. meningitidis*.
† *N. catarrhalis* is differentiated from *N. flavescens* by growth at 22° C. *N. flavescens* is reputed to fail to grow at that temperature.

medium is preferable, however, and is prepared from the carbohydrate broth base by adding 0.15 per cent agar.* A comparatively large amount of inoculum (several colonies) is required to establish growth. The inoculum should be placed in the top 0.5 cm. of the semi-solid agar column. In Table 1 are listed the carbohydrate fermentation reactions of the various species of *Neisseria*.

### 7. *Characteristics which aid most in distinguishing the gonococcus from other important members of the genus Neisseria*

1. The fermentation of the single carbohydrate glucose with the formation of acid is the most dependable criterion for differentiating the gonococcus from other members of the genus *Neisseria* (Table 1).

* Prepared by Difco Laboratories, Inc., Detroit, Mich.

2. A Gram-negative, biscuit- or coffee-bean shaped diplococcus, observed in films prepared from exudates from the genital canal or from "oxidase-positive" colonies from the same source usually proves to be the gonococcus.

3. Colony formation readily distinguishes most strains of *Neisseria gonorrhoeae* from the other species of *Neisseria* when grown on ascitic fluid or chocolate agar. After incubation for from 24 to 48 hours they are convex, transparent, from 1 to 3 mm. in diameter, and have undulate margins. They are grayish and slightly opaque by transmitted light. By their transparency and the character of their margins they can usually be differentiated from young colonies of streptococci and diphtheroids with which they are most frequently confused.

4. Growth on slants of an enriched medium is usually delicate.

5. The failure of a Gram-negative coccus to grow on plain agar, especially a meat extract agar, at room temperature, points to the gonococcus. Occasionally, a strain of *Neisseria gonorrhoeae* grows well on unenriched media at room temperature.

### 8. *Evaluation of findings*

Routinely, it may be reported that a culture of the gonococcus has been isolated from the specimen, if the following characteristics have been observed:

1. Colony shows typical morphology (see 3 above).
2. Colony is "oxidase-positive."
3. Organism is a Gram-negative, biscuit-shaped diplococcus.

It should be emphasized that in medicolegal and doubtful cases or when a Gram-negative gonococcus is isolated from sources other than the genital tract, final identification should be carried out on carbohydrate media.

### 9. *Reporting results*

Report, *The gonococcus (or Neisseria gonorrhoeae) was isolated in culture,* or . . . *was not isolated in culture.* If the original specimen was unsatisfactory for culture, this fact should be reported and a second specimen requested.

## D. DETERMINATION OF RESISTANCE TO PENICILLIN *in vitro*

The author has failed to date, January, 1949, to recover a strain of the gonococcus that would grow in a concentration greater than 0.08 units of penicillin per ml. of medium.[13] Yet several reports describing

cases of penicillin-resistant gonorrhea have been published.[14-16] Inasmuch as such strains may develop in the population, a description of a satisfactory technic employed to determine the resistance of the gonococcus to penicillin *in vitro* is described.

### 1. *Media*

Douglas' broth with 0.05 per cent potassium nitrate, 0.04 per cent potassium dihydrogen phosphate, and 5 per cent lapine blood is employed as the basic medium.

### 2. *Technic*

A culture is tested in varying concentrations of penicillin as follows: 0.2 ml. of a 48 hour blood broth culture of each strain of gonococcus is inoculated into a series of tubes of the blood broth containing respectively, 0.005, 0.01, 0.02, 0.04, and 0.08 units of crystalline penicillin G. A control tube without penicillin is likewise inoculated. The total volume of each tube is 3 ml. The culture is then incubated for 48 hours at 36° C.

After incubation for 48 hours, subcultures are made on chocolate agar plates and incubated for 48 hours at 36° C. under $CO_2$ tension. The plates are examined thereafter, and the first tube from which no subculture is obtained indicates the inhibiting concentration of penicillin.

## E. COMPLEMENT-FIXATION TEST

The complement-fixation test described herein is as satisfactory as any of the procedures now in use. It was developed in the research laboratories of the New York City Department of Health by Thomson and Hamann.[17]

From 5 to 10 ml. of blood are collected by venipuncture for the complement-fixation test for gonococcal infection. After the blood has coagulated, the serum is removed and inactivated in a water bath at 56° C. for ½ hour.

### 1. *Preparation and standardization of reagents*

The reagents used in the test are (1) gonococcal antigen, (2) antigonococcal human serum, (3) guinea pig complement, and (4) a hemolytic system comprised of sheep red blood cells and hemolysin prepared by the inoculation of rabbits with sheep cells. Human anti-

serum with titers of 4+, 3+, 2+, and + should be available for the standardization of the reagents. Either of the two antigens recommended is suitable for use in the procedure outlined. The method described is McNeil's technic recently modified by Agnes C. Hamann[17] of the Diagnostic Laboratories of the Department of Health of New York City.

The total volume of the test is 0.5 ml. and, therefore, sufficient 0.85 per cent solution of sodium chloride ("saline") is added to each tube to bring the final volume to this amount.

a. *Antigens*

(1) *Price's Antigen as Modified by Torrey*[18]

(a) *Cultures*—Two strains of *Neisseria gonorrhoeae* are selected according to sensitivity, antigenic activity, and absence of anticomplementary properties of their protein fractions.

(b) *Medium*—Hormone-Veal-Peptone-Agar.[18]

(c) Preparation:

(1) Inoculate surface of hormone-veal-peptone agar in Kolle culture flasks with cells from a 24 hour growth on slants of the same medium to which 1 part of ascitic fluid has been added to 4 parts of the agar. Better growth is obtained when the cells used for inoculation are suspended in ascitic fluid rather than in "saline." Drain excess fluid from the surface of medium 1 hour after inoculation and incubate cultures for 24 hours at 36° C.

(2) Wash growth from each flask with 100 ml. of "saline" and place in tall glass cylinder.

(3) Add 1.0 ml. of N/1 NaOH and place cylinder in water bath at 37° C. for 2 hours.

(4) Filter through sterile lint.

(5) Adjust reaction of the filtrate to pH 6.4–6.6 by adding from 1.5–2.5 ml. of 10 per cent trichloracetic acid.

(6) After incubation at 37° C. for 20 minutes, centrifuge and suspend precipitate in 4 ml. of "saline."

(7) Adjust to pH 7.5 with N/10 NaOH.

(8) Add merthiolate to yield a final concentration of 1:10,000.

(9) Combine the two strains used in equal proportions.

(d) *Standardization of antigen:*

(1) Make the following dilutions of the antigen: 1:5, 1:7.5, 1:10, 1:12.5, 1:15, 1:17.5, 1:20 and test each dilution as follows:

(2)

| | *Tube 1* ml. | *Tube 2* ml. | *Tube 3* ml. | *Tube 4* ml. | *Tube 5* ml. | *Tube 6* ml. | *Tube 7* ml. |
|---|---|---|---|---|---|---|---|
| Antigen dilution | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |
| Human serum 4+ | 0.02 | | | | | | |
| Human serum 3+ | | 0.02 | | | | | |
| Human serum 2+ | | | 0.02 | | | | |
| Human serum + | | | | 0.04 | | | |
| Human serum normal No. 1 | | | | | 0.04 | | |
| Human serum normal No. 2 | | | | | | 0.04 | |
| Complement, day's dilution | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

(3) Incubate in the water bath at 37° C. for 35 minutes.

(4) Add 0.2 ml. sensitized cells to each tube.

(5) Observe reaction after 30 minutes.

(6) Select that dilution of antigen which gives optimal activity with each of the positive sera and no reaction with the negative sera, and gives complete hemolysis in tube No. 7.

(2) *Cohn's antigen* [10]

(a) *Cultures*—12 strains of *Neisseria gonorrhoeae* to insure a wide range of antigenic activity.

(b) *Medium*—Chocolate agar.

(c) *Preparation:*

(1) Inoculate surface of medium in Petri dishes and incubate for from 48 to 72 hours.

(2) Add 5 ml. of "saline" to each Petri dish and suspend the organisms carefully to avoid removing agar.

(3) Collect suspensions in 1 liter bottles fitted with glass or rubber stoppers. Add phenol to yield a final concentration of 0.5 per cent.

(4) Age antigen at 4° C. for 3 months before use.

(d) *Standardization of antigen:*

The standardization is carried out in the same manner as described for the Price antigen.

b. *Complement and sheep cell suspension*

Best results are obtained from the use of a complement dilution between 1:15 and 1:20; therefore, that combination of complement

dilution and sensitized cell density is determined which gives complete hemolysis with the least amount of complement within this range of dilution.

(1) *Complement and sheep cell titration*—

| | *Tube 1* ml. | *Tube 2* ml. | *Tube 3* ml. | *Tube 4* ml. | *Tube 5* ml. | *Tube 6* ml. |
|---|---|---|---|---|---|---|
| Complement 1:20 dilution | 0.08 | 0.07 | 0.06 | 0.05 | 0.04 | 0.03 |
| Sensitized 5 per cent cells | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |

| | *Tube 7* ml. | *Tube 8* ml. | *Tube 9* ml. | *Tube 10* ml. | *Tube 11* ml. | *Tube 12* ml. |
|---|---|---|---|---|---|---|
| Complement 1:20 dilution | 0.08 | 0.07 | 0.06 | 0.05 | 0.04 | 0.03 |
| Sensitized 5 per cent cells (or 0.2 ml. sensitized 4 per cent cells) | 0.16 | 0.16 | 0.16 | 0.16 | 0.16 | 0.16 |

| | *Tube 13* ml. | *Tube 14* ml. | *Tube 15* ml. | *Tube 16* ml. | *Tube 17* ml. | *Tube 18* ml. |
|---|---|---|---|---|---|---|
| Complement 1:20 dilution | 0.08 | 0.07 | 0.06 | 0.05 | 0.04 | 0.03 |
| Sensitized 5 per cent cells (or 0.2 ml. sensitized 3 per cent cells) | 0.12 | 0.12 | 0.12 | 0.12 | 0.12 | 0.12 |

| | *Tube 19* ml. | *Tube 20* ml. | *Tube 21* ml. | *Tube 22* ml. | *Tube 23* ml. | *Tube 24* ml. |
|---|---|---|---|---|---|---|
| Complement 1:20 dilution | 0.08 | 0.07 | 0.06 | 0.05 | 0.04 | 0.03 |
| Sensitized 5 per cent cells (or 0.2 ml. sensitized 2.5 per cent cells) | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 |

(a) Incubate in water bath at 37° C. for 30 minutes. If complement is unusually active complete hemolysis will occur with the sensitized 5 per cent cells as well as with the sensitized 2.5 per cent cells. Therefore, use 0.2 ml. of sensitized 5 per cent cells and determine the correct dilution of complement: if tube No. 4 shows complete hemolysis, 0.05 ml. of complement of 1:20 dilution represents the minimal hemolytic dose, and 0.1 ml. of complement of 1:20 dilution (2 units) should be used in the test on the patient's serum.

If the complement is low in activity, use 3 per cent or 2.5 per cent sensitized cells and the correct dilution of complement.

## 2. *Technic of test*

a. Pipette serum and reagents as follows:

(1) *Test on patient's serum:*

| | *Tube 1* ml. | *Tube 2* ml. | *Tube 3* ml. |
|---|---|---|---|
| Patient's serum to be tested | 0.04 | 0.02 | 0.04 |
| Antigen, as standardized | 0.1 | 0.1 | 0 |
| Complement of day's dilution | 0.1 | 0.1 | 0.1 |

(2) *Controls:*

(a) *Positive human serum control—*

| | Tube 1<br>ml. | Tube 2<br>ml. | Tube 3<br>ml. |
|---|---|---|---|
| Positive human serum 2+ | 0.04 | 0.02 | 0.04 |
| Antigen, as standardized | 0.1 | 0.1 | 0 |
| Complement of day's dilution | 0.1 | 0.1 | 0.1 |

(b) *Antigen control—*

| | Tube 1<br>ml. | Tube 2<br>ml. | Tube 3<br>ml. | Tube 4<br>ml. |
|---|---|---|---|---|
| Antigen, as standardized | 0.1 | 0.2 | 0.3 | 0.4 |
| Complement of day's dilution | 0.1 | 0.1 | 0.1 | 0.1 |

(c) *Negative control—*

| | Tube 1<br>ml. | Tube 2<br>ml. | Tube 3<br>ml |
|---|---|---|---|
| Human negative serum | 0.04 | 0.02 | 0.04 |
| Antigen, as standardized | 0.1 | 0.1 | 0 |
| Complement of day's dilution | 0.1 | 0.1 | 0.1 |

(d) *Hemolytic system and cell control—*

| | Tube 1<br>ml. | Tube 2<br>ml. |
|---|---|---|
| Complement | 0.1 | 0 |

b. Incubate in water bath at 37° C. for 35 minutes.

c. Add 0.2 ml. of sensitized cells.

d. Incubate in water bath at 37° C. for 30 minutes.

e. Centrifuge at low speed all tubes except those showing complete hemolysis. Observe. The negative and hemolytic system controls should be completely hemolyzed.

The cell control should show no hemolysis.

The antigen control should show hemolysis in tubes 1 and 2 at least.

The positive serum control should give a 2+ reading.

The readings on the patient's serum should be based upon the degree of hemolysis of the cells:

No hemolysis in both tubes is recorded as 4+.

No hemolysis in 0.04 ml., and only a trace in 0.02 is recorded as 3+.

A trace of hemolysis in 0.04 ml., and about 50 per cent in 0.02 is recorded as 2+.

About 50 per cent hemolysis in 0.04 ml. and 75 per cent in 0.02 is recorded as +.

### 3. *Evaluation of findings and reporting results*

The complement-fixation test for gonococcal infection is a measure of the gonococcal antibody content of the serum of the patient.

a. *A positive complement-fixation test suggests one of the following—*

(1) Present or past gonococcal infection.
(2) The use of gonococcal vaccines.
(3) Cross-fixation with complement-fixing bodies resulting from meningococcal infection.

A positive complement-fixation test is considered by some investigators to indicate always the presence of gonococci in the tissues. Not infrequently the serum from some patients remains positive for weeks or even years after clinical and bacteriologic cure. Although the complement-fixation test for gonococcal infection is regarded as a specific reaction, nonspecific fixation does occur. In some instances this nonspecific fixation has been observed in serum from pregnant women and from patients with severe disease caused by infectious agents other than the gonococcus.

b. *A negative complement-fixation test indicates one of the following—*

(1) That the patient is free from gonococcal infection.
(2) That the serum was obtained in the early acute stage of the disease before the formation of complement-fixing bodies.
(3) Adequate drainage of inflammatory exudates with insuffficent antigen absorbed to stimulate antibody production.

Patients with vulvovaginitis, acute anterior urethritis, or infection of the lower birth canal may recover spontaneously or after chemotherapy without ever showing a positive complement-fixation test.

Occasionally the serum from a patient with a gonococcal abscess in the Fallopian tube will give a negative reaction.

It is important that the physician correlate the results of the complement-fixation test with the clinical and bacteriologic findings because of the unreliability of the test at this stage of development.

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# Hemophilus pertussis

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## I. INTRODUCTION

THE etiological agent of pertussis is generally recognized to be *Hemophilus pertussis* which was described by Bordet and Gengou[1] in 1906. Respiratory disease indistinguishable clinically from pertussis has been reported as having been associated occasionally with organisms other than *Hemophilus pertussis.* Brown[2] noted such symptoms associated with *Brucella bronchiseptica,* and Eldering and Kendrick[3,4] reported a series of cases in which a closely related organism, called by them a parapertussis bacillus, was apparently the cause; similar reports have been made by Bradford and Slavin,[5] by Miller, Saito, and Silverberg,[6] and others. These three organisms have much in common in their cultural and serological characteristics and deserve further taxonomic study. In this outline the discussion will be largely limited to *Hemophilus pertussis,* which undoubtedly is associated with clinical whooping cough in the majority of cases.

Laboratory tests are scarcely needed for the diagnosis of pertussis when the characteristic symptoms of whooping and vomiting are present. It is in the early stages of the disease, before paroxysms have appeared and in atypical cases without paroxysms, that laboratory results have diagnostic significance.

## II. THE VALUE AND PRACTICAL LIMITATIONS OF LABORATORY PROCEDURES

Diagnostic procedures which have been used by various laboratories include the lymphocyte count, microscopic examination of sputum, isolation of pertussis bacilli by the plating of washed sputum

or by the cough plate of Mauritzen, described by Chievitz and Meyer,[7] or by the plating of nasopharyngeal swabs as described by Bradford and Slavin,[8] and complement-fixation, agglutination, opsonic, and skin tests.

### A. Lymphocyte Counts

Lymphocyte counts have limited value. The characteristic rise in the total and relative number of lymphocytes usually occurs only when the paroxysmal stage is well established. The lymphocyte count cannot be relied upon in making an early diagnosis, but may give valuable information in differentiating an atypical attack of pertussis from a persistent cough due to other causes.

### B. Examination of Smears Microscopically

The direct microscopic examination of stained smears of sputum is without diagnostic value in pertussis. Gram-negative bacilli of proper morphology may be seen in such a specimen, but cannot be identified as *Hemophilus pertussis*. *Hemophilus influenzae* presents the same morphology and staining characteristics as the organism under discussion and is present as a secondary invader or as a part of the flora in many cases of whooping cough.

### C. Isolation of Hemophilus pertussis

The isolation of *Hemophilus pertussis* is of particular value in the early stages of the disease or in a persistent cough of questionable etiology. In general, it should be remembered that the organisms are not recoverable from any sources other than the trachea, bronchi, and lungs, and their secretions.

Fresh sputum expectorated by a patient suffering from pertussis may be washed in three changes of sterile salt solution, and the thick, tenacious strands coming from the lower respiratory tract plated on Bordet-Gengou medium. Where suitable specimens can be obtained and plated within a few hours, the results from this method are very satisfactory. There are certain limitations to its use: (1) very young children do not expectorate their sputum; (2) the specimen may be contaminated with vomitus; (3) the specimen may contain only saliva and mucus from the throat; or (4) the specimen may be overgrown with saprophytic organisms between the time of collection of the specimen and its examination in the laboratory.

The cultural methods which have given the most satisfactory results in the hands of most experienced workers are the cough plate procedure of Mauritzen in which the patient coughs on a plate of suitable medium, and the method described by Bradford and Slavin[8] in which the medium is inoculated by means of a nasopharyngeal swab. This latter procedure is recommended especially for use with young infants. Brooks, Bradford, and Berry,[9] and Saito, Miller, and Leach[10] report a higher percentage of positive findings with this method than with the cough plate. Cruickshank[11] in 1944 reported the successful use of postnasal swabs obtained through the mouth, but apparently this method has not been widely used. Cultural procedures are described in Section III.

### D. Serological Tests

Serological tests, *i.e.*, agglutination, complement-fixation, and opsonic tests, are of use in particular instances for the demonstration of antibodies in the patient's serum. Diagnostic information from these methods is limited, since antibodies usually do not appear in significant titer until the disease is in a moderately advanced stage. As in other diseases, occasional individuals fail to produce demonstrable antibodies. Serological tests have their place in the study of atypical coughs of long duration and in the investigation of individual immunity resulting from natural infection or artificial immunization.

### E. Intracutaneous Tests

Intracutaneous tests with various antigens and antigenic fractions of *Hemophilus pertussis* have been studied by numerous observers. Data are accumulating to suggest the usefulness of a skin test in the study of the immunity status of children following immunization (See Felton, et al.,[12] Sauer and Markley,[13] and Miller, Ryan, and Havard[14]); however, there is no indication of its value as a diagnostic procedure.

## III. THE ISOLATION OF *HEMOPHILUS PERTUSSIS*

### A. Diagnostic Medium

All individuals reporting success in the isolation of *Hemophilus pertussis* have used either the original medium of Bordet and Gengou[1] or a modification of this formula. Simplifications, such as those reported by Barksdale,[15] have been found incapable of supporting growth of *Hemophilus pertussis*. The original formula of Bordet and

Gengou called for 50 ml. of glycerin potato extract (liquid from 100 gm. of tender, cooked potatoes autoclaved with 200 ml. of 4 per cent glycerin), 150 ml. of "physiological solution of NaCl (0.6 per cent)," and 5 gm. of agar. For the final medium this base was mixed with an equal amount of rabbit or human blood. Since the medium did not contain peptone, it was considered less favorable to putrefactive saprophytes.

The use of 50 per cent blood in this medium is impossible in large-scale work. The Danish modification of the medium using 30 per cent blood has given excellent results. With this medium, however, it is impossible to detect the hemolytic zone which is caused by the growth of *Hemophilus pertussis*. It is an excellent medium for the storage of stock cultures as dissociation of the organisms is delayed for many cultural generations. For diagnostic work it is possible to reduce the blood content to between 15 per cent and 20 per cent if the salt balance is adjusted. A modified formula has given very satisfactory results (C.M. No. 34).

Penicillin is an effective agent in reducing the growth of Gram-positive organisms on diagnostic plates. McLean,[16] Fleming,[17] and Lawson[18] used crude culture filtrates of *Penicillium notatum*, spreading 8 to 12 drops over the surface of a previously inoculated plate. More recently penicillin has been incorporated in the medium in the proportion of one unit per ml. and has been used with success by Cruickshank,[19] Kendrick,[20] and others. In order to increase the number of positive findings, it is suggested that two plates be used for each patient, one with and one without penicillin.

### B. Directions for Obtaining a Cough Plate

1. Hold the uncovered plate of Bordet-Gengou medium 4 or 5 inches from the patient's mouth during several expulsive coughs. If necessary, induce a cough by tickling the throat with a swab, pressing on the larynx, pressing on the trachea through the suprasternal notch, or giving a drink of ice water.

2. Cover the plate as soon as possible after obtaining the specimen in order to avoid contamination.

3. Send the plate to the laboratory as soon as possible to facilitate early incubation and early reporting. Plates may be kept at room temperature for some time and still be of use for diagnosis, but this procedure is not to be recommended because of the resulting delay. On the history slip, the type of cough to which the plate is exposed

should be described as natural or induced, and the severity likewise indicated. Shallow coughs may give rise to positive culture findings, but should never be relied upon as negative evidence. It is best to mark such plates "Unsatisfactory" at the time they are taken and secure a second more suitable specimen.

In transporting the plates to and from the laboratory they may be held together by heavy rubber bands and placed in manila envelopes with accompanying history slips. Pyrex plates may be sent through the mail wrapped in corrugated paper and placed in tightly fitted boxes. In Denmark, aluminum plates have been used with considerable success in transporting medium and cultures through the mail; aluminum culture boxes are also used by Sauer.[21]

### C. The Nasopharyngeal Swab

In many instances, particularly in infants and in children with mild coughs, obtaining a satisfactory cough plate is difficult, and inoculation of the medium by means of a nasopharyngeal swab is much more successful. As described by Bradford and Slavin,[8] the swab consists of a small bit of cotton tightly wrapped about the end of a thin flexible wire.* The prepared swab should be carefully inspected before sterilization to make certain that the cotton is secure on the wire and that the swab is very small and slender and entirely smooth.

The sterile swab is passed gently through a nostril into the nasopharynx of the child while his head is immobilized to prevent injury. Often a cough is induced which increases the chances for successful results, especially if the swab is allowed to remain in place during a coughing paroxysm. If resistance is encountered because of a large turbinate, deviated septum or adenoids force should not be used. It is occasionally not possible to pass the swab through either nostril.

The inoculated swab is withdrawn from the nostril and streaked immediately over a portion of a plate of Bordet-Gengou medium. The inoculum is then spread over the entire plate with a platinum loop. If medium containing penicillin is used the swab may be streaked directly over the entire plate.

### D. Incubation and Examination of Plates

1. Incubate the plates at 37° C. and examine them several times during the first 48 hours to detect molds and other spreading colonies

* A wire that has been found satisfactory is braided bronze trolling line 2 No. 100B (300 ft. winders) manufactured by Edwards Mfg. Co., 2215 So. Michigan Ave., Chicago 16, Ill.

which might later overgrow the plate. With a sterile needle or scalpel remove the agar surrounding and supporting such spreaders.

2. Examine the plates after about 40 hours for the colonies of *Hemophilus pertussis*. With a bright light and a hand lens examine the plates by transmitted light to detect hemolysis and by reflected light to detect the typical colony appearance. Examine the plates twice daily until *Hemophilus pertussis* is found. Discard the plates after 5 or 6 days if no colonies have been identified.

## E. CRITERIA FOR IDENTIFICATION OF COLONIES OF HEMOPHILUS PERTUSSIS

### 1. *Colony appearance*

*Hemophilus pertussis* colonies appear smooth, raised, glistening, pearly, almost transparent, and not over 1 mm. in diameter. Colonies of Gram-positive cocci are generally duller, darker, and more opaque. Colonies of *Hemophilus pertussis* are surrounded by a characteristic zone of hemolysis, a zone which is not sharply delimited but which merges somewhat diffusely into the surrounding medium. This zone usually is absent when 30 per cent or more blood is used in the medium.

### 2. *Consistency of the growth*

The consistency of the growth and the manner of its diffusion into water are typical of *Hemophilus pertussis*. The growth is homogeneous and when placed in a drop of water or salt solution it spreads, showing first a momentary clumping effect which disappears with very slight agitation, and leaves a smooth homogeneous suspension.

### 3. *Morphology and staining reactions*

Stained by Gram's method *Hemophilus pertussis* decolorizes readily, much more readily than *Hemophilus influenzae*. Microscopic examination of a stained smear will show small faintly stained coccoid bacilli scattered evenly throughout the film. They occur for the most part singly and in pairs, occasionally in short chains. The organisms are almost invariably less than 1 micron in length. Smooth, freshly isolated organisms show little pleomorphism. Strains maintained for long periods on laboratory media may show smooth-to-rough changes including marked pleomorphism, threadlike filaments, and thick bacillary forms, but these atypical organisms are not encountered in diagnostic work.

#### 4. *Slide agglutination test*

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 to 500, or 1/10 of the titer of the serum. If the colonies are *Hemophilus pertussis,* there will be almost immediate agglutination. The result is significant only if the control suspension without serum remains smooth and shows no clumping. The technic of the quantitative agglutination test is described later.

#### 5. *Identification by subculture*

If there is insufficient growth for a slide agglutination test, colonies of *Hemophilus pertussis* may be transferred to another plate. After 24 hours' incubation there is usually sufficient growth for a slide agglutination test.

### F. The Parapertussis Bacillus and Brucella bronchiseptica

The parapertussis bacillus is occasionally encountered from patients with pertussis-like symptoms. Also *Brucella bronchiseptica* has been reported from human beings in a few instances. Colonies of both these species on Bordet-Gengou medium resemble *Hemophilus pertussis* in all respects except that they develop more rapidly and grow to a larger size. These organisms are also morphologically indistinguishable from *Hemophilus pertussis.* Differentiation is based on agglutination tests and biochemical characteristics. Since cross-agglutination may occur to a relatively high titer, care must be taken in performing slide agglutination tests to use serum dilutions beyond the range of the cross-reactions, or preferably, to use absorbed antisera for the three strains. The following chart indicates the differential cultural characteristics of the three organisms.

| *Characteristic* | *H. pertussis* | *parapertussis* | *Br. bronchiseptica* |
|---|---|---|---|
| Growth on agar without blood | − | + | + |
| Brown pigment produced in peptone medium | − | + | − |
| Motility | − | − | + |
| Reduction of nitrates to nitrites | − | − | + |

### G. Reporting the Results of Cough Plate Examination

#### 1. *Positive report*

Plates showing colonies with the cultural and morphological characteristics of *Hemophilus pertussis* and giving positive agglutination with specific antiserum should be reported as soon as found, "*Hemophilus pertussis* found."

#### 2. *Negative report*

Plates showing no colonies of *Hemophilus pertussis* by the 4th day should be reported "*Hemophilus pertussis* not found to date." Such plates should be incubated for an additional day or two before being discarded. In the rare instance of a positive finding after the first report, an additional report may be made, "*Hemophilus pertussis* found after further incubation."

#### 3. *Unsatisfactory plates*

If the cough plate shows a very scanty inoculation or overgrowth with molds or other saprophytic organisms making the report on *Hemophilus pertussis* unreliable, a negative report should not be made but the plate reported unsatisfactory and the reason given. Also, a negative report should not be made if the history slip indicates that the cough plate exposure was unsatisfactory. Another specimen should be obtained immediately.

## IV. PURE CULTURE STUDIES OF *HEMOPHILUS PERTUSSIS*

### A. Cultural and Staining Characteristics

To preserve their antigenic stability, cultures should be maintained on Bordet-Gengou medium containing at least 15 per cent blood. Some workers believe 30 per cent blood is preferable. It is recommended that for storage, cultures suspended in sterile skimmed milk be subjected to the lyophile process.

It is possible to adapt cultures of *Hemophilus pertussis* to other media such as blood agar, chocolate agar, brain-veal agar, or infusion agar; but in the process of adaptation, the organisms dissociate, lose their smooth characteristics, and become intermediate or rough forms. These latter usually are non-pathogenic, show increased electrophoretic migration velocities and are serologically and culturally altered as

compared with the original parent strain. In this outline we are concerned only with smooth cultures, the group designated Phase I by Leslie and Gardner.[22] These cultures invariably are isolated from patients with pertussis, whereas dissociated forms result from laboratory manipulation.

The colony appearance on Bordet-Gengou medium and the staining characteristics of *Hemophilus pertussis* have already been described. Capsules can be demonstrated by proper technic, perhaps the most satisfactory result being obtained by using the stain devised by Lawson.

*Hemophilus pertussis* is strictly aerobic and grows best at a temperature of 34 to 37° C. A formula based upon that of Bordet-Gengou (C.M. No. 34) seems to be essential in providing for the growth requirements and maintaining the characteristics of smooth strains. However, it has been demonstrated by several workers including Hornibrook,[23] Verwey,[24] and Cohen and Wheeler,[25] that smooth strains can be maintained for at least a few generations on defined liquid media which do not contain blood. With regard to the usual biochemical tests applied to bacteria, the pertussis organism is peculiarly inert. It does not ferment carbohydrates, form indol, or reduce nitrates; it does not liquefy gelatin. Litmus milk is slowly rendered alkaline. The production of alkali is a characteristic of all forms of *Hemophilus pertussis* whether smooth or dissociated, and all media will have a final alkaline reaction—approximately pH 8.0. Catalase is produced; tests for this enzyme may be made with $H_2O_2$ on washed, aerated suspensions of *Hemophilus pertussis* grown 48 to 72 hours on Bordet-Gengou medium, with a technic essentially as described by Farrell.[18]

## B. SEROLOGICAL REACTIONS

It is generally accepted that smooth cultures of *H. pertussis* form a serologically homogeneous group.

Results of agglutination tests reported by various workers, including Kristensen[26] and Kendrick and Eldering[27] support this view. However, the finding of occasional poorly agglutinating strains when tested with certain antisera, suggests the need for further study of antigenic relationships.

The following procedure for rapid determination of agglutinins is essentially the technic of Noble[28] applied to pertussis studies by Kendrick.[29-30]

### 1. *Technic of rapid agglutination test*

a. *Antigen preparation*

With a stiff bent needle, remove the growth of *Hemophilus pertussis* from Bordet-Gengou medium incubated for 36 to 72 hours and suspend in salt solution. If the suspension is not entirely smooth, filter it through a thin layer of cotton. A technic for preparing simple filters for this procedure has been outlined by Kendrick.[29] Adjust the turbidity of the suspension to approximately 20 billion organisms per ml. If a photometer is not available for adjustment of the turbidity of the suspension, it is sufficiently accurate for the agglutination test to consider McFarland density tube No. 3 as roughly equivalent to 10 billion *Hemophilus pertussis* per ml.

b. *Preparation of antiserum*

Immunize a rabbit against a smooth culture of *Hemophilus pertussis*. The antigen should be a suspension containing 10 billion organisms per ml. of culture grown on Bordet-Gengou medium (C.M. No. 34) for not more than 72 hours and killed by contact with a 1:10,000 solution of merthiolate or phenol 0.5 per cent for at least 48 hours at 4° C. Inject four doses of this vaccine—0.2, 0.4, 0.8, and 0.8 ml. respectively, per kg. of weight—intravenously, at 3 or 4 day intervals. One week after the fourth injection make a trial bleeding. If the agglutination titer against the homologous strain and several recently isolated strains is satisfactory, bleed the rabbit from the heart and collect the antiserum. The titer should be 1:4,000 or more by the rapid test. Occasionally the titer will be found satisfactory after the third injection, and sometimes a rabbit will require more than the usual four injections.

c. *Dilution of antiserum*

Dilutions may be prepared conveniently as follows:

| *Tube No.* | *Serum Dil. Required* | *Serum Dil. to be Used* | *Serum ml.* | *Saline* |
|---|---|---|---|---|
| 1 | 1:10 | undiluted | 0.3 | 2.7 |
| 2 | 1:50 | 1:10 | 1.0 | 4.0 |
| 3 | 1:100 | 1:50 | 2.5 | 2.5 |
| 4 | 1:250 | 1:100 | 1.0 | 1.5 |
| 5 | 1:500 | 1:100 | 0.5 | 2.0 |
| 6 | 1:750 | 1:100 | 0.5 | 3.25 |
| 7 | 1:1,000 | 1:100 | 0.7 | 6.3 |
| 8 | 1:1,500 | 1:1,000 | 2.0 | 1.0 |
| 9 | 1:2,000 | 1:1,000 | 1.0 | 1.0 |
| 10 | 1:2,500 | 1:1,000 | 1.0 | 1.5 |

d. *Agglutination test*

Mix 0.1 ml. of each serum dilution with 0.1 ml. of antigen in agglutination tubes, the measurements being made with graduated pipettes. For an antigen control, mix 0.1 ml. of saline with 0.1 ml. of antigen.

Shake the serum-antigen mixtures by hand for 3 minutes, rocking the racks at the rate of approximately 60 back-and-forth motions per minute, and in such a way that the contents flow about three-quarters of the length up the tube.

After the period of shaking, add 0.5 ml. of physiological salt solution to each tube to facilitate reading. The Hipple pipetting apparatus, which is frequently used in the Kahn test, set to deliver 0.5 ml. of saline, is convenient for large series of tests.

e. *Reading the results*

Read the reactions immediately after adding saline. Record each reaction as —, ±, 1, 2, 3, or 4 plus, according to the degree of agglutination. In determination of the titer, 2 plus is the lowest reading to be considered as an endpoint.

It is pointed out that the titers in this rapid test are not directly comparable to those obtained in other tests where the concentration of antigen, the total volume of reagents, and the time and temperature of incubation are dissimilar.[21, 22]

## C. REACTIONS IN ANIMALS

Whooping cough has been transmitted to monkeys by the injection of *Hemophilus pertussis* cultures, according to the reports of several workers, including Sauer and Hambrecht,[31] Shibley,[32] Culotta, Harvey, and Gordon,[33] and Rich, Long, Brown, Bliss, and Holt.[34] Certain characteristic reactions follow the intraperitoneal injection of smooth *Hemophilus pertussis* cultures in rabbits, guinea pigs, and mice; in mice, pertussis pneumonia may be produced by intranasal or intratracheal injections, and encephalitis by intracerebral injections. These reactions constitute helpful criteria for the study of *Hemophilus pertussis*.

### 1. *Skin reactions*

A skin reaction is produced in rabbits and guinea pigs by the intracutaneous injection of a living suspension of smooth *Hemophilus pertussis*. Within a few hours after injection there is an ischemic, indurated area at the site of injection. By 24 hours a purplish center

appears, surrounded by an ischemic ring and, beyond that, the outer part of the indurated zone appears inflamed and may show an arborization of capillaries. The central zone shows a dark purple necrotic area after 48 hours. Within a few days, depending upon the intensity of the reaction, a scab forms over the purple zone, and later a scar indicates the site of inoculation. A dose of 0.1 ml. of a 2 billion per ml. suspension gives a reaction about 1 cm. in diameter.

### 2. *Intraperitoneal injection*

The intraperitoneal injection of mice and guinea pigs with a suitable dose of smooth *Hemophilus pertussis* is followed by death and characteristic gross and microscopic pathology at autopsy. The fatal dose shows considerable variation with individual animals. Usually a dose of 1 ml. of a 10 billion per ml. saline suspension kills a 15 to 18 gm. mouse within 72 hours. A larger dose is usually required for guinea pigs. At autopsy there is observed a more or less extensive hemorrhagic area in the peritoneum at the site of injection, and occasionally an area of necrosis in the skin. The peritoneal fluid is increased; markedly, in the guinea pig. Frequently all organs and membranes are covered with a sticky mucoid exudate. Grossly, the heart, lungs, liver, and spleen show no changes. There may be marked congestion around the reproductive organs. *Hemophilus pertussis* may be recovered with ease from the peritoneal fluid. Cultures from the heart's blood of the mice usually show *Hemophilus pertussis.*

A report by Silverthorne [35] records the use of mucin for suspending the infecting dose of pertussis organisms.

### 3. *Intranasal injection*

The intranasal injection of susceptible strains of mice under anesthesia as described by Burnet and Timmins [36] is followed by death of a large percentage of the animals. The dose must be determined for a particular set of conditions but is usually about 1,000 million organisms, or 0.04 ml. of a 25 billion per ml. suspension. At autopsy, pneumonia is indicated by gross and microscopic pathology. The pathological picture has been described in detail by Bradford.[37] *Hemophilus pertussis* may be recovered in a large percentage of the animals from the pleural fluid, lungs, trachea, and heart's blood. Reports on the use of this method have been made by Lawson,[38] Miller,[39] and also by North, *et al.*[58] The intranasal infection of young

rats with the production of experimental pertussis has been reported by Hornibrook and Ashburn[40]; intraperitoneal and intratracheal routes have been used with success by Sprick.[41]

#### 4. *Intracerebral injection*

The course of disease in mice following intracerebral injection of an infective dose of *Hemophilus pertussis* has been described by Kendrick, Eldering, Dixon, and Misner.[42] There is an incubation period of several days after which the infected mouse shows a series of typical symptoms. Encephalitis is followed by death usually between 4 and 14 days after injection. In general, a dose of $10^7$ organisms of a smooth strain kills the majority of a group of 16 to 20 gm. white mice. The more virulent strains kill the majority of mice in a dose of $10^3$ or less.

The intracerebral route for infection of mice is useful as a method of determining the relative virulence of cultures; and also, in protection tests, for studying their relative antigenicity.

## V. SEROLOGICAL PROCEDURES IN THE DIAGNOSIS OF PERTUSSIS

The limitations of serological procedures have been discussed in the introduction. For those who wish to employ these methods under the conditions in which they are applicable, the following suggestions are made as to technic.

### A. AGGLUTINATION

For testing a patient's serum, the technic already described may be used except that lower serum dilutions should be included. A convenient series is 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, etc. The series may be extended or modified to meet the needs. A technique modified for testing children's sera has been described by Miller and Silverberg.[43] In two studies (Miller, Saito, and Humber,[44] and Sako[45]), both using this technique, it was found that vaccinated children carrying high titers of agglutinins were clinically immune but, on the other hand, children with low titers were not necessarily susceptible.

### B. COMPLEMENT-FIXATION

A description of the technic of complement-fixation is omitted as this procedure has not been studied sufficiently with newer antigens made from smooth organisms and with the modern technic of com-

plement-fixation. Smooth organisms were used in this test by a number of the earlier workers—prior to 1910; but in much subsequent work antigens of dissociated forms have been used. Both technic and interpretation need reinvestigation. Kristensen and Larsen[46] reported on the use of complement-fixation in 1926. Other authors include Doughtry-Denmark,[47] Weichsel and Douglas,[48] Paton,[49] and Mishulow, Siegel, Leifer, and Berkey.[50]

### C. OPSONIC REACTION

The method given here was used by Kendrick, Gibbs, and Sprick[51] in pertussis studies. Reports on the opsonic test in pertussis have been made also by Singer-Brooks and Miller[52] and by Bradford and Slavin.[53]

The technic follows.

#### 1. *Materials*

a. Antigen: a 20 billion per ml. suspension of *Hemophilus pertussis*, preserved with merthiolate 1:10,000
b. Sodium citrate solution, 4.0 per cent
c. Hastings' stain, filtered, or Wright's stain may be used
d. Lancet, for drawing blood
e. Blood pipette, 0.1 ml. graduated in hundredths
f. Test tubes, 3 x ⅜", with stoppers to fit
g. Capillary pipettes

#### 2. *Collection of blood*

Blood may be collected as for a blood cell count from the finger, toe, heel, or ear.

a. Draw 0.02 ml. of 4 per cent sodium citrate solution into the pipette and then blood, up to 0.1 ml. This makes a final concentration of 0.8 per cent sodium citrate.

b. Blow out the citrated blood into a test tube and mix gently but thoroughly. If the test is not to be completed immediately, the tube should be plugged to prevent evaporation. Tests have been found satisfactory up to at least 3 hours after collection of blood.

#### 3. *Preparation of antigen blood mixtures*

Add 0.1 ml. of antigen to the citrated blood and mix by rocking back and forth gently for 1 minute.

### 4. *Incubation*

Place the antigen-blood mixture at 37° C. for 30 minutes.

### 5. *Preparation of slides*

a. Rock the incubated antigen-blood mixture back and forth for 1 minute to redistribute the cells and organisms.

b. With a capillary pipette, remove some mixture from the tube and place a drop on the end of a clean slide. A blood drop 5 mm. in diameter is the correct size for a satisfactory film.

c. Touch the drop of blood with the edge of another slide so that the blood follows as the slide is pushed gently almost to the end of the first slide. The blood should have been completely used before the end of the slide is reached.

d. Dry the film in air.

e. Stain the film by flooding with 0.5 ml. filtered Hastings' stain. After 20 seconds add 1.0 ml. distilled water and allow to stand 10 minutes. Wash with distilled water and dry in air.

### 6. *Examination of stained films*

Select a field in which the organisms and cells are well distributed. Observe the first 25 polymorphonuclear neutrophiles encountered. Count the number of phagocytized organisms in each and classify the cells in the following groups: 0, 1–5, 6–20, 21–40, 41 or more, according to the number of bacilli in the cells. Exclude eosinophils from the count as well as disintegrated cells and cells covered by large clumps of bacilli when it cannot be determined whether the bacilli are phagocytized or merely on top of the cells.

### 7. *Reaction rating*

In general, the reaction is strong, moderate, slight, or negative, according to the relative amount of phagocytosis. A method for interpreting the findings quantitatively is given in the paper by Kendrick, *et al.*[39] and includes the following steps:

a. Multiply the number of cells in the groups 0, 1–5, 6–20, 21–40, and 41–60 or more by the assigned factors 0, 1, 3, 8, and 12, respectively, in series. Add the products.

b. Rate the reaction according to the following outline:

| *Sum of Products* | *Reaction Rating* | |
|---|---|---|
| 0– 25 | negative | — |
| 26–100 | weak | 1+ |
| 101–200 | moderate | 2+ |
| 201–300 | strong | 3+ |

### 8. *Interpretation*

It cannot yet be said how closely the results of the opsonic test correlate with protection, but the results of several workers, including Rambar, Howell, Denenholz, Goldman, and Stanard,[54] suggest that a high titer of opsonins is correlated with protection. It is known that there is a marked increase in opsonins following pertussis and also following injections of an active vaccine of *Hemophilus pertussis.* A high level is maintained for several months. The reactions may be slightly weaker after 6 months and relatively weaker as the interval after disease or immunization increases. The reaction in children with no record of a pertussis attack or immunization, usually is negative or weakly positive.

## VI. LABORATORY PROCEDURES IN THE DETECTION OF CARRIERS AND FOR THE REGULATION OF ISOLATION AND RELEASE

### A. DETECTION OF CARRIERS

There have been few reports of healthy carriers of *Hemophilus pertussis.* Luttinger's[55] frequently cited "Pertussis Pete," assumed by him to be a carrier, was convalescing from an attack of whooping cough. Miller,[56] using the nasopharyngeal swab of Bradford[8] found *Hemophilus pertussis* in three of twelve familial contacts to pertussis.

Convalescents may carry *Hemophilus pertussis* for some time. It is probable that 90 per cent of cases of whooping cough become non-infectious within the first 5 weeks of the disease. In rare instances the organisms have remained after the 8th week of the disease. Kristensen[57] has reported one positive cultural finding from a child in the 10th week of the paroxysmal stage. Kendrick and Eldering[20] have had positive findings in the 9th week of disease in 2 instances.

It is not uncommon to encounter mild cases of whooping cough which never reach the threshold of clinical recognition. These cases might never be suspected were it not for known familial exposure,

and their nature could never be proved without bacteriological findings. These individuals might be classed as carriers by some, as there is no definite clinical evidence of the typical disease other than a cough and a possible slight fever of short duration. To our minds, however, these individuals have mild or abortive attacks, similar in every respect to scarlet fever without a rash and the mild types of other communicable diseases which furnish an unsuspected reservoir of infection in the community. Second attacks of whooping cough generally assume this mild form and are usually undiagnosed by the clinician. Such mild attacks and early cases still in the catarrhal period are the most dangerous reservoirs of infection in the community and serve to keep the disease at endemic levels at practically all times. The finding of "carriers" therefore, is in reality a matter of finding cases.

### B. Isolation and Release

The use of laboratory procedures as a basis for isolation and release cannot be recommended for general use. In the first place, adequate laboratory facilities are not generally available. Also, the results of bacteriological studies indicate pretty clearly that very few patients with pertussis are infectious more than 5 weeks after onset of symptoms. This gives a sound basis for release based on an isolation period. Under certain conditions, however, when adequate laboratory service is available, advantage is gained by basing release on several consecutive negative cultures. It makes possible the release of children with short, mild attacks as soon as negative cultures indicate their non-infectiousness; the procedure lends itself particularly well to hospital and institutional practice.

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# The Diphtheria Bacillus

## *(Corynebacterium diphtheriae)*

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## I. INTRODUCTION

ALTHOUGH bacteriological data are of assistance to the physician as supporting evidence in diagnoses of diphtheria arrived at on clinical grounds, and often suggest a correct diagnosis when clinical evidence is not clear, the diagnosis of diphtheria in human beings is a responsibility solely of the physician. On this point he should, therefore, regard the bacteriological laboratory merely as a source of corroboratory evidence. 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 reasonably be expected to assume responsibility for furnishing the physician not only with accurate data as to the presence, type, and virulence of strains of *Corynebacterium diphtheriae,* but also with reliable toxin and control materials for the Schick test and methods for determining and interpreting the antitoxin content of the blood serum.

It is the purpose of this section to present: (a) Methods by which the bacteriological laboratory may aid the physician in the diagnosis of suspected cases of diphtheria; (b) means of assisting the epidemiologist by isolating pure cultures of *Corynebacterium diphtheriae* and determining their type and virulence; and (c) methods for estimating the susceptibility of various persons to diphtheria by (1) the Schick

test, and (2) accurate measurements of the amount of antitoxin in the blood serum. In order to give an added value to the protocols, they are preceded by a brief summary of some of the more important concepts upon which the methods are based. The whole is presented under two headings: Bacteriological Considerations, and Immunological Considerations. Bacteriological Considerations will be discussed first.

## II. BACTERIOLOGICAL CONSIDERATIONS

### A. CULTURES FROM PATIENTS AND CONVALESCENTS

In frank cases of diphtheria of the respiratory tract *Corynebacterium diphtheriae* is usually present in large numbers in the lesions and membranes, and experience has shown that the diphtheria organisms in such cases, when cultivated on a proper medium and stained in an appropriate manner, are quite easily recognized. Further, in such cases experience has shown that the presence of morphologically typical organisms in a typical lesion may usually be accepted as *presumptive evidence,* not only of the identity, but also of the virulence* of the organisms. On the basis of such evidence, the physician usually administers antitoxin if he has not previously done so on the grounds of his own clinical observations. *Completed evidence* of the identity, virulence, and type of the organism in question should be obtained later, after isolation of a pure culture of *C. diphtheriae* from the patient.

Due to the relatively large numbers of diphtheria bacilli nearly always found in cultures made from diphtheritic lesions, the isolation of these organisms by the use of the medium described herein is a relatively simple matter in clinically typical cases.[2, 3] Even in convalescent carriers the diphtheria organisms persisting in the throat or nose retain for weeks or months their characteristic growth form, morphology, and staining reaction, and often their relative numerical status, so that isolation is seldom difficult unless the convalescence is of very long duration. Further, the virulence of the organisms in convalescent carriers invariably remains unchanged with respect to the guinea pig, chick, or rabbit, so that frequent tests are not necessary except for administrative reasons.[4–6] In general, the same remarks apply to carriers in immediate contact with cases.

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* There is, however, some evidence that virulence for human beings is not always detected by the usual "virulence tests" in animals.[1]

### B. CULTURES FROM DOUBTFUL SOURCES AND HEALTHY CARRIERS

It is in dealing with cultures from doubtful cases, as a rule, or in examining those taken in the absence of any lesion, especially swabs from healthy, "casual" carriers (as opposed to *known* convalescent carriers or case-contact carriers), that difficulty may be encountered in demonstrating the presence of true *Corynebacterium diphtheriae* by means of the microscope, or in excluding from consideration, by microscopic methods, certain organisms (diphtheroids) which closely resemble this species. Further, it has been shown that, under conditions of low morbidity, virulent diphtheria organisms in *original* cultures from healthy carriers may occur in morphologically unrecognizable (coccoid) forms, and that a large proportion of strains from healthy carriers even when typical in morphological, tinctorial, and cultural properties, may be totally avirulent.[7, 8]* The proportion of these avirulent strains in any carrier group varies from a very few up to as high as 100 per cent.[9] Therefore, the isolation and study in pure culture of suspect organisms from doubtful sources, carriers, or convalescents of long standing, are an essential part of the work of the public health laboratory since they are the only means of identifying the organisms with certainty and establishing their virulence or avirulence beyond doubt.

### C. DETERMINATION OF TYPE

For several years considerable interest has centered around differentiation of the so-called *gravis*, *mitis*, *minimus*, and other types of *Corynebacterium diphtheriae*.[10-12] These differentiations are based entirely on cultural tests, such as starch fermentation, etc. Originally it was thought that the *gravis* type was related to severe cases which were refractory to serum therapy, and the *mitis* type to mild cases and to carriers. It is now clear that in the United States, at present, there is no correlation between any of these cultural types of diphtheria bacillus and any clinical form of the disease.[13, 14]

Type determination as at present performed can have little significance for clinical diagnosis as related to therapy because of the time (at least 1 week) required and because the toxins produced by all types appear to be identical.[15] The importance of typing to epidemiology, and hence, ultimately, to the practising physician and health officer, can be finally evaluated only when more exact data, *ob-*

* As measured by animal virulence tests.

***tained by comparable methods,*** are at hand. So far, very few comparable studies have been made.

## D. QUARANTINE AND RELEASE

In diphtheria, as in certain other infectious diseases, the problem of release from quarantine is often a complex one. "Release cultures" taken at intervals may indicate that an individual is harboring virulent diphtheria organisms over an indefinite period. Release then becomes a matter of judgment on the part of the health officer in charge. The question may involve medical, administrative, epidemiological, bacteriological, economic, and even police factors. It is obviously not practical to deal with all of these details in this discussion.

As to the need for prolonged quarantine, opinions vary as to the persistence of virulence in diphtheria organisms. However, *all* who have studied the question experimentally agree that a strain of *Corynebacterium diphtheriae,* once virulent (*as tested in animals*), and so long as it retains its typical morphology and cultural characters so as to be *recognizable as C. diphtheriae,* is always virulent, but is not necessarily always highly *infectious* or *pathogenic for human beings.* As a rough guide it may be said that in convalescent carriers of up to at least 4 weeks' duration, a dangerous degree of infectiousness and pathogenicity for human beings of the organisms may be assumed.[1, 4-6, 16-21] There is, however, reliable epidemiological experience which suggests that after an indefinite period, possibly about 4 weeks, *in the absence of any pathological condition* of the upper respiratory tract, the danger from the diphtheria convalescent is reduced.[22-24] The virulence of the organisms, as judged by the usual tests in guinea pigs, rabbits, and chicks, however, continues unchanged. On the other hand, questions have been raised,[1, 20, 25, 26] concerning the reliability of the animal virulence test as a guide to virulence for human beings. Further evidence is needed along these lines.

## E. SUMMARY

From the foregoing discussion it is evident that in bacteriological investigations of diphtheria at least two, and possibly three, sorts of evidence are required. These are:

### 1. *Presumptive Evidence*

a. Microscopical demonstration of the presence of morphologically and tinctorially typical *Corynebacterium diphtheriae,* after cultivation on some suitable medium, preferably Loeffler's, may be accepted as cor-

robatory evidence in the aid of, or confirmation of, diagnosis in frank or clinically suspicious cases. The absence of such organisms in smears does not exclude diphtheria.

### 2. *Completed Evidence*

a. Isolation of the organism in pure culture and demonstration of its identity as *Corynebacterium diphtheriae by*

(1) Morphological examination
(2) Fermentation of dextrose without gas *
(3) Failure to ferment saccharose *

b. Demonstration of virulence (or avirulence) by test with the pure culture, in animals with specific controls (see below). Virulence tests with mixed or " whole " or " field " cultures often give entirely erroneous results.

### 3. *Evidence as to Type (optional; for special studies)*

a. Completed evidence as to identity and virulence (or avirulence) as outlined above.

b. Demonstration of the presence or absence of the properties characteristic of various biochemical types; e.g.—

(1) Glycogen fermentation
(2) Starch fermentation
(3) Colony form
(4) Pellicle formation on broth
(5) Absence of hemolysin production (Hammerschmidt qualitative method)
(6) Rapid (4 to 5 days) reversion of pH of broth cultures from acid to alkaline

## III. BACTERIOLOGICAL METHODS

The bacteriological procedures outlined below, while not the only ones available †, ‡ have been selected because, if carefully followed, they are known to yield satisfactory results in the collection of ***presumptive***

* Strains of virulent *C. diphtheriae* which ferment saccharose or which fail to ferment dextrose, or both, have been encountered. While they are not common in the United States at present (1947),[27, 28] their existence robs these fermention tests of much of their value.

† For example, the modification of the Folger-Solé method, devised for original diagnostic cultures by Brahdy, et al.,[30] seems to have definite value in certain situations, especially in hospital diagnosis, and in individual instances. The older Loeffler method is here recommended because it is more familiar, is more generally applicable to health department practice, and has advantages, especially with respect to moisture content and the danger of drying out, not possessed by the serum-swab method.

‡ The use of the direct smear in obtaining *presumptive evidence* of diphtheria is not recommended, on the grounds that it is *usually misleading*. It is of value, however, in giving a clue to Vincent's angina when the culture is negative for diphtheria, and should not be neglected.

and *completed evidence* and in the *determination of type* in diphtheria studies. The use of this outline by diphtheriologists will have the added advantage that results obtained in different laboratories may be reasonably comparable and, therefore, possess enhanced value for large scale epidemiological investigations.

## A. Collection of Specimens

Routinely, material may be collected from nose or throat on wooden or aluminum applicators padded with absorbent cotton. It is convenient, in preparing swabs, to pad some of them with a minimum of cotton for use in the nose. Swabs are best sterilized and kept in sterile tubes until used.

Two sterile swabs should be used for each person cultured, one to obtain material from the throat lesions or tonsillar crypts, the other to be passed to the nasopharynx through one nostril.

For clinical diagnostic purposes, the swabs should be prepared, sterilized, and kept separately. In large carrier surveys both swabs may be sterilized together and kept in the same tube after use.

## B. Preparation of Original Cultures

Slants of Loeffler's medium (C.M. No. 55) should be inoculated immediately after collection of the specimen if possible; in any case, within 2 hrs. thereafter. Long delays in transporting swabs to the laboratory are known to cause a considerable reduction in numbers of viable organisms, especially on swabs from convalescent and casual carriers.[29]

Primary inoculation of swabs onto *slants* of tellurite media intended for morphological diagnosis may give misleading results as tellurite tends to distort the morphology of *C. diphtheriae*. However, *plates* of cystine tellurite medium (C.M. No. 36) [2, 3] should be streaked with the initial swab, as these plates often yield pure cultures 24 hrs. earlier than plates inoculated from the Loeffler slant next day.

For *carrier surveys*, each pair of swabs (one throat swab and one nose swab) may be stroked gently several times over the entire surface of a single slant rotating the swabs between the thumb and the forefinger as the swabs glide back and forth over the medium.

The streaking of a tellurite plate with initial swabs is of little advantage in carrier surveys.

For *diagnostic* purposes, the swabs should be cultured separately on two slants and on a tellurite plate.

Under *no circumstances* should a diagnosis be based on smears made directly from the throat or membrane. There are many non-diphtherial membranes which closely resemble those of diphtheria, and there are many actinomyces occurring in both the normal and diseased throat which are morphologically indistinguishable from *C. diphtheriae* The error is frequent and the result confusing.

### C. INCUBATION OF ORIGINAL CULTURES

Original cultures should be *finally* examined only after 18 hrs.' incubation at 35° to 37° C. However, preliminary examinations are permissible after 8 hrs.' incubation, and if positive, are of great

| LABEL HERE | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| | 6 | 7 | 8 | 9 | 10 |

FIGURE 1—Microscopic slide (3 X 1) marked for ten smears and label

assistance and value. Re-incubation of " negative " cultures after the 18 hr. examination is also permissible, but when a culture is "positive" only after re-incubation, completed evidence of the identity of the organism should be required before its significance is evaluated for clinical purposes. A second specimen is preferable to re-incubation of the first one.

### D. PREPARATION OF SMEARS

The entire growth on each slant should be thoroughly mixed on the surface of the slant by means of a sterile wire. A small portion is then emulsified in a drop of sterile distilled water on a slide, for staining and microscopic examination. A slide may be ruled into 10 squares to receive 10 smears (Figure 1). Care must be taken to avoid spattering from one square to the adjoining ones.

## E. STAINING

It is recommended that all smears prepared in connection with this work be stained with Loeffler's methylene blue solution and examined with an oil immersion lens at a magnification of not less than 900 or more than 1,000.*

## F. ISOLATION OF PURE CULTURE

### 1. *Inoculation of Plates*

Although tellurite plates inoculated with original throat swabs often yield pure cultures, colonies are usually not evident at the time microscopic examination of the 18 hr. Loeffler slant is made since 48 hr. incubation of the plates is necessary. It is therefore desirable to

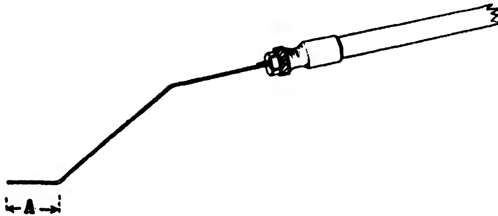


FIGURE 2—Inoculating needle bent for streaking plate. The "foot" which comes into contact with the agar is shown at A.

inoculate a second plate directly from the Loeffler slant in case the first plate fails of its purpose; a frequent occurrence.

The growth on "original" (see B above) Loeffler's (C.M. No. 55) slants may be used for the inoculation of tellurite-blood-agar (C.M. No. 36 or 36a). These tellurite media give comparable results.[31] The entire growth on the slant should be well mixed and only a very small portion used.

Best results are obtained by using one whole plate for each culture. As nearly as possible, the *entire* surface of each plate should be used, care being taken to distribute the inoculum so as to obtain well isolated colonies. This can be accomplished readily after a little practice. A convenient method of streaking plates is to prepare a stiff platinum-iridium or nichrome wire so that a "foot" (Figure 2) can be passed

* Granule stains used with Loeffler's medium do not yield reliable results.

back and forth over the medium in wide swaths. Material is distributed as shown in Figure 3.

### 2. *Incubation of Plates and Selection of Colonies*

Inoculated plates should be incubated in an inverted position, not over two plates to a pile, at 37° C. for from 45 to 50 hrs. and then

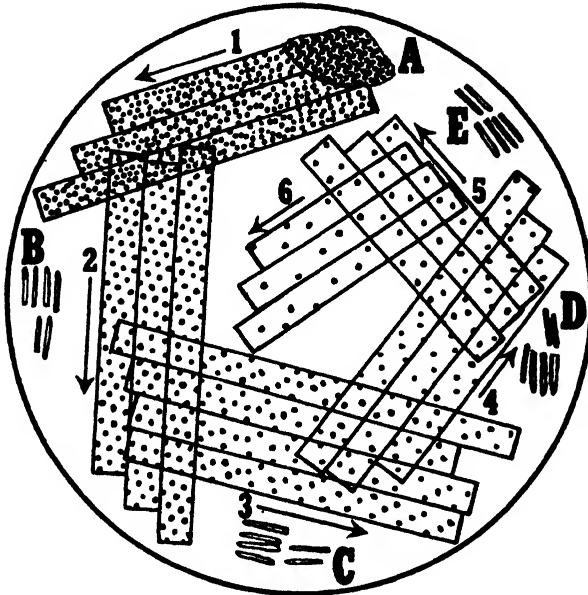


FIGURE 3—Method of distributing material on plate for isolation of pure cultures. Material is first deposited at A and the needle flamed. The foot of the inoculating needle (shown in Figure 2) is passed across the areas shown at 1, in the direction indicated. The needle is then jabbed deep into the agar at B several times to remove excess material and then passed across the areas shown at 2, in the direction indicated. This process is repeated at 3, C, 4, D, 5, E, and 6. Well isolated colonies are almost invariably found at 5 and 6.

examined for colonies of *Corynebacterium diphtheriae*. Tellurite is somewhat toxic for *C. diphtheriae* and will kill the colonies if incubation is prolonged much beyond 50 hrs.

Colonies of *Corynebacterium diphtheriae* (except colonies of the *minimus* type) on the media recommended are circular, soft, and

butyrous in consistency, smooth and usually polished or glistening, and somewhat 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*. They are usually opaque from rim to rim, but there is sometimes a very narrow, translucent zone at the edge. They never produce any zone of hemolysis or other change in the appearance of these media.

Colonies of the minimus type are exceedingly minute (0.1 to 0.5 mm.), usually flat, with a black center and brownish periphery, or entirely black. They may be rough or smooth in appearance. A hand lens is necessary in examining them. Diphtheria bacillus colonies are never leathery, brittle, or membranous in texture on this medium.

It must be remembered that tellurite media are not absolutely selective for *Corynebacterium diphtheriae*, especially with carrier-cultures. Diphtheroids and staphylococci are the organisms which cause most difficulty. Diphtheroid colonies are usually light grey in color, or dark in the center, and whitish or grey and definitely translucent at the periphery, especially when well isolated. When crowded they often have an appearance indistinguishable from colonies of *Corynebacterium diphtheriae*. Usually, however, even when crowded they are rather flat, and are of a slightly lighter grey color.

Staphylococcus colonies of some species are usually flatter and thinner than those of *Corynebacterium diphtheriae* and are of an intense glistening, jet-black color. However, other species of staphylococcus (e.g., *Staphylococcus aureus*) or *Micrococcus* colonies tend to a slaty tint, and often closely resemble the colonies of *Corynebacterium diphtheriae*. Usually, the colonies are large, often showing a center, or concentric rings of a lighter grey color.

White colonies are almost invariably yeasts. Other types of colony sometimes appear, but nearly all of them are readily distinguishable from those of *Corynebacterium diphtheriae*. A little experience soon serves as a guide to what colonies to "fish." *When there is doubt, fish the colonies!*

### 3. *Fishing Colonies*

Colonies thought to resemble those of *Corynebacterium diphtheriae* may be fished from any plate containing them and transferred to slants of Loeffler's (C.M. No. 55) or Pai's[32] medium,* or a convenient

* The morphology of cells stained directly from tellurite medium (C.M. No. 36 or 36a) is often entirely atypical and misleading; hence the transfer from tellurite plates to Loeffler's or Pai's slants before microscopic examination, is recommended.

modification (C.M. No. 54) of the latter.[33] These are incubated for from 12 to 18 hrs. at 37° C.

## G. Examination of Pure Cultures

### 1. *Morphology*

The growth on the Pai or Loeffler slants inoculated with individual colonies is smeared, stained, and examined as outlined in D and E above. Any cultures showing mixtures of typical or suspected *Corynebacterium diphtheriae* with other organisms should be discarded if pure cultures from the same plate are available. Otherwise, they may be replated as in F. Cultures which are morphologically typical or suspicious may be tested as to cultural characters and virulence as indicated below.

### 2. *Cultural Characteristics*

Because fully virulent strains of *C. diphtheriae* which ferment both dextrose and saccharose, or neither, as well as those fermenting only dextrose, have been discovered in various parts of the United States,[27, 28] the former practice of discarding as diphtheroids all cultures which ferment saccharose or fail to ferment dextrose is no longer permissible. The virulence test is the only remaining criterion of identity of true *C. diphtheriae.* Fermentation tests are of value only for corroboratory evidence, for special studies, or type determination. If the fermentation tests are made, a reliable procedure is as follows.

Pure cultures showing suspicious or typical morphology are used to inoculate tubes containing dextrose (1 per cent) infusion broth (C.M. No. 5) colored with brom-cresol-purple and having a pH of from 7.4 to 7.8. Similar tubes of 1 per cent saccharose broth (C.M. No. 5) should also be inoculated, as well as control tubes of plain infusion broth (C.M. No. 5) (with indicator only) of the same lot or lots as those used for the dextrose and saccharose.

The plain broth controls should show no significant change in the color of the indicator during 5 days' incubation at 37° C. If they do, the tests must be repeated with a batch of broth not subject to this error. Broth rendered "sugar-free" by *Escherichia coli* or *Clostridium perfringens* is not recommended. Yeast-fermented broth is satisfactory.

The broth used must support a satisfactory growth of *Corynebacterium diphtheriae,* and only tubes in which such growth occurs can be regarded as satisfactory fermentation tests.

### 3. *Virulence*

Virulence tests with mixed or "field" cultures are not recommended. Experience shows that they are often misleading.

*Method using rabbits or guinea pigs—*

(Adapted from Fraser, D. T., *A.P.H.A. Year Book, 1936–1937*) [84]

a. *Principle of test*

The method permits the same animal to be used for the "test" and "control" injections. A suspension of the culture to be tested is injected intracutaneously in the non-immune animal; 5 hrs. later, diphtheria antitoxin is given intravenously, and the same suspension is again injected as a control into a different area of the skin of the same animal. The antitoxin does not obliterate the characteristic reaction of the skin produced in response to the first injection of virulent bacilli because the damage done during the time interval between the first and second injections when the animal was not immune, is not repaired. The animal is, however, rendered immune by the antitoxin, and the tissues are specifically protected against the second (control) injection.

b. *Suspension of culture and intradermal injection of animal*

A heavy, even suspension of microörganisms is made by adding an appropriate amount, 2 to 3 ml., of meat infusion broth (C.M. No. 5), pH 7.2, to an 18 hr. growth on a slope of Loeffler's serum (C. M. No. 55) or other appropriate medium. With a syringe graduated in 0.1 ml. and needle of 26 gauge, 0.2 ml. of the suspension is injected into the skin of the back of a guinea pig or rabbit. The suspension may be retained in the syringe and stored in the cold for 5 hrs. at which time it is again injected into the animal, which has been rendered immune immediately before by the injection of antitoxin.

c. *Preparation of animal*

The animal is prepared by clipping the hair with an appropriate electric clipper.* Squares of approximately 3 cm. are marked off with indelible pencil, or by other suitable means, upon the moistened back and flanks of the animal. By drawing a line along the middle of the back of the animal and subsequent lines parallel and at right angle thereto, squares are formed which do not overlie the vertebrae. Non-pigmented areas of skin only are used. It is desirable to avoid inject-

* Hand clippers are not satisfactory. Depilatory is unnecessary and undesirable.

ing skin areas anterior to the shoulder girdle, and over the sacrum. Ten intradermal injections may conveniently be made when a guinea pig is used for the test, and 20 in the case of the rabbit. Non-pregnant animals should be chosen.

d. *Injection of diphtheria antitoxin and second injection of suspension*

Five hours after the primary injection of suspensions of culture, 500 to 1,000 units of diphtheria antitoxin are injected intravenously (rabbit), intraperitoneally or intracardially (guinea pig). Immediately thereafter, in the case of the intravenously or intracardially injected animals, 0.2 ml. of the original suspension may again be injected intradermally into fresh areas. In the case of the intraperitoneally injected animal, ½ hour may profitably be allowed to elapse before this injection is made.

e. *Control cultures*

A "virulent," toxigenic strain which is known by previous experience to elicit characteristic reactions (Park 8 strains are not satisfactory) is incorporated in the series of suspensions injected and serves as a "positive control." Similarly a suspension of *Corynebacterium hoffmannii* or *Corynebacterium xerosis* may be used as a "negative control" (but is not necessary).

f. *Reading and interpretation*

Lesions reach their height in the guinea pig after 48 hrs. and in the rabbit after 72 hrs. at which times readings may be made. The lesion produced by the "positive control" suspension of *Corynebacterium diphtheriae* injected 5 hrs. before giving the antitoxin, is characterized typically by a central necrotic area 5 to 10 mm. in diameter, surrounded by a zone of redness 10 to 15 mm. in diameter in the guinea pig, with inhibition of growth of hair. A central hemorrhagic area is usually present. The lesions are typically larger and more intense in the rabbit (Figure 4).

The response induced by the positive control suspension injected 5 hours later, and following the administration of antitoxin as described, exhibits a small pinkish papule about 5 mm. in diameter in the guinea pig and a similar though somewhat larger area in the rabbit. A given suspension may present, in the areas injected before and after the administration of antitoxin, reactions similar to the corresponding injec-

tions of the known positive control. The interpretation is then that the suspected culture contains virulent (toxigenic) *Corynebacterium diphtheriae.* If, on the other hand, the suspected culture does not elicit the typical response characteristic of virulent strains, the interpretation is obviously that the culture does not contain virulent diphtheria bacilli.

Difficulties of interpretation may arise if " field " cultures, as opposed to pure cultures, are used in the test. (The use of field cultures is *definitely not recommended.*) In case their use is for any reason

FIGURE 4—Intradermal virulence tests on a rabbit.
Courtesy Communicable Disease Center, Atlanta, Ga.

*necessary,* the guinea pig should be used as the test animal and not the rabbit, since atypical reactions due to the presence of contaminants are less likely to occur with the former. When field cultures are 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 with some assurance interpret this difference in size as due to the presence of toxigenic *Corynebacterium diphtheriae.* If the first and the second injections result in lesions of the same size, and if the characteristics of the lesions are definitely not those of the known positive control, one may hazard that the suspension did not contain toxigenic *Corynebacterium diphtheriae.* Both the first and second injections of a suspension of a field culture may produce a very large skin lesion due to the presence of contaminating microörganisms (streptococcus, pyocyaneus, etc.). In such cases, to obtain an unequivocal

result, it is necessary to have the microörganism morphologically like *Corynebacterium diphtheriae* in pure culture and to repeat the test.

g. *Discussion*

*Density of suspension*—The margin of safety, in so far as the density of suspension is concerned, is great. . . . Although satisfactory results may be obtained with emulsions equal in density to a McFarland suspension 3,[35] . . . it is not desirable to make a test with a suspension more dilute than that represented by 3 of the McFarland scale. There is no evidence that the suspensions may be too dense, such as are obtained when a minimum of diluent is added to the slope of an 18 hour growth of the diphtheria bacillus. . . .

*Injection of antitoxin and second injection of suspension*

Tests were carried out by Fraser and Weld[34] in which the time interval between the first injection of suspension and the giving of antitoxin intravenously was respectively 1, 3, 4, and 7 hrs., followed immediately by the second injection of suspension. The 3 hr. interval allows of an interpretation of the test but the lesions are relatively small and lack the characteristic necrosis. The reactions obtained at the 4, 5, and 7 hr. interval show practically no difference.

There is a wide margin of safety in so far as the quantity of diphtheria antitoxin is concerned. The amount of toxin contained in the Schick test, even when diluted fourfold, or the suspension of *Corynebacterium diphtheriae* diluted to McFarland 3, and injected intradermally elicited a suitable skin reaction in rabbits even after the giving of 7,000 units intravenously, provided only that the time interval between the injections of the antitoxin and the injection of toxin or culture was 5 hours. Storage of the suspensions for 5 hours at 5° C. did not diminish their power of eliciting characteristic lesions in the skin.

*Tests of single cultures subcutaneously*—Cultures have occasionally been encountered which gave doubtful readings when tested in the skin of the rabbit or guinea pig as described above. In order to eliminate as much uncertainty as possible regarding such cultures, recourse may be had to the injection of a large dose of the living culture subcutaneously (*not intraperitoneally*) into a test guinea pig, and an antitoxin-protected control.

For this test, one may use 2 to 5 ml. of a heavy broth (C.M. No. 5) culture 48 to 72 hrs. old, or the entire growth from a Loeffler (C.M. No. 55) or Pai slant (C.M. No. 54) inoculated 18 to 24 hrs. previously.

Emulsify the organisms from the solid media in about 5 ml. of sterile broth.

Areas about 3 cm. in diameter on the guinea pig abdomens are shaved or clipped, and wiped with alcohol. The control pig then receives 2 to 4 units of antitoxin per gm. of body weight *intraperitoneally.* Two hours later, each pig receives 4 to 5 ml. of the test material *subcutaneously* in the previously prepared area. If the culture is wholly avirulent, neither pig should show any evidence of local necrosis or intoxication in 10 days, or paralysis later. If the culture has any virulence, the unprotected pig may die in 1 to several days or show an area of necrosis or inflammatory reaction.

The interpretation of very low grades of virulence as evidenced only by paresis, etc., is an extremely difficult, if not impossible task in the light of present knowledge. Further studies on low grades of virulence are needed.

*Method using chicks* [36]

a. *Selection and maintenance of stock*—Plymouth Rock or Rhode Island Red chicks approximately 10 to 20 days old are used by preference. Leghorn chicks have been found not so well adapted to laboratory handling as the larger breeds. Sound, *Pullorum*-tested, guaranteed stock is usually available at from 10 to 30 cents per chick.

Chilling from open windows or cold rooms must be avoided. It is absolutely essential that the chicks have warm, dry quarters (85° F.). A $50 mail-order brooder will house about 100 chicks, and occupies little more space than a good-sized steamer trunk.

Never use chicks if the stock is showing evidence of epizoötic disease or numerous deaths due to unknown cause. Error may arise when chicks arrive from dealers without food or water late in the day and remain overnight with neither, and are in a weakened condition when desired for use. Preferably, newly arrived chicks, even if at least 10 days old, should be given a 24 to 48 hr. rest and feeding period before being used in an experiment. Commercial chick feeds are satisfactory. Day-old chicks may be obtained by mail or express and are then held for at least 10 days before using.

b. *Method of testing virulence*—Only pure cultures are tested. About 4 ml. of a *well grown* 48 hr. infusion-broth culture are used. The injections may be made intraperitoneally or into the subcutaneous areolar tissues dorsally between the wing insertions. A few drops of alcohol are used to moisten and control the feathers. Methods of holding and

injecting the birds properly are shown in Figures 5 and 6. The chick is held in the left hand, legs between little and ring fingers, right wing between first and middle fingers, left wing folded in the palm of the

FIGURES 5 and 6—Injecting chick for virulence test.

Courtesy Communicable Disease Center, Atlanta, Ga.

hand in its natural position. The chick's head protrudes through the collar formed by the thumb and index fingers. The bird is quite comfortable.

About ½ hour before injecting test material into chicks, antitoxin controls are prepared by injecting 50–100 units of antitoxin intraperitoneally or into the same area that is to receive the culture. The

serum is quickly absorbed. Protected chicks may be designated by a spot of eosin on top of the head. Dyes of different colors may be used on head, tail, wings, etc., so that large numbers of combinations are available. Leg bands or web punches may be used, but are not necessary. Equal amounts of the same culture are injected into test and control birds.

c. *Results*—When the tested strains are virulent, 80 per cent of the unprotected chicks die within 24 hrs. Twelve per cent survive for 48 hrs., and relatively few are found alive 72 hrs. after inoculation. When they survive for 48 hrs. or over, wing or leg paralysis is well advanced at this time, leaving no doubt as to the virulence of the culture.

*The in vitro Method*

a. *Special medium necessary*—A method devised by Elek has been tested and improved by King, Frobisher, and Parsons[37] whereby most virulent strains may be determined by a test involving only cultivation on serum agar of special composition (C.M. No. 37).

b. *Preparation and inoculation of plates*—Diphtheria antitoxin is diluted to contain 500 units per ml. Into this is dipped a strip of sterile filter paper about 1.5 cm. by 7 cm. The paper is then placed in the center of a dish of warm, fluid agar and allowed to settle to the bottom. After the agar has hardened, the plate is held 4 hrs. at 37° C. to dry the surface. Inoculations are then made in lines across the plate at right angles to the paper strip, ½ inch apart. Well-grown broth culture or broth suspensions from solid media may be used as inoculum.

c. *Incubation and reading the plates*—A positive result consists in the appearance of 1 or more whitish lines of precipitate in the agar, about 1 cm. away from the paper, beginning at, and extending out from, the line of inoculation at an angle of 45° away from the filter paper. The lines can be seen only with oblique light, against a dark background. A hand lens and considerable practice are usually required unless the lines are very marked (Figure 7). Often the lines are visible in 24 hours at 37° C., but they may require 72 hours for full development. Lines appearing after 72 hours, or in different locations than described or in multiple, are of doubtful significance. *A negative reaction does not exclude virulence. Negative and doubtful reactions should be verified in animals as described herein.*

4. *Determination of Type*

Pure cultures, obtained as described in (F, 3) above, should be used as inoculum for the tests required in type determination, as soon as possible after isolation. Strains may undergo alteration in some of their type characteristics if subcultured frequently or held too long on

FIGURE 7—The *in vitro* virulence test.
Courtesy Communicable Disease Center, Atlanta, Ga.

one culture. One or more markedly variant strains sometimes develop in a culture under such circumstances.

The *minimus* type is characterized by delay or absence of dextrose fermentation and by minute colony size. Fermentation tests with dextrose are described in Section G, 2, and the *minimus* colony form is described in Section F, 2. The following details apply to differentiation of the *gravis* and *mitis* types only.

a. *(Character A) Fermentation of glycogen*—Glycogen, "Eastman highest purity" or equivalent, is best prepared in a 5 per cent aqueous solution and autoclaved. One ml. is added to 5 ml. of infusion broth (C.M. No. 5), such as described under (G, 2) above, just before inoculation of the culture. Incubation of the culture at 37° C. for at least 1 week is desirable unless fermentation occurs sooner.

b. *(Character B) Fermentation of starch*—This is determined in the same manner as fermentation of glycogen except that "soluble Starch according to Lintner" of highest purity obtainable is prepared in a 2 per cent aqueous solution. Only freshly prepared starch solutions should be used, as the starch may hydrolyze slowly under some conditions.

c. *(Character C) Colony form*—The minute size of *minimus* type colonies is evident on any of the tellurite media (C.M. No. 36, 36a or 62) or on plain blood infusion agar. For differentiation of *mitis* and *gravis* types only the agar of McLeod (C.M. No. 36a) is reliable.

(1) Inoculation and incubation of McLeod medium (C.M. No. 36a) plates:

(a) When the McLeod agar is firm, invert the plates and divide the bottom surface into halves with a wax pencil.

(b) One culture is then streaked on each half of the agar, taking care to use very small inocula and spreading widely so as to secure well isolated colonies.

(c) Incubate the plates at 37° C. for about 48 hrs.

(2) Evaluation and recording of results:

(a) Not infrequently several kinds of colonies are observed on plates of McLeod's medium, inoculated with one pure culture. Only experience and judgment can enable the investigator to arrive at a reasonable estimate of the tendency of the culture as a whole. Usually it is best to examine several isolated colonies and to form a sort of mental average of the group. In any event, colonies will vary between the *mitis* type of perfectly smooth, circular glistening form; and the irregular *gravis* type, which may be thin and rugose, with feathery, etched edges; or rather thick, showing scalloped edges and radial ridges and often concentric marks.

It has been found most convenient to report colony form simply as *smooth* (or *almost smooth*) and *definitely* irregular or *rough*.

Degrees of "roughness" seem to have little significance and to depend on little known factors difficult to control.

d. *(Character D) Pellicle Formation*—

(1) Medium—Ordinary infusion broth is inoculated in the usual manner and observed for the development of a scum or definite surface membrane. Any fermentation or other broth cultures should also be examined for pellicle formation, as only one out of several cultures made may show a pellicle.

(2) Method—

Technical precautions of importance in studies of pellicle formation are: (a) observation of the cultures on each of 3 or 4 days, and (b) avoidance of agitation of the culture tubes. As regards (a), pellicles often break up and sink quite early in the age of the culture, while with respect to (b), slight shaking will often cause the precipitation of a heavy pellicle to the bottom of the tube, resulting in a totally erroneous notation. A thick growth of smoothly suspended organisms just *under* the surface of broth, or a fine "veil" over the surface, may be mistaken for a pellicle. The true nature of these appearances becomes apparent as soon as the tube is agitated in the least degree, when they disperse as a smooth, cloudy suspension.

It is also of great importance that well buffered, carbohydrate-free broth,* such as is used for fermentation tests, be selected for these investigations, as even a slight acidity tends to lower the surface tension and pellicles may not then be so well supported upon the surface.[11]

e. *(Character E) Hemolysin production*

(1) Method—

(a) A tube of infusion broth (C.M. No. 5) similar to that used for fermentation tests and pellicle studies is inoculated and incubated for 48 hrs.

(b) Place 1 ml. of the culture in a clean serological tube.

(c) Add 1 ml. of a 2 per cent suspension † of thrice washed human ‡ erythrocytes in 0.85 per cent saline solution.

(d) Place in a 37° C. water bath for 1 hr. and then in the icebox overnight.

(e) *Avoid agitating the tubes.* Read hemolysis as *present* or *absent,* depending on the appearance of a clear, ruby-red color

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* Infusion broth (C.M. No. 5) is satisfactory.

† The use of a smaller amount of more concentrated cell suspension does not yield comparable results.[11]

‡ Cells of other animals give discordant results.[11]

above the cells in the bottom of the tube. The test is wholly qualitative, the amount of hemolysis seeming to have no particular significance.

(f) A control tube prepared with sterile broth (C.M. No. 5) and washed red cells of the same lots must show absolutely no hemolysis.

f. *(Character F) Rate of reversion in reaction*—Although all infusion broth cultures (initial pH between 7.4 and 7.8) tend to become somewhat acid within 48 hrs., some will revert within 4 days to a pH of 7.6 or above, while others do so much more slowly.

(1) Method—

(a) Inoculate a tube of infusion broth as in 4, e, (1) above.

(b) After 96 hours incubation, determine the pH. The colorimetric method of Brown[38] is satisfactory. Electrometric methods, while permissible, involve unnecessary accuracy and trouble.

g. *Evaluation and reporting of results*—If one is to follow the definition of those who originated the terms *gravis* and *mitis*[10] a strain of *Corynebacterium diphtheriae,* in order to qualify as one of the *gravis* type, must have at least the 6 principal characteristics described in this outline; i.e., it must ferment starch and glycogen, have definitely irregular and rough colonies, produce a pellicle, fail to cause hemolysis, and revert to a reaction of pH 7.6 or over in broth cultures in 96 hours. A *mitis* strain fails to ferment starch or glycogen, produce a pellicle, or revert to a pH of 7.4 or more. It produces hemolysin and has a smooth regular colony. However, it may sometimes be expedient to adopt 1, 2, or 3 characteristics as " selectors " for purposes of tentative identification, and experience indicates that about 80 per cent of cultures which ferment glycogen *and* starch, and have rough colonies on McLeod's agar (C.M. No. 36a), possess the other 3 principal *gravis* characteristics.[11]

In reporting, cultures having the 6 principal characteristics of *Corynebacterium diphtheriae* var. *gravis,* as outlined above, may be identified as such. Cultures lacking *all* of these characteristics may be reported as *Corynebacterium diphtheriae* var. *mitis.* Other cultures may be designated as indeterminate, giving a list of the *gravis* characteristics which they possess.

Studies show that the various cultural types of *C. diphtheriae* were of no clinical significance in the United States in 1935–1947.[11, 14]

## IV. IMMUNOLOGICAL CONSIDERATIONS

In routine immunological investigations of diphtheria, evidence is sought as to:

1. The reactivity of the superficial layers and blood capillaries of the skin to an intracutaneous injection of 1/50 m.l.d. of a suitably diluted diphtheria toxin of high potency (the Schick test) or,

2. The quantity of antitoxin in the circulating blood, or both.

The results of these determinations are interpreted in terms of the ability of the patient to resist diphtheria, and have been used advantageously in the study of natural immunity and in the development of methods of artificial immunization.

3. A fundamentally sounder method of estimating ability to resist diphtheria is to measure the antitoxic response of an individual to primary and secondary antigenic stimuli.[40] This method is recommended where facilities and time for its use are available.

### A. SCHICK TEST

With respect to the Schick test, no final decision has been reached as to what constitutes the best diluting fluid, a sufficiently potent toxin, or the best type of control material. Neither is there complete agreement as to the most reliable method of reading the Schick test or the full significance of the various types of reaction observed. These problems are still being investigated and the writer therefore feels that final definite recommendations should await more exact information.

However, as a result of recent studies, the field of choice in both materials and methods has been somewhat narrowed and attention may profitably be called to those which seem at present to hold most promise of yielding satisfactory results.

#### 1. *Diluting Fluids*

In the original Schick test physiological salt solution was used to dilute diphtheria toxin so that the intracutaneous dose contained about 1/50 m.l.d. This is free from any objection due to the addition of foreign protein material to the toxin, and is the method of choice when the test can be done ***immediately*** after diluting the toxin. It was soon

found, however, that the toxin deteriorated rapidly in this material, and stabilizing agents have therefore been developed which overcome this difficulty to a great extent. At present three substances, peptone, human serum albumin,[41] and gelatin, are under consideration for this purpose. All are satisfactory as stabilizers.

The most recent diluent is that devised by Edsall and Wyman[41] and containing human serum albumin in a concentration of 0.05 per cent. It is ideal when considered from the standpoint of nonspecific reactions. Merthiolate 1:10,000 is used as a preservative, as phenol robs the albumin of its stabilizing property. The fluid base is the borate buffer solution of Glenney, Pope, and Waddington.[42]

A very simple and useful diluent is that devised by White, Bunney, and Malcolm.[43] Peptone, preferably Witte's "Peptonum Siccum," is dissolved in 0.25 per cent concentration in normal saline solution. The mixture is adjusted to pH 7.8, boiled, sterilized by filtration, and 0.5 per cent phenol is added.

Gelatin diluents may be prepared by one of several methods, a simple formula being that of Moloney and Taylor.[44]

Whatever the diluent selected, toxic filtrate is added to the diluent so that 5 ml. of the mixture contains 1 m.l.d. of the toxin. Final tests on potency, sterility, and specificity are necessary before use. When toxin is properly diluted, "exposure to 37° C. for 24 hours shall cause no appreciable loss in toxicity."[46]

### 2. *Toxin*

The toxic filtrates used for Schick testing should be highly potent so that a minimum of extraneous protein (beef, bacillary, etc.) from the broth itself will be added to the diluent along with the toxin *per se*. Synthetic, protein-free media[47] for Schick-toxin production may be permissible, and very highly purified toxins have become available for immunization purposes[48] but complete data are not yet available concerning the suitability and stability of such toxins for Schick testing.

No minima of potency have been established by law in the United States, but one competent worker[49] has stated that toxins for use in the Schick test should have a value of at least 300 m.l.d.* per ml. The question is a very complex one and is related to the toxoid, toxone, etc., content of the filtrate. Opinion is divided as to the need for exact

* The m.l.d. is "the smallest amount of toxin which when diluted to 3 ml. and injected subcutaneously into guinea pigs weighing 250-280 gm. shall cause death in less than 96 hrs. in 75 per cent of the guinea pigs."[46]

statement or regulation of the maximum content of these nonspecific antitoxin-neutralizing substances permissible in toxic filtrates used in the Schick test, but it would seem that the recommendations of the League of Nations Health Organization might be followed with benefit.* Final conclusions on this point are therefore believed unwarranted at present. Further information is needed.

### 3. *Test*

In performing the Schick test a sharp, short-bevel needle (B. & S. gauge 26) ¼" to ⅝" long is used. It may be of steel, although platinum-iridium is preferable. A syringe of 1 or 2 ml. capacity may be used, provided that the dose of 0.1 ml. may be accurately read upon it. Specially graduated and marked syringes, with different colored plungers for toxin and control are available and are recommended. An area on the flexor surface of the left forearm, previously wiped clean with alcohol, is selected midway between wrist and elbow. With the syringe full, the point of the needle is introduced between the superficial layers of the skin just far enough to cover the point and bevel of the needle. The opening in the end of the needle should be just visible through the skin when the injection is made. A white, pitted "button" about 0.5 to 0.8 cm. in diameter should appear immediately. Platinum-iridium needles may be sterilized by heating the tip momentarily to a dull redness. Others should be replaced by a sterile needle for each patient.

### 4. *Control*

Some workers omit the control in performing the Schick test in large groups of infants and children.† If the test is done, however, it is recommended that the control accompany it. The control injection is made in the right forearm in an area and manner corresponding to that of the test on the left arm.

There is some disagreement as to the most desirable control material. Probably the most widely used (and generally complained of) is pre-

* The League of Nations Health Organization has recommended that the 0.1 ml. dose (1/50 m.l.d.) shall produce no skin reactions in the presence of 1/750 unit of antitoxin but shall give a positive Schick reaction in the presence of 1/1,250 unit. Further, if the skin dose is to be 0.2 ml. (1/40 m.l.d.), then 1/25 of this dose shall produce a positive reaction, while 1/50 should not do so.

† Others omit the Schick test altogether in routine institutional and city-wide immunization campaigns, believing the added work, expenses, and unfavorable effect of 1 or 2 extra "needlings" on the children and their parents, as well as occasional rather severe, allergic reactions, to be uncompensated for by the small number of immunizations saved. This view has much to recommend it.

pared by heating some of the material used for the test itself. There is no agreement as to the most desirable period or temperature of heating, but one combination which gives practicable results is an exposure at 75° C. for 10 minutes[49] Temperatures of 80° C., or even 70° C., and time periods ranging from 5 to 30 minutes have also been used.

Other control materials are under discussion. The highly purified toxoid of Pappenheimer[48] would seem ideal but is still (1948) in an experimental phase. The use of formalin toxoid, suitably diluted* as in the Moloney test,[50] has the advantage that those who react to it are also apt to react unfavorably to immunizing doses of toxoid, and the person responsible for immunization is thus warned and may take suitable precautions against excessive dosage, etc. An objection to formalin toxoid is that, chemically, it is not so close a parallel to the test material as the heated control. Also, since different lots are diluted in varying degrees, the foreign protein content is variable. It has been stated that Moloney reactors are often (about 80 per cent correlation) Schick-negative.[51-54]

### 5. *Reading the Test*

The true Schick reaction results from the effect of diphtheria toxin, un-neutralized by any antitoxin in the patient's tissues, upon the superficial layers of the skin, and the neighboring capillary blood vessels. It consists of an erythematous area varying in diameter from 1 to 4 or more cm., appearing only after 2 days, reaching its maximum intensity in from 5 to 7 days, and fading slowly over a period of 1 to 3 weeks, often desquamating slightly, and usually being discolored by a slight brownish pigmentation. Necrosis rarely occurs.

There should be no reaction whatever at the site of the control injection. However, confusing reactions sometimes occur, probably due to an allergic condition of the patient with respect to the stabilizing agent, the materials used in preparing the medium, or proteins derived from the diphtheria bacilli themselves. These "pseudo-reactions" appear on both arms as red, often indurated areas with ameboid extensions, developing rather rapidly (sometimes within 5 minutes) and fading in from 1 hr. to 4 or 5 days. Pseudo-reactions vary in size and color, but characteristically fade before the true Schick reaction is fully developed, and leave no pigmentation.

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* The "suitable" dilution is determined by comparative tests with each new lot and may vary from batch to batch.

Since the erythematous area of the true Schick reaction persists only on the test arm and, if well developed, for at least 7 days, readings made after this interval are less apt to be in error due to prolonged pseudo-reactions than if earlier observations are made. However, faint but genuine reactions may fade between the 5th and 7th days, and for this reason some workers prefer to read the tests on the 5th day. On the other hand, pseudo-reactions occasionally persist until the 5th and even 6th day. More reliable data would doubtless be obtained by making readings on both the 5th and 7th days.

### B. QUANTITATIVE DETERMINATION OF ANTITOXIN IN THE SERUM

Observations over a period of several decades of endemic and epidemic diphtheria, and of the antitoxin content of many human sera under varying circumstances have led many to the view that the Schick test frequently fails to yield sufficiently exact information as to the true immunity status of the person tested. This is due in part to the fact that the Schick test cannot measure an important factor in resistance, i.e., the ability of various individuals to respond to diphtheria infections, or to antigens injected for the purpose of active immunization. Further, the test itself acts as an effective stimulus to antibody production in many persons and thus introduces a considerable error into such studies. It is well known, as a result of the researches of Fraser, Jensen, and others[55-59] that individuals vary greatly, not only in their response to the antigens commonly used in routine immunization, but in the rate at which the antitoxin disappears from their blood stream. Thus, the Schick test may be positive in many persons who are in reality quite resistant to diphtheria.

For these and other reasons, a simple means of measuring the actual antitoxin content of the circulating blood, as well as for evaluating changes following immunization by natural or artificial processes is of value.

Kasawara and Schick,[60] v. Groer and Kassowitz,[61] Michiels and Schick,[62] Kolmer and Moshage,[63] Zingher,[64] Kellogg,[65, 66] Flood,[67] Glenney and Llewellyn-Jones,[68] Messeloff and Karsh,[69] Fraser,[70] Jensen,[71] and others, have proposed or tested methods of making such antitoxin determination. Most of these involve the mixture of graded quantities of the patient's serum with a previously standardized dose of toxin and then testing for neutralization by injecting 0.1 or 0.2 ml. of the mixtures intradermally into guinea pigs or rabbits. All of these

methods are based on the original work of Römer and his coworkers.[72]

Some of the modifications of the original method overlook the facts that: (1) There is no constant relationship between dilutions of a dose of toxin previously standardized against a certain amount of antitoxin (e.g., the L+ dose) and corresponding dilutions of the amount of antitoxin (say 1 unit) against which that dose was originally standardized. (2) The fraction of a dose of toxin giving an erythematous zone 1 cm. in diameter on intracutaneous injection into guinea pigs or rabbits is not by any means constant, differing greatly from toxin to toxin.

The reasons for these facts are found in the peculiar composition of diphtheria culture filtrates which contain various quantities of substances, other than the toxin itself, supposedly with differing avidities for antitoxin. Much of this difficulty may be eliminated by the use of highly purified toxin.[48]

It must be constantly borne in mind, therefore, that once a dose of toxin of the ordinary sort is adjusted to an arbitrary endpoint against a certain amount of antitoxin, e.g., as in adjusting the L+ or Lr (*vide infra*) dose, any alteration in the absolute amount of toxin thus used causes a disproportionate alteration in the quantitative relationship between toxin and antitoxin which cannot be compensated for by a corresponding arithmetical alteration in the amount of antitoxin. An entirely new adjustment between the two reagents is necessary when the dosage of toxin is altered. The relationships, furthermore, are different for each new lot of toxin.*

### 1. *Principles of the Method*

The method of titration outlined here is based chiefly on those described by Glenney and Llewellyn-Jones,[68] Fraser,[70] and Jensen.[71] The accuracy of the test depends to a large extent on the potency and stability of the toxin used and on the accurate determination of its Lr dose at the dilution level selected for work.

#### a. *The Lr dose*

The Lr dose is, in all respects, analogous to the L+ dose: differing principally in that the end reaction, when 0.2 ml. of a mixture containing 1 ml. of the tested toxin dilution and 1 ml. (1 unit) of antitoxin are injected intradermally, is not the death of the pig, but merely a zone of erythema in the skin about 1 cm. in diameter.† If the toxin

* According to Jensen[71] the departure from the law of multiple proportions is insignificant. According to Glenney and Llewellyn-Jones[68] it is a matter of critical importance, and the experience of the writer strongly supports the latter view.

† Since as many as 40 such reactions can be borne on the skin of a single rabbit without ill effect, the advantage of using rabbits in place of guinea pigs is obvious.

is diluted 1 in 7,500, it is said to have a *potency* of 7,500 Lr although actually only 0.1 ml. (1/75,000 ml. of original toxin) is injected. Any "unknown" serum which, when substituted for the standard antitoxin in such mixtures in the same amount and dilution, accomplishes the same degree of neutralization, obviously is as potent as the standard.

b. *The titration level*

Since, in studies of human immunity, it is sometimes desirable to tests for amounts of antitoxin as small as 1/500 unit per ml., the toxin dose must be so adjusted against a 1/500-unit-per-ml. standard that serum dilutions containing an excess of this amount of antitoxin per ml. will prevent or diminish the toxic effect, while those containing less will permit the development of a zone of erythema due to the unneutralized toxin. This is spoken of as titration at the "1/500th level."

The unknown sera may be mixed in various dilutions with this particular dose of toxin and suitable injections made.

2. *Diluent and Temperatures*

In such high dilutions of toxin its natural tendency to deteriorate spontaneously is greatly increased. It has been found that the reaction of the diluting fluid plays a very important role, and it is recommended that the toxin be diluted in a suitable mixture such as the diluent of Moloney and Taylor.[44]

The *temperature* of *all* fluids in the test should be held as low as possible at all times except during the period of incubation of the toxin-antitoxin mixtures.

3. *Standardization of Toxin at the 1/500th Level*

This is the most difficult step in the entire procedure. Laboratories burdened with routine work may do well to obtain a toxin already standardized at the 1/500th or other desired level. The remaining procedures of the serum antitoxin titrations are very simple.

As toxins vary greatly in potency and combining power, no definite quantitative directions can be given which will cover all cases, but a sample protocol with a hypothetical toxin will serve to illustrate the principles and steps involved. Assume that, by means of the technic outlined below, preliminary titrations in the presence of standard antitoxin diluted so that 1 ml. contains 1 unit, have shown the *potency* of this hypothetical toxin to be 9,680 Lr. For convenience it is so diluted that the potency is 1,000 Lr (1 ml. + 8.68 of diluent). This is labeled

"stock toxin" and keeps well at 4° C. Further preliminary titrations have shown that sufficient toxin to give the desired endpoint of erythema *at the 1/500 level* (i.e., when 1 ml. is mixed with 1 ml. containing 1/500 unit of antitoxin, and 0.2 ml. of the mixture injected) *is present in 1 ml. of some dilution lying between 1 in 40,000 and 1 in 80,000 of the stock.*

*With this information in hand, the procedure is as follows:*

(a) Withdraw 1 ml. of the stock and dilute it to a potency of 1 Lr.

(b) Of this material prepare various dilutions, at intervals of 5, ranging from 1 in 40 to 1 in 80.

(c) Add 1 ml. of each dilution to 1 ml. of standard antitoxin so diluted that each ml. contains 1/500 unit. Mix by gentle agitation.

(d) Incubate the mixtures at 37° C. for 1 hr. and then store overnight in the icebox. Since toxins and antitoxins vary greatly in the rapidity with which they combine, it is necessary to allow ample time for this reaction to occur. Otherwise the mixtures may be too toxic and erroneously low antitoxin titers may be obtained.

(e) The animals may be prepared as directed under "Virulence test" (III, G, 3, c).

(f) Inject 0.2 ml. of each mixture into the closely clipped dorsal or flank skin of a mature rabbit. Guinea pigs may be used for preliminary work if decided savings can be effected thereby, but their skin is very tough.

(h) Readings are best made after 48 hrs. The 0000 clipper may be used 1 hr. before reading.

(i) That dilution of toxin which produces a zone of erythema most nearly 10 mm. in diameter in the presence of antitoxin diluted as indicated may be selected as the proper dose for use in antitoxin titrations of sera containing as little as 1/500 unit per ml.

### 4. *Tests for the Presence of 1 Unit, 0.02 Unit, and 0.002 Unit of Antitoxin in an "Unknown" Serum at the Lr/500 Level*

Suppose, for purposes of this discussion, the best end reaction is found to have resulted from injection of the mixture containing "stock" toxin diluted 1 in 65,000. In the performance of routine tests, all that is necessary is (a) to dilute the stock 1 in 65,000; (b) to 1 ml. amounts of this dilution add 1 ml. amounts of the "unknown serum" dilutions, and (c) to incubate and inject as directed in (d), (e), and (f) above.

The following protocol shows the details of such a test for 3 different antitoxin values in an unknown serum:

| *Tube* | *Patient's Serum* | | | *Toxin Diluted as Already Determined (in This Case 1 in 65,000 of Stock of potency 1,000 Lr.) (ml.)* | *Incubation* | *Amount Injected (ml.)* | *Reading after 48 Hours* |
|---|---|---|---|---|---|---|---|
| | *Dilution* | *Fraction of Unit Tested for* | *Amount in Tube (ml.)* | | | | |
| 1 | 1:500 | 1 unit | 1 | 1 | 1 hour | 0.2 | 2 cm. zone of eryth. |
| 2 | 1:10 | 1/50 | 1 | 1 | at 37° C. | 0.2 | 1.5 cm. zone of eryth. |
| 3 | Undiluted | 1/500 | 1 | 1 | followed | 0.2 | no reaction |
| 4 | Standard (1/500 unit per ml.) | - | 1 | 1 | by 18 hrs. in icebox | 0.2 | 1 cm. zone of eryth. |

Since 1 ml. of undiluted serum (tube 3) completely neutralizes an amount of toxin which gives an erythematous reaction in the presence of 1/500 unit, the serum obviously contains more than 1/500 unit of antitoxin per ml. On the other hand, since being diluted 1 in 10 it fails to neutralize this amount of toxin to the same extent as the control, it must contain less than 0.02 (1/50) unit per ml.

The following protocol illustrates the reactions obtained with a more potent serum:

| *Tube* | *Patient's Serum* | | | *Toxin diluted as Previously Determined (as above) (ml.)* | *Incubation* | *Amount Injected (ml.)* | *Reading after 48 Hours* |
|---|---|---|---|---|---|---|---|
| | *Dilution* | *Fraction of Unit Tested for* | *Amount in Tube (ml.)* | | | | |
| 1 | 1:500 | 1 unit | 1 | 1 | as above | 0.2 | 1 cm. zone of erythema |
| 2 | 1:10 | 1/50 | 1 | 1 | | 0.2 | no reaction |
| 3 | Undiluted | 1/500 | 1 | 1 | | 0.2 | no reaction |
| 4 | Standard (1/500 unit per ml.) | — | 1 | 1 | | 0.2 | 1 cm. zone of eryth. |

Here, when diluted 1 in 500, the serum neutralized, to the same extent as the control, the amount of toxin which, in the presence of 1/500 unit of standard antitoxin, is shown by tube 4 (control) to give the usual endpoint. Obviously then, it must be equivalent to the undiluted standard or contain 1 unit per ml.

By varying the dilutions of the serum, other values are found in a similar manner. For example, the quantity of antitoxin regarded by some authorities[56, 57, 73] as the minimum required to produce a negative Schick reaction (1/100 unit) may be tested for if a dilution of 1 in 5 is included. However, since opinions differ on this point,[74] a protocol is given showing proper dilutions for determining other fractions of a

TABLE OF DILUTIONS OF PATIENT'S SERA, AND UNITS REPRESENTED, IN TITRATION AT THE 1/500TH LEVEL

| *Serum (ml.)* | *Saline (ml.)* | *Dilution* | *Fraction of Unit Tested for by Using 1 ml. of This Dilution with 1 ml. of Toxin Adjusted to Give Lr Endpoint in Presence of 1 ml. of Standard Antitoxin Dilution Containing 1/500 Unit per ml.* |
|---|---|---|---|
| 1.0 | 0.0 | 0 | 1/500 |
| 0.5 | 0.5 | 1 in 2 | 1/250 |
| 0.2 | 0.8 | 1 in 5 | 1/100 |
| 0.1 | 0.9 | 1 in 10 | 1/50 |
| 0.1 | 1.6 | 1 in 16.6 | 1/30 |
| 0.1 | 2.4 | 1 in 25 | 1/20 |
| 0.1 | 4.9 | 1 in 50 | 1/10 |
| 0.1 | 9.9 | 1 in 100 | 1/5 |
| 0.1 | 49.9 | 1 in 500 | 1.0 |

unit. These differences of opinion may result from the use by the various workers of toxins having widely different combining powers, or of insufficient incubation periods. Thus, a toxin containing large amounts of nonspecific combining substances, or low combining rate, would tend to give positive reactions in the presence of minimal antitoxin titers.

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# The Tubercle Bacillus

## (*Mycobacterium tuberculosis*)

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## I. INTRODUCTION

PULMONARY disease can be detected clinically and radiographically but the demonstration of tubercle bacilli in the sputum or body fluids of a patient is the surest method of diagnosing tuberculosis. As other diagnostic methods find increasing employment, a greater need arises for the demonstration of the causative microörganism. The finding of the tubercle bacillus admits of no further uncertainty or equivocation in the diagnosis.

With the advent of mass radiography many patients have been classified as "tuberculosis suspects." The physician is confronted with the problem of deciding first: does this lesion in the lungs represent tuberculosis? and second, if this is tuberculosis, is it "active" or "inactive"? His decision determines whether or not the patient will be sent to a sanatorium or tuberculosis hospital. Unless repeated x-rays show definite changes, it is almost impossible to answer either one or both of the above questions without bacteriological study.

Modern laboratory methods of searching for the tubercle bacillus can greatly assist in the diagnosis of suspected tuberculous pulmonary lesions. The three procedures most widely used are: (1) microscopic search for bacilli, (2) cultural techniques, and (3) animal inoculation. Nearly all types of pathological material can be examined by one or all of these methods.

## II. COLLECTION OF SPECIMENS

### A. SPUTUM

If a patient with symptoms of pulmonary tuberculosis consults a physician and expectorated material is available, the sputum should be

treated and cultured as soon as possible. The patient first needs an explanation of what sputum is, and how to expectorate so that a proper sample can be sent to the laboratory. The clinician should first examine the specimen to determine whether it is really sputum or merely saliva or nasal secretions. Obviously the latter type of material is not satisfactory for culture.

The specimen should be collected in a sterile, widemouth, glass container with a tightly fitted rubber stopper or screw cap. *No preservative* should be added since these substances are frequently inhibitory to growth of tubercle bacilli and many actually render them non-viable.

### B. GASTRIC WASHINGS

Careful study of gastric washings from tuberculous patients whose sputum has given negative results will yield tubercle bacilli in 20 to 25 per cent of cases if cultural methods are employed. Pinner has shown that culture of specimens of sputum and gastric washings that were negative by direct or concentrated smear examination raised the number of positive results by an added 30 to 40 per cent.[1]

The use of gastric lavage is indicated in two types of patients:

1. All patients with roentgenological lesions suggestive of tuberculosis but with sputum negative by other methods of examination.

2. All patients who raise no sputum, or those who swallow their sputum.

The specimen is best taken in the early morning from a fasting stomach. There is some evidence that peristalsis occurs with the first physical efforts and the tubercle bacilli, if present, may be carried away by the emptying of the stomach. Several techniques of securing the specimen are available. A sterile Levine tube passed into one nostril and directed through the esophagus is the technique most widely employed. The patient is asked to drink 50 to 100 ml. of water and then the gastric contents are aspirated with a sterile 50 ml. syringe.

The use of a large French catheter passed through the mouth into the esophagus and then into the stomach is preferred by many European workers. The catheter has a funnel attachment and through this a large amount of sterile distilled water is poured into the stomach. It is stated that this gives a more vigorous washing of the stomach and frees the microörganisms which cling in the rugal folds. The specimen is collected by lowering the funnel to the floor and letting the gastric contents empty into a large sterile flask.

The specimen is sent to the laboratory promptly for culturing since there is some evidence that the tubercle bacilli disappear from the specimen in proportion to the hours the specimen stands. This deleterious effect on the bacilli has been attributed to the acid in the gastric juice and to the enzymes which are present. There is some evidence that neutralization of the specimen offsets this action to some degree. We have been able to culture tubercle bacilli in gastric washings 72 hours after lavage, using the French catheter technique. This is probably due to the large amount of water used in the lavage process (250 ml.) which acts to dilute both the acid and the enzymes present.

Gastric specimens should not be examined microscopically for two reasons:

1. Bacilli are usually scarce and difficult to find.

2. Not uncommonly, non-pathogenic acid-fast bacilli are present in the gastric washings and such microörganisms are not distinguishable under the microscope from pathogenic bacilli having the same tinctorial qualities.

### C. URINE SPECIMENS

Demonstration of tubercle bacilli in the urine is naturally of the greatest importance. It is most important in the search for them to employ methods that yield reliable results.

Smegma bacilli normally inhabit the external genitalia and skin of man. Indistinguishable morphologically and tinctorially from tubercle bacilli, they may occasionally become a troublesome factor in the diagnosis of genitourinary tuberculosis. For this reason dependence upon the ordinary smear preparation is unreliable, and culture, animal inoculation, or both procedures should be carried out. Careful cleansing of the external genitalia and the use of the three-glass tests* will help eliminate the possibility of confusing the smegma bacillus with the tubercle bacillus. The urine collected in the second vessel should be that saved for examination. *Catheterized specimens should be used whenever possible.*

Twenty-four hour specimens are desirable for several reasons:

1. Tubercle bacilli may be eliminated intermittently with the consequence that a single specimen may contain but few or no microorganisms.

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* After the external genitalia have been thoroughly washed urine is collected in three separate sterile vessels.

2. The sediment from a large quantity of urine concentrates the bacilli and facilitates their detection.

Bacilluria has been reported in miliary tuberculosis, in tuberculous meningitis, and in a few patients with pulmonary but without urogenital tuberculosis.

### D. LARYNGEAL SWAB

Laryngeal swabs may be used to search for tubercle bacilli in patients from whom no sputum is obtainable.

The swab is prepared by using a flexible wire or sound about 8 inches in length which has a roughened or hooked end to which a small cotton ball may be attached. The sound is bent into a gentle curve and may be sterilized in a large test tube or in gauze or paper. Before use the cotton is immersed in sterile water and pressed dry with sterile forceps. The operator holds the patients tongue forward with gauze and then with the use of a laryngoscope mirror the swab is passed into the region of the larynx. The patient is instructed to cough, following which the sound is removed. It is then placed in a sterile test tube ready to be submitted to the laboratory.

### E. OTHER BODY FLUIDS

Pleural, pericardial, ascitic, synovial fluids and other body fluids are aspirated using sterile technique. The specimen is placed in a sterile test tube or other suitable container to be submitted to the laboratory. Oxalate bottles may be used to prevent clotting.

### F. PUS AND TISSUES

This type of material may be submitted to the laboratory in sterile test tubes *without preservatives.* Tissues may also be collected in sterile Petri dishes.

### G. FECES

Widemouth, glass containers *without preservative,* such as are used for sputa, may be employed for collecting fecal specimens.

## III. MICROSCOPIC EXAMINATION OF SPECIMENS

While other means are available for detecting tubercle bacilli in pathological material, none is so easy, rapid, or readily applicable as that based on the tinctorial reactions of this bacterium. Microscopic techniques, however, are not sensitive enough to be reliable when small

numbers of bacilli are being sought. It has been estimated that between 10,000 and 100,000 bacilli per ml. of sputum must be present before their detection in smear preparations is likely. In addition, it is extremely difficult, or impossible, to differentiate microscopically non-pathogenic acid-fast microöorganisms from tubercle bacilli.

However, since acid-fast bacilli found in sputum usually are tubercle bacilli, microscopic examination of sputum should be performed since it affords a method of giving the clinician a prompt report.

## A. Technique for Making Smears

1. *Direct Smear.* This is of use chiefly for sputum specimens. The specimen is poured into a Petri dish on a *black background.* Several flecks of *caseous* particles or *thick masses* of the sputum are removed with wooden applicators or wire loops. These are placed on a micro-slide and a smear is made by rubbing the material with another slide. A wire loop or spade may be used to prepare the smear. After the smear has dried in the air, it is passed through a low flame two or three times for fixation. Electrical heating trays are available and serve this purpose well. The use of excessive heating is to be avoided, since the morphology of tubercle bacilli may be altered by high temperature. The specimen is then ready for staining.

2. *Concentrated Smear.* A smear may be made from the concentrated and homogenized specimen. (See techniques of concentration below.) Using a wire loop or a capillary pipette, the liquid material is placed on a slide. Serum or egg albumen may be used as a fixative, since thin smears tend to wash off during the staining procedure. A ring made on the center of the slide with a glass-marking pencil tends to keep the material from washing off. The preparation is heated gently before carrying out the staining process.

*Direct smears are as efficient as concentrated smears if they are well made and especially if careful selection of choice particles is carried out. This selection procedure is a concentration technique in itself.*

## B. Staining Methods

Many staining methods have been devised but the Ziehl-Neelsen technique still remains the most reliable and widely used. Therefore,

while references are given to some alternative techniques, only the Ziehl-Neelsen procedure is given in detail.

1. Ziehl-Neelsen method[2]

*Procedures*

Cover smear with carbolfuchsin stain *
Steam gently 3 to 5 minutes
Wash in tap water
Add acid alcohol † until preparation is colorless (app. 1 minute)
Wash in tap water
Counterstain with methylene blue ‡ five seconds or longer, depending upon the thickness of the smear
Air-dry with gentle heat

*Other counterstains*

Brilliant green and picric acid have been used as counterstains. It is only by trial that one can select the counterstain of preference. It has been noted that counterstaining with picric acid is useful to individuals who are color-blind.

*Brilliant Green* may be used as 1 per cent solution in 0.01% sodium hydroxide.
*Picric acid* may be used in strengths from ½ per cent to saturated aqueous solution (1%).

2. Other methods

Cooper[3] has suggested a modification in staining based on the fact that sodium chloride and several other chemical substances in proper amounts precipitate the dye material from carbolfuchsin. The precipitate is formed at room temperature but disappears at 28 to 30°C. Presuming that such a deposit might occur in the body of the acid-fast bacillus when brought in contact with warm carbolfuschin and sodium chloride, he devised a method employing the addition of 10 per cent sodium chloride to carbolfuchsin. The stain is warmed to 30°C., or above, before it is applied to the smear. After staining, either by steaming or in the incubator, the smear is allowed to cool before being

* *Carbolfuchsin*
Alcohol 95% .......... 100 ml.
Basic fuchsin .......... 3.0 gm.
This makes a saturated solution.
Carbolfuchsin is then prepared from this solution as follows:
Saturated alcoholic solution .......... 10.0 ml.
5% phenol (aq.) .......... 90.0 ml.

† *Acid alcohol* is prepared by adding 3 ml. of concentrated hydrochloric acid to 97 ml. of 95 per cent alcohol.

‡ *Loeffler's alkaline methylene blue*
1% methylene blue in 95% Ethyl alcohol .......... 30 ml.
0.01% aq. solution of KOH .......... 100 ml.
Dilute with distilled water 1:20 before use.

washed and decolorized. In all other points the procedure is like Ziehl-Neelsen staining.

Gabbett [4] has devised a method of combined destaining and counter-staining. This is a rapid technique but has not proved as satisfactory as the Ziehl-Neelsen method.

Steenken [5] has described a 24 hour method of staining in which the slides are placed in staining trays containing modified carbolfuchsin.* The trays are placed in a wire rack and allowed to stand in the incubator in a humidor overnight. The tray described by Steenken has the advantage of not allowing cross-contamination from positive smears to negative ones. The latter consideration is more theoretical than practical when dealing with smears made from sputum but should be considered when cultures or thin specimens are being stained.

Muller and Chermock [6] have described a rapid staining method which employs the use of a detergent.†

Fluorescent microscopy in the hands of some workers has been reported to yield good results. It has the advantage of enabling the microscopist to examine smears rapidly, using low powered objectives. However, the danger of false-positives because of fluorescent particles other than tubercle bacilli, has been pointed out by many workers.[7]

The examination is performed in a dark room. The microscope is equipped with an ultra-violet lamp, an aluminized mirror, and a yellow ocular filter. Magnification with a 20 X ocular and an 8 mm. objective is desirable. The well stained smear shows fluorescent bacilli against a dark background. The technique has been best described by Richards and Miller,[8] and Richards and Kline.[9] The staining solutions which have proved to be most useful have been reported by Thompson.[10]

## C. Recording of Findings

There is a difference of opinion as to the proper interpretation of the number of bacilli which are found by the microscopist. Some laboratories still prefer the use of the Gaffky scale [11] for reporting bacilli. Gaffky assigned an arbitrary evaluation to numerals so that each numeral represented an average number of bacilli found in an oil immersion field. The present Committee of Evaluation on Laboratory Procedures of the American Trudeau Society [12] condemns this scale as being unduly cumbersome and so detailed as to be misleading. There-

* Modified carbolfuchsin (Cooper): To 100 ml. Ziehl-Neelsen carbolfuchsin add 0.5 ml. 10 per cent sodium chloride.

† One drop of tergitol No. 7 to each 25–40 ml. of carbolfuchsin.

fore, they recommend a simplified method of reporting, referring to the number of bacilli found as follows:

| | | |
|---|---|---|
| Numerous | 3+ | (10 or more acid-fast bacilli in most oil-immersion fields) |
| Few | 2+ | (10 or more acid-fast bacilli in entire smear) |
| Rare | 1+ | (3–9 acid-fast bacilli in entire smear) |

1 or 2 bacilli to be so recorded

The time of the examination of each slide, and more important the amount of each slide examined, gives a more reliable index as to the number of organisms present. It is, therefore, recommended that each smear have the same number of lines examined, either three horizontal lines or 9 vertical lines (3 x 1 slide), regardless of how long this procedure takes. This conduces to uniformity of reporting since some individuals may be able to examine a given area of smear in a few minutes whereas another microscopist may require a much longer time for the same area. Using this method of examination the number of bacilli found are reported by our laboratory as follows:

| | | |
|---|---|---|
| Innumerable | = | 100 or more bacilli found |
| Many | = | 50 to 100 bacilli found |
| Few | = | 10 to 50 bacilli found |
| Rare | = | 5 to 10 bacilli found |

1–4 acid-fast bacilli found are so recorded

This method is easily adaptable to evaluation studies on microscopic material.

Clinically, the finding of acid-fast bacilli on a smear is more important than the number present. Before the final diagnosis is made the worker will probably want recourse to culture or animal inoculation.

## IV. CULTURAL METHODS FOR THE DEMONSTRATION OF TUBERCLE BACILLI IN PATHOLOGICAL MATERIAL

With the development of relatively specific and fairly sensitive culture media, small numbers of tubercle bacilli can be detected in pathological material by methods of cultivation. The steps in the preparation of specimens for cultivation are: (1) digestion and decontamination (2) homogenization and (3) concentration. Several acceptable methods of cultivation are listed below.

### A. SODIUM HYDROXIDE METHOD (Petroff) [18]

Equal parts of 3 or 4 per cent sodium hydroxide are added to the sputum. The mixture is shaken by hand or in a shaking machine for

15 minutes or until complete homogenization is secured. Paint-conditioning machines have been adapted for this purpose. After homogenization is complete, the specimen is centrifugalized at 3,000 r.p.m. for 15 minutes. The supernatant fluid is poured off and one drop of phenol red indicator solution added to the sediment. Enough normal hydrochloric acid is then added for neutralization. The concentrated material can then be seeded on tubes of a reliable culture medium using a capillary pipette, or it can be inoculated into animals. This method of cultivation is serviceable for nearly all types of pathological material especially specimens such as sputum, pus, tissues, and thick tenacious material.

### B. HYDROCHLORIC ACID CONCENTRATION (McNabb)[14]

An equal volume of 3 per cent hydrochloric acid containing brom cresol purple as an indicator is added to the specimen. The mixture is then shaken in a paint shaker for 15 minutes. The specimen is then neutralized using 3 per cent sodium hydroxide. The specimen is centrifugalized at 3,000 r.p.m. for 15 to 30 minutes. The supernatant is decanted and the sediment is inoculated onto culture media using a capillary pipette with a rubber bulb.

Five per cent sulfuric acid may be used in place of 3 per cent hydrochloric acid. The acid concentration methods are particularly useful for thin, clear body fluids where strong digesting agents are not required.

### C. ALUM FLOCCULATION METHOD (Hanks)[15]

Equal volumes of specimen and 4 per cent sodium hydroxide which contains 0.2 per cent potassium alum and 0.002 per cent thymol blue are mixed. After digestion add 2.5 N hydrochloric acid, drop by drop, until the color denotes neutrality. Shake the specimen for 30 seconds or until flocculation occurs. If flocculation does not occur in 5 minutes, add 0.2 ml. of 1 per cent ferric chloride and shake again. The mixture is centrifugalized for 10 minutes at 3,000 r.p.m. The supernatant fluid is decanted and the sediment is ready for cultivation or animal inoculation. This technique is useful because many tubercle bacilli which ordinarily might not be in the sediment are carried down by the flocculation. It is most desirable for pleural fluid and urine specimens.

### D. TRISODIUM PHOSPHATE CONCENTRATION METHOD (CORPER & STONER)[16]

The specimen is added to 10 per cent trisodium phosphate (23% $Na_3PO_4 \cdot 12\ H_2O$) in equal volumes. After homogenization is com-

plete the specimen is centrifugalized at 3,000 r.p.m. The supernatant fluid is decanted and the sediment is washed with 10 ml. of sterile physiological saline. The mixture is recentrifugalized, the supernatant poured off once more, and the sediment is ready for planting on culture media or for animal inoculation. Because trisodium phosphate is relatively nontoxic to tubercle bacilli this method is useful for specimens which are shipped through the mail. The digestant may be added to the collecting container and digestion is usually complete when the specimen arrives in the laboratory.

## E. INCUBATION AND INTERPRETATION

The culture tubes are incubated at 37°C. and are examined at weekly intervals for a total of 6 to 8 weeks. From the color and morphology of a colony the trained bacteriologist can usually decide whether it is formed by tubercle bacilli or non-pathogenic acid-fast bacteria. Smears should be made of all cultures to determine acid-fastness.

On a complex solid egg medium such as the Jensen modification of Lowenstein's medium[17] (¾ per cent glycerol) human, bovine, and avian strains will grow with certain colony characteristics which assist the bacteriologist in determining their type.

Colonies of human tubercle bacilli generally appear in 12 to 25 days and, because of their luxuriant growth, are termed "eugonic." They are dry, friable, somewhat rough and, after a few weeks, generally measure from 8 to 12 mm. in diameter. They assume a "cabbage" appearance and are easily detached from the surface of the medium. They emulsify with difficulty.

Colonies of the bovine type do not grow as rapidly as those of the human type. They usually appear in 25 to 40 days. They are tiny, pale, smooth, pyramidal colonies. Their growth characteristics have been termed "dysgonic." They adhere to the surface of the medium and emulsify readily.

Avian type colonies generally appear in two or three weeks. They are smooth and hemispherical and often have a faint yellow or grey pigment. They are somewhat larger than bovine type colonies.

Colonies of acid-fast saprophytes appear in a few days and are usually chromogenic. Their appearance differs from that of the pathogenic types in that they are soft, creamy, and usually smooth. Most saprophytic strains grow well at room temperature whereas tubercle bacilli require 37–38°C. for optimal growth.

Although some workers will not rely upon cultural characteristics to differentiate types, it is usually because they are not fully conversant with these differences. With a modest amount of training one may tentatively identify types of acid-fast bacilli by their colony appearance on Lowenstein-Jensen medium. *Proper animal studies to confirm the cultural diagnosis should always be performed.*

## V. CULTURE MEDIA FOR THE ISOLATION OF TUBERCLE BACILLI FROM PATHOLOGICAL MATERIAL

Reliable cultural results depend on attention to certain details of technique, as well as upon the use of the best available medium. It is important in this connection that:

1. The glassware used should be well cleaned and sterile. Hard glass such as Pyrex and Jena are preferable to soft glass, since soft glass tends to give off alkali during sterilization.

2. The chemicals and nutrient material should be pure and fresh.

3. The heating necessary for coagulation and sterilization should be gentle to prevent the release of substances which may inhibit growth of microörganisms.[18]

4. After inspissation the medium should be stored carefully in well stoppered tubes to prevent evaporation.*

5. The culture medium should support the growth of small numbers of bacilli; should promote rapid growth and should permit easy identification of typical colonies. The medium should also be capable of inhibiting the growth of contaminating microörganisms.

### A. PREPARATION OF GLASSWARE

Frequently new glassware contains traces of alkali and often contains spores of various bacteria. New glassware, therefore, should be allowed to stand overnight in a weak solution of hydrochloric acid (2 per cent). The glassware is then washed in running water and rinsed twice in distilled water. After this, it is allowed to drain and dry at room temperature. Old glassware, particularly test tubes in which cultures remain, should be sterilized in an autoclave. The bulk of the old media is removed and the glassware is boiled in soapy water under a hood for 30 minutes. This glassware is then ready for cleaning with

* Paraffined cotton plugs and screw cap tubes are equally desirable.

the above mentioned hydrochloric acid technique or it may be cleaned as follows:

1. The glassware is placed in a cleaning solution (prepared by dissolving 50 gm. of technical grade sodium dichromate in 400 ml. water and adding one liter of concentrated sulfuric acid). After removal from this solution, the glassware is thoroughly rinsed in tap water and then rinsed again in distilled water to remove all traces of the cleaning solution.

2. After the tubes are dried, non-absorbent cotton plugs are placed in the mouth of the tubes to a depth of about 2 cm.

3. After plugging, the tubes are placed in a hot-air sterilizer where they are maintained for a period of 1 hour at 170°C. This is usually sufficient to destroy all bacteria. Petri dishes and pipettes may be wrapped in paper or placed in metal containers prior to sterilization.

Sterilization by autoclave may be used for material which does not rust. Material in closely packed baskets should be kept at 15 lbs. pressure and 121° C. for 20 minutes. When tubes containing liquid are autoclaved, care must be taken to prevent the blowing out of the cotton plugs by rapid reduction of pressure.

## B. CULTURE MEDIA

The choice of a culture medium to be employed for isolation of tubercle bacilli from pathological material is usually decided by personal preference. There are now, however, enough evaluation studies to indicate that certain media are more desirable than others for primary isolations. In general liquid media are not suitable because one cannot determine whether visible turbidity is due to tubercle bacilli or non-pathogenic acid-fast bacilli. Even smears of these cultures are of little help because morphologically one cannot differentiate reliably between tubercle bacilli and saprophytes. It is therefore recommended that a solid egg medium be employed for primary isolations. The liquid media are reserved for transfers of pure cultures and for research purposes.

In the experience of the Tuberculosis Evaluation Laboratory of the U. S. Public Health Service the Jensen modification of Lowenstein's medium is at present the most efficient culture medium for routine diagnostic use. It has the advantage of supporting the growth of human, bovine, and avian strains and the trained bacteriologist can differentiate, with a fair degree of certainty, non-pathogenic from pathogenic acid-fast bacilli.

FIGURE 1—Photograph demonstrates the use of the capillary pipette with a rubber bulb. The inoculum is spread evenly over the surface of the slant by employing a serpentine motion.

1. *Modified Lowenstein's Medium* [19]

| | |
|---|---|
| Salt Solution: | |
| Monopotassium phosphate | 2.4 gm. |
| Magnesium sulfate | 0.24 gm. |
| Magnesium citrate | 0.6 gm. |
| Asparagine | 3.6 gm. |
| Glycerol | 12 ml. |
| Distilled water | 600 ml. |
| Potato flour | 30 gm. |
| Homogenized whole eggs | 1,000 ml. |
| Malachite green, 2% aqueous solution | 20 ml. |

The salt solution is heated in a flask in a 56°C. water bath for 2 hours, or until all of the salts are dissolved. Thirty gm. of potato flour are added to the flask, and the mixture is boiled, with continual shaking or stirring, in a boiling water bath, until the potato flour assumes a "cooked" appearance, usually about 30 minutes. The flask is then placed in a 56°C. water bath for 1 hour, during which time the eggs may be prepared.

Fresh country eggs, not more than 1 week old, are employed, and are cleaned by vigorous scrubbing in a solution of approximately 5 per cent soap and soda solution. They are then left in the soap and soda solution for 30 minutes, after which time they are placed in running cold water, until the water becomes perfectly clear. They are broken into a sterile flask, homogenized completely by shaking, and filtered through sterile gauze.

One liter of homogenized whole eggs is added to 1 flask of the potato flour-salt solution, and to this are added 20 ml. of malachite green solution. After thorough mixing, the medium is tubed by means of a sterile aspirator bottle with a funnel and bell attachment, or similar tubing device. Between 5 and 6 ml. are delivered into 150 mm. Pyrex test tubes, and these tubes are solidified by inspissation at 85°C. for 40 minutes. The cotton stoppers are then trimmed and paraffined, to prevent evaporation.

It is advisable to keep the medium stored in a refrigerator until it is used. The medium should be used as soon as possible after preparation and should be discarded after 4 weeks since it loses sensitivity after that time.

2. *Trudeau Committee Egg-Yolk Potato Flour Medium* [20]

| | |
|---|---|
| Egg Yolk | 500 ml. |
| Potato flour water (2% glycerol) | 500 ml. |

a. The potato flour water is made by adding 20 gm. of potato flour to 500 ml. of 2 vol. per cent glycerol water in a flask or beaker. Heat to boiling with constant stirring. Cool to 50° C.
b. Egg Yolk: Fresh hens' eggs are carefully cleansed with wet gauze, rinsed in alcohol, and flamed. The egg white and yolks are separated. A proportion of one whole egg to eleven egg yolks is used and 500 ml. of this combination is prepared.
c. The 500 ml. of egg yolks are poured into the 500 ml. of potato flour water and to this is added 20 ml. of 1 per cent malachite green solution in 50 per cent alcohol. All ingredients are thoroughly mixed and tubed.
d. The medium is coagulated in a slanted position and sterilized by a single one hour stay in an inspissator heated to 90°C.* The tubes are incubated for 48 hours; contaminations are rare if aseptic technique is maintained throughout the procedure. An egg-yolk medium prepared as indicated above will be definitely acid in reaction, with pH in the neighborhood of 6.5. If the tubes contain too much water of condensation the excess should be removed.

### 3. *Liquid Media for Subcultures and Research*

a. ***Basal Medium*** (Dubos) [21]

| | | |
|---|---|---|
| $KH_2PO_4$ | 1.0 gm. | |
| $Na_2HPO_4$ | 6.3 gm. | heat in 100 ml. distilled water to dissolve |
| Asparagine | 2.0 gm. | |
| *Add:* | | |
| Distilled water | 850 ml. | |
| Enzymatic digest of casein | 2 gm. | (40 ml. of a 5% autoclaved solution in distilled water) |
| Ferric ammonium citrate | 0.05 gm. | (1 ml. of a 5% stock solution in distilled water) |
| $MgSO_4 \cdot 7\ H_2O$ | 0.01 gm. | (1 ml. of a 1% stock solution in distilled water) |
| $CaCl_2$ | 0.0005 gm. | (1 ml. of a 0.05% stock solution in distilled water) |
| $ZnSO_4$ | 0.0001 gm. | (1 ml. of a 0.01% stock solution in distilled water) |
| $CuSO_4$ | 0.0001 gm. | (1 ml. of a 0.01% stock solution in distilled water) |

Adjust *p*H to 6.5–6.8

Distribute medium in flasks or Blake Bottles as described below—or in test tubes 25 mm. in diameter (5 ml. per tube).

* We employ 85° C. for 40 minutes.

b. *Tween-Albumin Medium*
(for submerged diffuse growth)

| | | |
|---|---|---|
| Basal medium | 900 ml. | |
| Tween 80 | 0.5 ml. | (5 ml. of a 10% stock solution in distilled water; keep this solution in the refrigerator and do not use after one month) |
| Autoclave | | |
| *Add:* | | |
| Bovine Albumin (serum Fraction V) | 5 gm. | (100 ml. of a 5% stock solution in 0.85% saline neutralized with NaOH and sterilized by filtration) |
| Glucose | 5 gm. | (10 ml. of a 50% stock solution in distilled water sterilized by autoclaving) |

(Cultures of Mycobacteria in this medium are said to be "fully grown" when macroscopic "creeping" of clumps can be seen on the sides of the tube or flask; at this time each ml. of medium will contain approximately 0.2 mg. dry weight of bacilli.) The albumin solution should be neutralized and heated at 56° C. for 30 minutes to prevent hydrolysis of the Tween.

c. *Oleic Acid-Albumin Medium* (Dubos) [21]

"IMPORTANT: In order to facilitate isolation from material contaminated with other bacteria an attempt has been made to render the medium as selective as possible by limiting the variety and concentration of nutrients in the basal medium. Thus glucose and glycerine are entirely omitted. It is advisable to reduce asparagine from 2 gm. to 1 gm., Ferric ammonium citrate from 0.05 gm. to 0.005 gm. Satisfactory growth of tubercle bacilli is obtained at pH 6.5, a reaction which depresses the growth of many contaminants.

"Substitution of oleic acid for Tween 80 causes the growth to be granular instead of diffuse; this may facilitate detection of large clumps of acid-fast bacilli under the low power lens."

Basal medium 900 ml.
Autoclave
*Add:*
100 ml. of the following preparation of oleic acid-albumin complex:

(1) Dissolve 0.12 ml. of oleic acid (0.1 gm.) in 10 ml. of N/20 NaOH by shaking with a rotary motion in a small flask.

(2) Add 5 ml. of this solution to 95 ml. of a neutral 5 per cent solution of Fraction V albumin in 0.85 per cent saline.

(3) Sterilize by filtration through bacteriological filters, preferably glass or porcelain filters.

d. *Egg Yolk-Water* (Besredka) [22]

| | |
|---|---|
| Egg yolks | 10 |
| Redistilled water | 3,500 ml. |
| Sodium hydroxide, 1% | 60–70 ml. |
| Malachite green, 2% (aq.) | 5 ml. per 1,000 ml. medium |

Add 10 egg yellows to 500 ml. of redistilled water in a 2 liter Erlenmeyer flask. Shake well. Filter through gauze into a 1 liter graduate cylinder. Measure the volume. Add 60 to 70 ml. of 1 per cent sodium hydroxide (pH 9). Make up to 3.5 liters with redistilled water. Five ml. of 1–10,000 malachite green are added to each liter of egg media (2 per cent malachite green). Fill test tubes with 6 ml. Autoclave 20 minutes at 110° C. The pH should be 8.1 after autoclaving. This medium supports submerged growth of tubercle bacilli and is particularly useful for isolating bovine strains of tubercle bacilli.

## VI. ANIMAL INOCULATION

Animal inoculation is sometimes necessary to differentiate between pathogenic and non-pathogenic acid-fast bacilli. Also, when dealing with specimens that are repeatedly contaminated on culture, it is sometimes possible to recover tubercle bacilli following animal inoculation.

The guinea pig is most widely used. Subcutaneous inoculation in the groin is preferred to other routes of injection. The concentrated material prepared as for culturing is used for animal inoculation. One ml. of concentrate is inoculated with a Luer-Lok or tuberculin syringe and a 20 to 21 gauge needle.

A tuberculin test with 5 mg. of old tuberculin is performed before inoculation and in three or four weeks after inoculation. The animal is sacrificed if the test is positive. If the test is negative the animal is allowed to live for six weeks at which time it is autopsied. If viable tubercle bacilli were present in the specimen, one will see regional lymph node involvement with spread usually to the spleen, liver, and lungs. Saprophytes may produce a local abscess and regional lymphadenitis.

Impression smears should be made of all organs showing evidence of gross pathology. These should be stained by the Ziehl-Neelsen technique and examined microscopically for the presence of tubercle bacilli. Cultures should be made from organs of those positive guinea pigs which were inoculated with specimens having negative cultures. All guinea pigs injected with specimens and not cultures, are inoculated subcutaneously in the right groin with 1 ml. of the suspension prepared according to the procedure previously outlined. They are killed and autopsied at the end of 6 weeks.

## VII. TYPING OF TUBERCLE BACILLI

Animals used for typing should be young and healthy. Prior to use they should be tested with 5 mg. of Old Tuberculin and only negative reactors should be used. If possible the animals which are

injected with pathological material or cultures should be kept in individual cages.

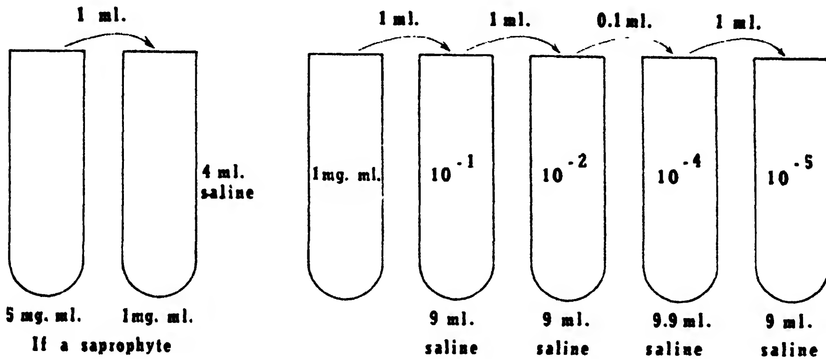
### A. Preparation of Cultures for Typing by Animal Injection

1. Place a sterile watch glass in a sterile Petri dish with sterile forceps. (Or have watch glasses sterilized in Petri dishes.)

2. Weigh the watch glass on an analytical balance, taking care not to contaminate it.

3. With a wire loop, sciape some of the colonies from the culture and place in the watch glass.

4. Weigh the watch glass with the culture, taking care to avoid contamination.




5. Mark 5 test tubes with the culture number and the dilutions: 1 mg. per ml., $10^{-1}$ mg., $10^{-2}$ mg., $10^{-3}$ mg. and $10^{-4}$ mg.

6. Measure sterile physiological saline into the first tube so that when mixed with the weighted culture 1 ml. will contain 1 mg. If the culture is a saprophyte, make the dilution so that 1 ml. will contain 5 mg. of bacteria.

7. Using the sterile loop, scrape the colonies from the watch glass into a sterile mortar; add one drop of saline from the tube marked 1 mg./ml. and grind the culture thoroughly; add another drop of saline; grind, continue adding saline a few drops at the time, and grinding the bacteria in it. When a homogeneous mixture is obtained add the remaining saline. Using a sterile Pasteur pipette, transfer the mixture to the test tube. From this tube the other dilutions are made.

8. Make dilutions as follows: $10^{-1}$ mg./ml., $10^{-2}$ mg./ml. $10^{-4}$ mg./ml. and $10^{-5}$ mg./ml.

Use a graduated pipette to transfer indicated amounts to next tube. Do not mix with this pipette. Take a new pipette and mix solution by drawing liquid up and down. Use this pipette to transfer desired amount. Discard pipette and proceed with the next dilution in a similar manner.

### B. Typing of Cultures by Animal Inoculation

If the colony characteristics suggest:

1. Dysgonic human or bovine type, inoculate 1 ml. of $10^{-4}$ mg. into a guinea pig intraperitoneally or 1 ml. of $10^{-1}$ mg. subcutaneously in the right groin and 1 ml. of $10^{-1}$ mg. into the marginal vein of the ear of a rabbit.

2. Avian type, inoculate 1 ml. of 1 mg. into one chicken intraperitoneally and into another intramuscularly deep in the pectoral region or intravenously in the wing.

3. Saprophyte, inoculate 1 ml. of 5 mg. into a guinea pig subcutaneously in the right groin.

Rabbits are killed after 3 months.

Guinea pigs are killed after 2 months (intraperitoneal inoculations) or after 6 weeks (subcutaneous inoculations).

Chickens are killed after 3 months.

### C. Autopsy Findings

1. *Rabbits:* Those inoculated with *bovine* tubercle bacilli usually die in 3 weeks with a generalized tuberculosis. Those receiving *human* tubercle bacilli survive and are sacrified at the end of 3 months. There are usually a few small tubercles in the lungs and kidneys *only*. *Avian* tubercle bacilli inoculated intravenously into rabbits give rise to the Yersin type of disease without the formation of macroscopic tubercles.

2. *Guinea pigs:* A skin test with 5 mg. O. T. may be performed at 4 weeks. If positive (at least 5 mm. of induration in 48 hours), the animal can be killed and autopsied. Human and bovine bacilli both give rise to a progressive, usually fatal, disease in guinea pigs with lesions in regional lymph nodes, abdominal nodes, spleen, liver, and lungs. Saprophytes (acid-fast) do not progress beyond the site of inoculation and regional lymph nodes. Avian bacilli often produce no lesions at all, or only a local abscess.

3. *Chickens:* After injection with avian tubercle bacilli chickens die in a variable time, usually greatly emaciated. The disease is marked in the spleen, liver, kidneys, and mesenteric lymph nodes. After inocu-

lation with mammalian tubercle bacilli, chickens respond, usually with no recognizable lesions but occasionally an abscess forms at the site of inoculation.

## VIII. DISCUSSION

From the point of view of the laboratory a given specimen should never be called negative for tubercle bacilli unless it has been examined by culture or animal inoculation or both. Carefully performed cultures are as efficient as animal inoculations and have the advantages of economy and rapidity.

Modern methods of searching for the tubercle bacillus can greatly assist in making the diagnosis of tuberculosis. Indeed there are some clinicians who would never make the diagnosis of active tuberculosis unless tubercle bacilli can be demonstrated by the laboratory. As with all other laboratory investigations, the results must always be correlated with the clinical findings. However, the finding of tubercle bacilli in a patient's sputum or body fluids unequivocally establishes the diagnosis of tuberculosis.

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# Salmonella and Shigella

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# I. INTRODUCTION

PROCEDURES are outlined for the examination of specimens for evidence of infection with Salmonella * and Shigella and for the detection of carriers. The methods have been selected in so far as practicable on the basis of comparative studies and in the light of helpful criticisms and suggestions from many laboratories in the United States and Canada. They are essentially those in use in the Division of Laboratories and Research of the New York State Department of Health [1a] and in the approved local laboratories throughout the state.[2]

The identification of individual Salmonella species other than *Salmonella typhosa* is not included since this procedure is specialized and can be efficiently undertaken only in a laboratory where a relatively large number of strains are examined. Consult Kauffmann,[3-6] Kauffmann and Edwards,[7] and Edwards and Bruner [8] for information on this subject.

The designation *Shigella paradysenteriae* as used in this chapter includes all the types or races that have been discussed by Boyd,[9] Weil,[10, 11] and Wheeler.[12, 13] Since a standard classification has not been generally accepted, recommendation of methods for their differentiation is impracticable.

The differentiation of types of *Salmonella typhosa* with bacteriophage as outlined by Craigie and Felix [14] provides information that may aid the epidemiologist in establishing the source of infection in typhoid fever. A national reference center for bacteriophage typing of Salmonella under the direction of Dr. P. R. Edwards has been established at the Communicable Disease Center of the United States Public Health Service at Chamblee, Ga. The bacteriophage preparations and standard type strains of *Salmonella typhosa* were supplied by Dr. A. Felix, Central Enteric Reference Laboratory, London. These preparations have been redistributed to a few laboratories in the United States, a list of which can be obtained from Dr. Edwards.

The failure of a microörganism to fulfil the criteria established for the identification of recognized pathogenic species is not sufficient reason for regarding it as of no diagnostic significance. Aberrant strains

* The nomenclature adopted in *Bergey's Manual* has been employed to conform with the editorial policy of the American Public Health Association.

of certain species are sometimes encountered, and hitherto unrecognized species may be found to incite enteric disease.

## II. COLLECTION OF SPECIMENS

### A. SPECIMENS TO AID IN DIAGNOSIS

Evidence of infection with bacillary incitants of enteric disease may be demonstrated by examination of:

1. Blood for Salmonella during the early stages of the illness.

2. Feces for Salmonella and Shigella at any stage of the illness and often during convalescence. The study of a series of fecal specimens is important if bacillary incitants of enteric disease are not isolated and clinical and epidemiologic data suggest their presence. They may be found in extremely small numbers or rendered nonviable by bacteriophage. Rectal swabs have been recommended by Hardy and his associates [15, 16] for the collection of specimens to be examined for dysentery bacilli, especially from inmates of institutions and hospitalized patients. Shaughnessy, Friewer, and Snyder [17] reported that fecal specimens collected after the administration of a cathartic were more reliable than rectal swabs for demonstrating the presence of *Salmonella typhosa* and other Salmonella, especially in the case of intermittent carriers of these microörganisms.

3. Urine for Salmonella and possibly certain types of Shigella when infections are localized in the genitourinary tract.

4. Blood serum for agglutinative properties for *Salmonella typhosa* after the first week or 10 days of illness. Agglutination tests with other Salmonella are usually not practicable because of the close antigenic relation of individual types within the several groups.

The agglutination test with dysentery bacilli has been found of little value as an aid in diagnosis during the acute stages of the illness.

### B. SPECIMENS FOR CARRIER DETERMINATION

Carriers of bacillary incitants of enteric disease may be determined by examination of:

1. Feces for Salmonella and Shigella.

2. Urine and duodenal drainage for Salmonella.

The examination of duodenal drainage is recommended (1) in case typhoid bacilli or other Salmonella are not found in feces or urine from an individual whom serologic tests or epidemiologic data indicate to be

a carrier; (2) to confirm the assumption that the infection is localized in the gall-bladder when cholecystectomy is contemplated; and (3) for release of a carrier after cholecystectomy.

Bacteriologic examination of gall-bladders and their contents is recommended regardless of the reasons for their removal.

3. Blood serum for agglutinative properties for *Salmonella typhosa.*

Blood serum from typhoid carriers may be expected to agglutinate *Salmonella typhosa* in a floccular manner. This type of agglutination, however, may be demonstrated for years in the blood of individuals who have recovered from typhoid fever and who are not carriers, and also in that of persons who have had vaccine. Vi agglutination occurs in the serum of from 80 to 90 per cent of typhoid carriers and from 5 to 10 per cent of individuals in whom no evidence of infection can be demonstrated, following typhoid vaccination,[18, 19] and in malaria.[20] Vi agglutination tests may, therefore, aid but are not infallible in the detection or exclusion of the carrier condition.

## C. OUTFITS FOR THE SUBMISSION OF SPECIMENS

When cultural examinations can be undertaken within a short time (1 or 2 hours) after collection, the only requirement for the outfit is that it shall be a tightly covered container that can be safely transported and destroyed or sterilized after use. Instructions for collecting the specimens and history forms for recording pertinent data regarding the patient should also be provided. When fecal and urine specimens are to be sent by mail, outfits should conform to the postal laws and regulations and should contain a diluent that provides conditions favorable to the viability of the pathogenic microörganisms.

1. *Feces*: Buffered 30 per cent glycerol solution * is recommended, in 8–10 ml. amounts in tightly stoppered or sealed glass tubes or jars with

* *Buffered Glycerol-Sodium Chloride Solution.*

| | |
|---|---|
| Sodium chloride | 4.2 gm. |
| Water, distilled, to make | 700 ml. |
| Glycerol | 300 ml. |
| Dipotassium phosphate, anhydrous | 3.1 gm. |
| Monopotassium phosphate, anhydrous | 1 gm. |

a. Dissolve the sodium chloride in part of the water and make up to 700 ml. Add glycerol and phosphates. The phosphate concentration is about 0.025 molar.

b. Determine the reaction which should be pH 7.2. If necessary, adjust the pH with *N* 1 HCl or *N* 1 NaOH.

c. Filter through paper and cotton.

d. Dispense in 8–10 ml. amounts in the tubes or jars used in the outfits to be distributed for collection of fecal specimens.

e. Cap loosely and autoclave for 40 minutes.

f. After removing from the autoclave, allow the jars or tubes to cool to 50°–60° C., fasten the caps or stoppers securely, and leave standing overnight in an inverted position. Discard leaky jars or tubes.

a capacity of 20–30 ml. About 1 gm. of feces should be added to this solution. A paper or wooden spoon or swab should be provided, which, after collection of feces, should be burned.

2. *Urine*: Urine specimens should be collected, preferably with a catheter, but if the examination is undertaken promptly or the specimen is combined with an equal volume of buffered 30 per cent glycerol, a voided specimen should be satisfactory.

3. *Duodenal drainage*: Outfits for the submission of duodenal drainage should provide a sterile, tightly stoppered or sealed glass container with a capacity of 10–15 ml. Specimens should be examined promptly after collection. Criteria of the suitability of duodenal drainage for bacteriologic examination are given by Forsbeck and Hollon.[21]

4. *Clotted blood*: Clotted blood specimens are recommended, since they provide material for both cultural and serologic tests. A sterile, tightly stoppered glass container with a capacity of 10–15 ml. should be provided.

5. *Gall-bladders*: Special arrangements may be necessary for an appropriate container when gall-bladders are submitted by mail. A gall-bladder should not be placed in fixative until after the contents and a portion of the wall have been removed for bacteriologic examination. Unless cultural examination is undertaken promptly, the specimen should be preserved by immersion in buffered 30 per cent glycerol solution.

## III. CULTURE MEDIA AND REAGENTS

### A. Bacteriologic Culture Media

The formulae for the preparation of culture media employed in these procedures will be found in the section on Culture Media. Many of these media can be obtained in dehydrated form.

For demonstration of acid production from single carbohydrates, any sugar-free base, such as peptone solution (C. M. No. 1), extract broth (C. M. No. 4), or agar (C. M. No. 4a) containing a satisfactory indicator can be employed. An extract-broth base (C. M. No. 4) is recommended for determination of gas production. These media should always be tested before use by inoculating both with microörganisms that ferment the carbohydrates present and others that fail to do so.

Russell's double-sugar agar (C. M. No. 18) and other media for determining gas production should not be stored in a refrigerator where they may absorb air.[22]

Appropriate tests should be made with each new lot of medium prepared to insure its suitability.

### B. Turbidity Standards

The turbidity of suspensions of bacillary incitants of enteric disease should approximate that of barium sulfate standard No. 3 [23] or Pyrex glass standard No. 2.5.[1b] Turbidity can also be determined by means of a silica standard or a photoelectric colorimeter. The Luximeter reading of such a suspension is 74, and the Klett-Summerson 250.

### C. Stains

Directions for staining by Gram's method are given on page 87. If a flagella stain is desired, Gray's method [24] is recommended.

### D. Agglutinating Sera

The following sera should be available:

*Salmonella typhosa for somatic and flagellar antigens*
Salmonella H-d (for the flagellar antigen of *Salmonella typhosa*)
Salmonella Vi (for the Vi antigen of *Salmonella typhosa*)
Multivalent Salmonella (one or more that will agglutinate all the common types of this genus)
Multivalent *Shigella paradysenteriae* (one or more for the mannitol-fermenting dysentery bacilli)
*Shigella dysenteriae*
*Shigella ambigua*
*Shigella sonnei*
*Shigella alkalescens*

Sera for other species may also be helpful: non-mannitol-fermenting Shigella,[25, 26] *Bacterium enterocoliticum* [27, 28] and other Gram-negative bacilli that have not been named.[29]

#### 1. *Preparation*

Agglutinating sera can usually be satisfactorily produced in rabbits by any one of a number of methods.[1c]

The H-d antigen of *Salmonella typhosa* can be demonstrated by floccular agglutination in an unabsorbed serum produced with a motile strain of this species. The reaction may be more clear-cut, however, if the somatic or granular agglutinative properties are absorbed. The

following procedure is recommended: Heat for 2 hours at 100° C. in streaming steam, a suspension in salt solution* of a culture of *Salmonella typhosa* grown on infusion agar (C. M. No. 5a) for 20–24 hours. Suspend the growth from agar in a 1 liter Roux bottle, or its equivalent, in 15–20 ml. of salt solution. Combine equal parts of the heated suspension and a 1:5 dilution of serum, and incubate at 48°–52° C. for 18–24 hours, shaking occasionally. Refrigerate for 18–48 hours and centrifuge. Decant the supernate and add 0.01 per cent merthiolate. Filter through infusorial earth in a Büchner funnel and through a filter candle. Test for sterility. This serum is satisfactory if it agglutinates only *Salmonella typhosa* and other Salmonella possessing the H-d antigen in a floccular manner.

Salmonella Vi agglutinating serum can be prepared by inoculating rabbits with either a living or a formalin-treated suspension of a pure Vi strain of *Salmonella typhosa* or another Salmonella with a strong Vi antigen.[8] Do not filter or add preservative. This serum is satisfactory only when a pure Vi strain of *Salmonella typhosa* is agglutinated in it and a strain with no Vi antigen is not agglutinated. Should agglutinative properties for the latter be present, absorb with a suspension of this species that has been heated in streaming steam at 100° C. for 2 hours. Combine equal parts of the absorbing suspension and a 1:5 dilution of the serum. Incubate at 35°–37° C. for 2–6 hours, shaking occasionally. Refrigerate for 18–48 hours. Centrifuge, decant the supernate, and retest.

Multivalent Salmonella serum can be prepared by combining equal parts of sera produced with *Salmonella paratyphi, Salmonella schottmülleri, Salmonella hirschfeldii, Salmonella enteritidis, Salmonella sp.* (Type London), and *Salmonella sp.* (Type Senftenberg).

Methods for the production of sera for differentiating Salmonella other than *Salmonella typhosa* are given by Kauffmann,[3-6] Kauffmann and Edwards,[7] and Edwards and Bruner.[8]

Three or more multivalent sera may be necessary for identifying *Shigella paradysenteriae*. These sera can be produced by inoculating each rabbit with several types, or by immunizing with individual types and combining the immune sera. The latter method yields lower titered sera but seems preferable since portions of the individual sera can be reserved for testing strains that react in a pool. Sera for the gas-producing types—*Shigella paradysenteriae* (Type Newcastle) [30, 31] and

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* Throughout these methods, "salt solution" signifies 0.85 per cent sodium chloride solution.

*Shigella paradysenteriae* (type Manchester) [32]—should be available and can be included in one of the pools.

Prepare *Shigella sonnei* serum with a combination of phase I and phase II [33] strains.

The growth of contaminating microörganisms can be prevented by combining sera with equal parts of glycerol of tested purity or by adding 0.01 per cent merthiolate.

### 2. *Standardization*

Prepare a series of dilutions of 1:10 through 1:1,000 or higher. Test with the strain or strains with which the serum was produced, using dilutions of 1:10 through 1:50 or 1:100 in macroscopic slide-agglutination tests and 1:100 through 1:1,000 or higher in tube tests. When the titer with the homologous strain has been determined, test a series of dilutions, including one or two greater than the titer, with freshly isolated strains of the homologous species and representative strains of antigenically related species. Select for use in tests for identification (1) the lowest dilution in which a specific reaction occurs in the macroscopic slide test, and (2) two or three of the lowest dilutions in which specific reactions occur in the tube test.

### 3. *Tube agglutination test*

Adjust the turbidity of all suspensions employed in tube agglutination tests as specified under Turbidity Standards (III B). Combine in small tubes (10–11 mm. x 75–80 mm.) equal volumes (0.3–0.5 ml.) of each serum dilution and broth culture or suspension in salt solution. For purposes of control, combine the suspension being tested with salt solution and with a low dilution of serum from a normal animal of the same species as that in which the agglutinating serum was produced. Also test the agglutinating serum with a strain of the homologous species. Incubate the tests at from 48°–52° C. for 15–18 hours and record the reactions. If no agglutination occurs, repeat the test in duplicate; incubate one test at 48°–52° C. for 15–18 hours and the other at 35°–37° C. for 2 hours followed by refrigeration overnight.

Read and record the reactions as follows:

| | |
|---|---|
| Definite clumping with | |
| Perfectly clear supernatant fluid | 4+ |
| Very slightly turbid supernatant fluid | 3+ |
| Turbid supernatant fluid | 2+ |
| Slight or indefinite clumping | + |
| No clumping, uniform turbidity | — |

#### 4. *Macroscopic slide agglutination test*

When slide agglutination tests are performed with only two or three sera, emulsify with a needle or loop a small amount of growth from an agar slant in a loopful of serum dilution on a glass slide. If tests are to be performed with several sera, prepare a suspension in salt solution. The growth from one agar slant in 0.3 ml. usually provides satisfactory turbidity, but this may vary with different species or strains. Combine one loopful of suspension with one loopful of serum dilution.

### E. AGGLUTINATING SUSPENSIONS[34-36]

#### 1. *Formalin-treated suspension for demonstrating floccular agglutination*

An actively motile, "smooth" strain of *Salmonella typhosa* is essential. Motility can often be enhanced by growing the culture either in broth or on agar at room temperature for a few days. Streak a plate of beef-infusion agar (C. M. No. 5a) poured 24 hours previously. If, after 18–24 hours' incubation, other than perfectly smooth colonies are noted, suspend two or three smooth colonies in small amounts of salt solution or broth and replate immediately. Repeat this process until an entirely smooth culture is obtained that is actively motile and is agglutinated in a floccular manner in appropriate serum. Examine a slide preparation stained by Gram's method and determine the reaction in double-sugar medium (C. M. No. 18). Use a suspension of an 18–24 hour beef-infusion agar culture to inoculate 2 per cent beef-infusion agar (C. M. No. 5a) in Roux or other bottles that provide adequate surface. If water of condensation is present on the agar, remove it with a pipette before inoculation. After 18–24 hours' incubation, pipette into each Roux bottle, or its equivalent, 10 ml. of buffered salt solution * to which 2 per cent of formalin has been added, and suspend the growth in this. Make Gram-stained preparations and discard suspensions that show the presence of contaminating microörganisms. Pool the suspensions in a sterile bottle, place in a refrigerator, and after 48 hours prepare subcultures. Hitchens's semifluid agar (C. M. No. 56)

---

* *Buffered Sodium Chloride Solution.*

| | |
|---|---|
| Sodium chloride | 6.8 gm. |
| Sodium phosphate, $Na_2HPO_4$ | 2 gm. |
| Water to make | 1,000 ml. |

a. Dissolve the ingredients and make up to volume.
b. Check pH, which should be 8.4–8.6.
c. Filter through hard paper.
d. Dispense in bottles or flasks and autoclave 30 minutes.

This solution, with the addition of 2 per cent formalin, is useful for suspending organisms to be used as "H" antigens in agglutination tests.

is recommended for this purpose, since it provides conditions favorable to both aerobic and anaerobic microörganisms. Should growth of *Salmonella typhosa* occur, retest, after additional refrigeration, until no evidence of viability is obtained. If considerable material settles out, decant the supernatant suspension and discard the sediment. Adjust the suspension to a turbidity 10 times that of barium sulfate standard No. 3 or its equivalent and a formalin content of 2 per cent. Determine the turbidity by comparison of diluted portions with the standard and with a similar suspension previously standardized. Calculate the required amount of diluent as in the following example:

Assuming that 400 ml. of concentrated suspension are to be adjusted and it is found that this needs to be diluted 1:12 to equal the standard, then $\frac{400 \times 12}{10} = 480$ ml., the volume of adjusted suspension, or 80 ml. of buffered formalinized salt solution are required to be added to the 400 ml. of the original suspension.

For agglutination tests, combine 1 part of concentrated suspension with 9 parts of salt solution, thus obtaining a suspension equivalent in turbidity to the standard and containing 0.2 per cent formalin.

Test the agglutinability of the suspension in comparison with a previous lot that has proved satisfactory, using, if possible, a series of human sera giving various degrees of agglutination as well as agglutinating sera produced in animals. Store in a refrigerator.

The technic of agglutination tests has been described under Agglutinating Sera (III, D).

2. *Alcohol-treated suspension for demonstrating granular agglutination*

An entirely "smooth" strain of *Salmonella typhosa* should be employed. Follow the procedure outlined for the formalin-treated suspension to insure smoothness and purity of the culture and inoculate 2 per cent infusion agar (C. M. No. 5a) in Roux or other bottles. After 18–24 hours' incubation, pipette into each Roux bottle, or its equivalent, 10 ml. of salt solution containing 0.5 per cent phenol and suspend the growth. Make Gram-stained preparations and discard suspensions that show the presence of contaminating microörganisms. Measure the suspension and pool it in an Erlenmeyer flask. Add 50 per cent by volume of absolute alcohol or 54 per cent of 95 per cent alcohol.

Rotate the flask slowly as the alcohol is added or stir with a sterile glass rod. Pour the alcohol-treated suspension into sterile cylinders

and incubate overnight at 35°–37° C. The following morning, decant the supernatant suspension into a sterile bottle and discard the sediment. Mix thoroughly and prepare subcultures to determine the presence of contaminating microörganisms. Hitchens's medium (C. M. No. 56) is recommended since it provides both aerobic and anaerobic conditions.

Adjust the turbidity of the suspension to 10 times that of barium sulfate standard No. 3, or its equivalent, and its alcohol content to approximately 30 per cent. The diluent consists of salt solution containing 0.5 per cent phenol combined with 50 per cent of its volume of absolute alcohol or 54 per cent of 95 per cent alcohol.

For agglutination tests, combine 1 part of the concentrated suspension with 9 parts of salt solution, to obtain a suspension equivalent in turbidity to the standard and containing approximately 3 per cent alcohol and 0.05 per cent phenol.

Test the agglutinability of the suspension in comparison with a previous lot that has proved satisfactory, using, if possible, a series of human sera giving various degrees of agglutination, as well as agglutinating sera produced in animals. It may be desirable to test the sera at the same time with a living suspension of a freshly isolated strain of *Salmonella typhosa.* Store in a refrigerator.

### 3. *Living suspension for demonstrating Vi agglutination*[37]

Maintain a pure Vi strain of *Salmonella typhosa* on inspissated egg medium (C. M. No. 54). Inoculate this medium with a single stroke of the needle from the base to the top of the slant. Incubate for 15–18 hours at 35°–37° C., seal, and store immediately in a refrigerator. Make a number of transplants at 6 month intervals, and discard those made previously. One egg medium slant will suffice for subcultures on beef-infusion agar on several different days. After 18–20 hours' incubation at 35°–37° C., suspend the growth from the agar slant in salt solution and adjust the turbidity as outlined under "Turbidity Standards." Prepare living suspension each day that tests are performed and keep in a refrigerator if not used immediately. The Vi antigen is apparently altered fairly soon if kept at room temperature. Pure Vi strains of *Salmonella typhosa* should also be stored in a dry state[38] so that they will be available in case the cultures on egg medium become unsatisfactory.

## IV. EXAMINATION OF SPECIMENS

### A. BACTERIOLOGIC EXAMINATIONS

The type of specimen and the clinical and epidemiologic data must be considered in the choice of procedures for the isolation of bacillary incitants of enteric disease.

#### 1. *Fluid enrichment media*

Many selective and enrichment fluid media have been recommended to aid in the isolation of typhoid bacilli and other Salmonella. Of these, peptone-tetrathionate enrichment broth (C. M. No. 28) and bile-glycerol-peptone broth (C. M. No. 27) are among the most valuable. Blood clots are partially dissolved in the latter. Either may be employed for unclotted blood, urine, duodenal contents, and gall-bladders or their contents. Peptone-tetrathionate broth increases materially the percentage of isolations of Salmonella from feces.

#### 2. *Plating media*

Employ at least 4 plates for each fecal specimen and, unless a diagnosis has been established, at least 2 plating media, one of which facilitates the isolation of dysentery bacilli. For purposes of release or determination of carriers, choose media that favor the isolation of the species sought. Usually only one or two plates are necessary for plating urine, blood, duodenal drainage, and gall-bladders and their contents, either before or after enrichment.

Bismuth sulfite agar (C. M. No. 23) markedly inhibits most strains of the coliform group and is favorable to the development of nearly all strains of Salmonella. Bile-salts-citrate agar (C. M. No. 25) and desoxycholate-citrate agar (C. M. No. 24a or C. M. No. 24b) also inhibit the coliform group and are particularly helpful in the isolation of Salmonella and dysentery bacilli. The use of a differential but non-inhibitive medium, such as Endo's agar (C. M. No. 22) is also recommended.

#### 3. *Colonial characters*

On bismuth sulfite agar (C. M. No. 23) well isolated colonies of ***Salmonella typhosa*** are usually flat and black, surrounded by a characteristic dark halo, which imparts a metallic cast to the medium in

reflected light. Colonies of the other Salmonella are generally convex and larger than those of *Salmonella typhosa*. Occasional strains of either are entirely inhibited; others may develop only after 36–48 hours' incubation, or produce colorless or green colonies. If the medium is too heavily seeded, the colonies may not be typical.

Colonies of dysentery bacilli and Salmonella are translucent and usually colorless or a delicate pink on Endo's agar (C. M. No. 22), bile-salts-citrate agar (C. M. No. 25) and desoxycholate-citrate agar (C. M. No. 24a or C. M. No. 24b). Those of the coliform species are opaque, from pink to deep red, usually with a metallic sheen on Endo's agar.

### 4. *Testing plating media*

When new lots of dyes are employed in media or a new lot of dehydrated medium is obtained, a series of plates should be inoculated with representative strains of bacillary incitants of enteric disease preferably recently isolated, and, if possible, with specimens known to contain these microörganisms. The media should also be tested with representative strains whenever they are inoculated with specimens.

### 5. *Examination of feces*

If a fecal specimen is satisfactorily diluted with buffered 30 per cent glycerol solution when received, plate directly and pipette approximately 4 ml. into 12 ml. of peptone-tetrathionate broth (C.M. No. 28) to which 0.3 ml. of aqueous iodine solution has been added immediately preceding use. If undiluted, suspend 3–5 gm. in tetrathionate broth and approximately 1 gm. in 8–10 ml. of buffered 30 per cent glycerol solution. After the coarse particles have settled, streak the latter on plates. Incubate the tetrathionate broth for 18–20 hours before plating.

a. *Streaked plates*—Using a loop approximately 5 x 12 mm. bent at an angle of 30° or 40°, transfer 2 to 4 loopfuls from the top layer of the suspension in glycerol solution to each plate of bismuth sulfite (C. M. No. 23) bile-salts-citrate (C. M. No. 25) and desoxycholate citrate (C. M. No. 24a or C. M. No. 24b) medium and 1 loopful to Endo's agar (C. M. No. 22). The best distribution of colonies is usually obtained by drawing the bent loop through the inoculum and streaking the plates in parallel rows 2 or 3 mm. apart.

After tetrathionate broth inoculated with feces has been incubated for 18–20 hours, streak 1 or 2 loopfuls (5 x 12 mm.) on one plate of

bismuth sulfite agar (C. M. No. 23) and one of bile-salts-citrate agar (C. M. No. 25).

b. *Poured plates*—If poured plates of bismuth sulfite agar are employed, inoculate at least 2 with each specimen. First dilute the specimen by combining 1 part of the suspension in glycerol solution with from 1 to 4 parts of broth (C. M. No. 4) depending upon the consistency of the specimen. The coarse particles of feces can be conveniently removed by drawing the suspension into a pipette or Asepto syringe (Becton, Dickinson & Co., Rutherford, N. J.), the tip of which has been wound with nonabsorbent cotton before sterilization. Use a rubber bulb for drawing the specimen into the pipette or syringe. Place from 2 to 4 drops in one Petri dish and from 1 to 3 ml. in a second. Add at least 20 ml. of melted bismuth sulfite agar that has been cooled to approximately 45° C. and mix by tilting and rotating the plate. When very large numbers of *Salmonella typhosa* are present, visible colonies may not develop on the plate inoculated with the larger amount.

Poured plates of bismuth sulfite agar may also be made from the tetrathionate broth inoculated with feces.

c. *Fishing colonies*—Examine the plates with a microscope or hand lens after 18–24 hours' incubation. Reincubate bismuth sulfite, bile-salts-citrate, and desoxycholate citrate agar plates so that they will be available for reëxamination if necessary. Inoculate double-sugar medium (C. M. No. 18) or a satisfactory modification[39, 40] from colonies characteristic of those of bacilli inciting enteric disease. Stab the butt to the bottom of the tube and streak the surface of the slant. Record the reactions after 18–24 hours' incubation.[41, 42]

The reactions in double-sugar medium provide a means of differentiating the microörganisms at present recognized as incitants of enteric disease from those of the coliform group, although some nonpathogenic species may ferment lactose slowly or not at all and hence fail to produce acid in the slant of the agar. On the other hand, very rarely strains of typhoid and dysentery bacilli may give an acid reaction in the slant as well as the butt.

If a medium to demonstrate the production of $H_2S$ [39, 40] is employed, it should be kept in mind that this property is not entirely reliable.

d. *Study of morphology*—Prepare films from the growth on the double-sugar medium or, if slide agglutination tests are performed, allow these preparations to dry; fix them and stain by Gram's method. All the bacillary incitants of enteric disease are relatively small, evenly staining

Gram-negative bacilli without spores. Examine a hanging drop of a 1–3 hour broth culture for motility. If none is observed and other properties are those of Salmonella, reëxamine after maintenance at room temperature. Motility can also be demonstrated by dispersion of growth in semisolid agar (0.3 to 0.4 per cent). A flagella stain may be helpful; Gray's [24] method is recommended.

e. *Macroscopic slide agglutination test*—Perform a macroscopic slide agglutination test in specific dilutions of appropriate sera (see "Agglutinating Sera") with the growth from a colony on the plating medium or from double-sugar agar. A report should not be based on the results of this test unless the species indicated has been isolated from a previous specimen from the same patient or other patients involved in the outbreak.

f. *Macroscopic tube agglutination test*—Perform macroscopic tube agglutination tests with broth cultures or suspensions in salt solution and specific dilutions of appropriate agglutinating sera (see "Agglutinating Sera"). Maintenance at room temperature enhances the agglutinability as well as the motility of some strains.

If the patient's serum is available, tests for agglutinative properties for the microörganisms isolated may often be of value, especially when the properties of the latter are not perfectly typical of a species that is of diagnostic significance.

g. *Fermentative properties*—Record at frequent intervals for 3 weeks the reactions in milk (C. M. No. 16) and in media containing single carbohydrates and an indicator (C. M. No. 1 or C. M. No. 4). Test for gas production by inoculating extract broth containing the desired carbohydrate in fermentation tubes (C. M. No. 4). If gas production is demonstrated in double-sugar medium (C. M. No. 18) or a satisfactory modification,[39, 40] no further tests for this property are necessary. If this is questionable, however, inoculate glucose broth in a fermentation tube. Tests for gas production from other carbohydrates are seldom necessary. Anaerogenic strains of Salmonella other than *Salmonella typhosa* are found more frequently than is generally appreciated.

h. *Indole production*—Test for indole by adding 0.5 ml. of Kovac's reagent * to 4 day cultures in peptone broth (C. M. No. 2). As a con-

* *Indol Test, Kovacs's Reagent for*

| | |
|---|---|
| Para-dimethylaminobenzaldehyde | 5 gm. |
| Amyl or butyl alcohol | 75 ml. |
| Concentrated HCl | 25 ml. |

a. Mix the alcohol and aldehyde and heat in a water bath or incubator at 50°–60° C. until the aldehyde is dissolved.
b. When cool, add the hydrochloric acid slowly.
c. Store in the dark in a brown bottle with a glass or rubber stopper.

trol, always incubate and test at the same time one tube of uninoculated medium, one inoculated with *Escherichia coli,* and one with *Salmonella typhosa.*

i. *Gelatin liquefaction*—To test for gelatinolytic activity, inoculate beef-extract gelatin (C. M. No. 15) and incubate at 35°–37° C. Determine liquefaction by placing the cultures in the refrigerator until a tube of the uninoculated medium is solidified. Incubate for 2 weeks unless liquefaction is demonstrated earlier.

j. *Urea decomposition*[43]—Inoculate the surface of a slant of urea agar (C. M. No. 31) and incubate. Decomposition of urea is indicated by a change in the color of the medium from yellow to violet-red that is usually apparent within a few hours. Some strains produce urease very slowly, however, and the failure to obtain the characteristic color change should not be considered final until the cultures have been incubated for 4 days. The majority of Proteus species decompose urea very rapidly, but some proteolytic microörganisms that have other properties of this genus apparently do not produce urease. *Proteus morganii* gives a delayed reaction. *Bacterium enterocoliticum* is the only one of the pathogenic species included in Table 1 that has thus far been found to decompose urea.

k. *Reporting results*—The criteria for identification of species are summarized in Table 1. Unless the identity of the incitant has been established previously, a complete study is necessary before reporting the species. Under certain circumstances it may be advisable to send a preliminary report on the tentative identification of the microörganism and state that a supplementary report will follow.

Report the isolation of *Salmonella typhosa* when the morphology and fermentative properties are typical of this species and agglutination (macroscopic tube method) occurs in Salmonella H-d serum as well as in either multivalent Salmonella or *Salmonella typhosa* serum. Unless all these tests are performed, anaerogenic strains of other Salmonella may be mistaken for typhoid bacilli. Freshly isolated strains may be inagglutinable because of the Vi antigen. Such strains may be tested with Vi agglutinating serum. They usually become agglutinable after being incubated at room temperature for a few days.

Report the isolation of Salmonella other than *Salmonella typhosa* only when agglutination in multivalent Salmonella serum as well as biochemical properties indicate a member of this genus. For information regarding the differentiation of types of Salmonella consult Kauffmann,[3–6] Kauffmann and Edwards,[7] and Edwards and Bruner.[8]

TABLE 1

*Criteria for identification of species**

| Species | Reaction in double-sugar agar | Motility | Acid in media containing: Glucose | Maltose | Mannitol | Lactose | Sucrose | Xylose | Rhamnose | Dulcitol | Indole | Reaction in milk | Serologic reactions |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| nella | ⊕ or (+) | Motile† | A | A | A | — | — | A (—) | A (—) | A (—) | Not produced | A± or Alk± or A to Alk | Agglutination in multivalent Salmonella serum |
| *nella typhosa* | + | Motile† | A | A | A | — | — | A (—) | — | — | Not produced | A± or Alk± | Agglutination in multivalent Salmonella, *S. typhosa* and *S.* H-d sera |
| lla | + or (⊕) | Nonmotile | A | — or A | — or A | — (A±) | — (A±) | — (A±) | — or A | — (A±) | V | V | Agglutination in multivalent and/or univalent sera |
| *lla dysenteriae* | + | Nonmotile | A | — or A± | — | — | — | — | — | — | Not produced | A± or Alk± | Agglutination in *Shigella dysenteriae* serum |
| *lla ambigua* | + | Nonmotile | A | V | — | — | — | — | A | — | Produced | A± or Alk± | Agglutination in *Shigella ambigua* serum |
| *lla sonnei* | + | Nonmotile | A | A or A± | A | A± | A± | — or A± | A or A± | — | Not produced | A or AC | Agglutination in *Shigella sonnei* serum |

TABLE 1 — (Continued)

*Criteria for identification of species**

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *ella radysenteriae*‡ | + | Nonmotile | A | V | A | — | — (A±) | — (A±) | — (A±) | — (A±) | V | A± or Alk± | Agglutination in multivalent and/or univalent sera for members of this group |
| *ella radysenteriae* pe Newcastle) | + or ⊕ | Nonmotile | A | — (A±) | — | — | — | — (A±) | — | — (A±) | Not produced | A± or Alk± | Agglutination in multivalent and/or univalent sera for members of this group |
| *ella radysenteriae* pe Manchester) | + or ⊕ | Nonmotile | A | — (A±) | A | — | — | — (A±) | — | — (A±) | Not produced | A± or Alk± | Agglutination in multivalent and/or univalent sera for members of this group |
| *ella alkalescens* | + | Nonmotile | A | A | A | — | — | A | A or A± | A (A±) | Produced | A to Alk | Agglutination in *Shigella alkalescens* and some *Shigella paradysenteriae* sera |
| *terium iterocoliticum* | + or ⊕ | Motile† | A | A | A | A± | A | A | — | — | Produced | A± or Alk± | Agglutination in *Bact. enterocoliticum* serum |
| *teus morganii* | + or ⊕ | Motile† | A | — | — | — | V | — | — | — | Produced | — or Alk | Of questionable value |
| iform group | ✪§ | V | A | V | A | V | V | V | V | V | V | A or AC | Of questionable value |

*Only those species which do not liquefy gelatin are included.

†Nonmotile strains sometimes found, especially when freshly isolated.

‡The mannitol-fermenting group.

§If lactose is fermented slowly, the reaction may be ⊕.

+ = Acid butt, neutral or alkaline slant, no gas.

⊕ = Acid and gas in butt, neutral or alkaline slant.

✪ = Acid and gas throughout.

A = Acid.

Alk = Alkaline.

C = Coagulation.

— = No perceptible change.

V = Variable.

± = Weak or delayed reaction.

( ) = Rare occurrence.

TABLE 1

*Criteria for identification of species**

| Species | Reaction in double-sugar agar | Motility | Acid in media containing | | | | | | | | Indole | Reaction in milk | Serologic reactions |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | *Glucose* | *Maltose* | *Mannitol* | *Lactose* | *Sucrose* | *Xylose* | *Rhamnose* | *Dulcitol* | | | |
| Salmonella | ⊕ or (+) | Motile† | A | A | A | — | — | A (—) | A (—) | A (—) | Not produced | A± or Alk± or A to Alk | Agglutination in multivalent Salmonella serum |
| *Salmonella typhosa* | + | Motile† | A | A | A | — | — | A (—) | — | — | Not produced | A± or Alk± | Agglutination in multivalent Salmonella, *S. typhosa* and *S.* H-d sera |
| Shigella | + or (⊕) | Nonmotile | A | — or A | — or A | — (A±) | — (A±) | — (A±) | — or A | — (A±) | V | V | Agglutination in multivalent and/or univalent sera |
| *Shigella dysenteriae* | + | Nonmotile | A | — or A± | — | — | — | — | — | — | Not produced | A± or Alk± | Agglutination in *Shigella dysenteriae* serum |
| *Shigella ambigua* | + | Nonmotile | A | V | — | — | — | — | A | — | Produced | A± or Alk± | Agglutination in *Shigella ambigua* serum |
| *Shigella sonnei* | + | Nonmotile | A | A or A± | A | A± | A± | — or A± | A or A± | — | Not produced | A or AC | Agglutination in *Shigella sonnei* serum |

TABLE 1 — (Continued)

*Criteria for identification of species**

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Shigella paradysenteriae*‡ | + | Nonmotile | A | V | A | — | — (A±) | — (A±) | — (A±) | — (A±) | V | A± or Alk± | Agglutination in multivalent and/or univalent sera for members of this group |
| *Shigella paradysenteriae* (Type Newcastle) | + or ⊕ | Nonmotile | A | — (A±) | — | — | — | — (A±) | — | — (A±) | Not produced | A± or Alk± | Agglutination in multivalent and/or univalent sera for members of this group |
| *Shigella paradysenteriae* (Type Manchester) | + or ⊕ | Nonmotile | A | — (A±) | A | — | — | — (A±) | — | — (A±) | Not produced | A± or Alk± | Agglutination in multivalent and/or univalent sera for members of this group |
| *Shigella alkalescens* | + | Nonmotile | A | A | A | — | — | A | A or A± | A (A±) | Produced | A to Alk | Agglutination in *Shigella alkalescens* and some *Shigella paradysenteriae* sera |
| *Bacterium enterocoliticum* | + or ⊕ | Motile† | A | A | A | A± | A | A | — | — | Produced | A± or Alk± | Agglutination in *Bact. enterocoliticum* serum |
| *Proteus morganii* | + or ⊕ | Motile† | A | — | — | — | V | — | — | — | Produced | — or Alk | Of questionable value |
| Coliform group | ✪§ | V | A | V | A | V | V | V | V | V | V | A or AC | Of questionable value |

*Only those species which do not liquefy gelatin are included.

†Nonmotile strains sometimes found, especially when freshly isolated.

‡The mannitol-fermenting group.

§If lactose is fermented slowly, the reaction may be ⊕.

+ = Acid butt, neutral or alkaline slant, no gas.

⊕ = Acid and gas in butt, neutral or alkaline slant.

✪ = Acid and gas throughout.

A = Acid.

Alk = Alkaline.

C = Coagulation.

— = No perceptible change.

V = Variable.

± = Weak or delayed reaction.

( ) = Rare occurrence.

The identification of dysentery bacilli should be based on a study of biochemical properties as well as agglutination in known sera. *Shigella alkalescens* is antigenically related to some types of *Shigella paradysenteriae* and may be mistaken for the latter if the biochemical properties are not studied.

The species designated as *Bacterium enterocoliticum* has been included in Table 1 because it has many properties in common with recognized bacillary incitants of enteric disease and has been shown to be an incitant of enterocolitis.[27, 28] The fact that this species hydrolyzes urea and ferments lactose and sucrose should be kept in mind. It is extremely pathogenic for mice when freshly isolated, but loses this property quickly when grown on culture media.

Until the status of *Shigella alkalescens* and *Proteus morganii* has been established, it would seem advisable to report their isolation and add that their diagnostic significance is questionable.

When a microörganism is isolated that cannot be readily or definitely identified, it is usually desirable to suggest that further specimens be sent for bacteriologic study, as well as blood for serologic tests.

### 6. *Examination of blood*

When clotted blood is received, the clot can be added to 8–10 ml. of bile-glycerol-peptone solution (C. M. No. 27), or it can be comminuted by forcing through a 10 or 20 ml. sterile syringe[44] into semifluid agar (C. M. No. 56), peptone-tetrathionate broth (C. M. No. 28), or other satisfactory medium.

In case the blood is submitted in a buffered 30 per cent glycerol solution* or contains an anticoagulant, add 5–10 ml. to bile-glycerol-peptone solution (C. M. No. 27), semifluid agar (C. M. No. 56), or other satisfactory enrichment medium. After 44–48 hours' incubation, streak one plate each of bismuth sulfite agar (C. M. No. 23) and another plating medium such as blood agar (C. M. No. 33). If no growth is obtained, reëxamine after 4 days' additional incubation. Continue with the procedure as outlined for feces. If microörganisms of possible significance other than bacillary incitants of enteric disease are isolated, attempt their identification.

### 7. *Examination of urine and duodenal drainage*

Streak plates as directed for feces, using one or two loopfuls on bismuth sulfite agar and one of the other plating media. Transfer 2 or 3 ml. to bile-glycerol-peptone solution or peptone-tetrathionate broth.

* See Footnote p. 215.

After 15–18 hours' incubation, streak the fluid medium on bismuth sulfite agar, and, unless contraindicated by the examination of the plates streaked directly with the specimen, on one of the other plating media. The presence of large numbers of contaminating microorganisms may render the results of the examination of questionable significance. Continue with the procedures as outlined for feces.

### 8. *Examination of gall-bladders and their contents*

Open the gall-bladder with sterile instruments. Remove the bile with a pipette or a syringe and needle. Examine the bile as outlined for urine and duodenal drainage. Macerate a small portion of the gall-bladder wall in salt solution, and place the remainder in a suitable fixative for histologic study. Examine slide preparations of the bile and gall-bladder stained by Gram's method. Streak bismuth sulfite agar and one other plating medium and inoculate broth and bile-glycerol-peptone or peptone-tetrathionate broth. Plate the fluid cultures on bismuth sulfite agar and one other plating medium after 18–24 hours' incubation, unless contraindicated by examination of the plates streaked directly with the specimen.

Wash gallstones, first in 70 per cent alcohol and then in salt solution, and crush them in a mortar in a small amount of salt solution. Follow the procedures outlined for the examination of bile and the gall-bladder wall.

When microörganisms other than typhoid bacilli or Salmonella are found, attempt their isolation and identification. The presence of contaminating microörganisms may render the results of the examination of questionable significance.

## B. SEROLOGIC EXAMINATIONS [1a, 35, 36]

Blood should be considered unsuitable for agglutination tests when an anticoagulant has been added or the serum is chylous or markedly hemolyzed. Hemolysis may be due to exposure to extreme heat or cold, or the growth of microörganisms, any one of which might alter the agglutinative properties. Serum is unsatisfactory unless conditions under which it is submitted preclude the possibility of subjection to conditions that might affect the agglutinative properties.

### 1. *Test for floccular and granular agglutinative properties*

Follow the general directions outlined under "Agglutinating Sera," for performing the tests and recording the reactions. Remove the serum

from the clot with an Asepto syringe or pipette and prepare dilutions of 1:10, 1:20, 1:40, 1:80, 1:160, and higher if desired. To one series of dilutions, add a formalin-treated (see "Agglutinating Suspensions") or other suspension of *Salmonella typhosa* that has been shown to be satisfactory to demonstrate the floccular type of agglutination; and to a second series add an alcohol-treated (see "Agglutinating Suspensions") or other suspension of *Salmonella typhosa* suitable for demonstrating the granular type of agglutination. If considered advisable, tests may also be performed with actively motile, living cultures in 3 per cent peptone-bile solution.* For purposes of control, test each suspension in serum known to possess agglutinative properties and in normal animal or human serum. Shake the tubes to insure thorough mixing and incubate at 48°–52° C. for 15–18 hours.

A tentative report may be made when reactions are obtained in tests incubated for 15 minutes and centrifuged for 10 minutes at approximately 2,000 r.p.m. This can be considered only a presumptive test and a report of the findings by the method recommended should follow. Centrifuging tests after incubation for 15–18 hours may demonstrate agglutination where it was not apparent before. When this occurs, the test should be repeated and another specimen requested.

Report the highest dilution in which definite granular or floccular agglutination occurs.

When agglutination is obtained in dilutions no higher than 1:40, report the reaction "partial" and of questionable significance.

An explanation of the significance of the reactions may be helpful to the physician. This may be printed on the report form or on a separate sheet to be enclosed with the report. Unless vaccine has been administered very recently, both granular (somatic) and floccular (flagellar) agglutination of typhoid bacilli in a 1:80 or higher dilution are seldom observed in sera other than those from patients having typhoid fever. Floccular agglutination in a 1:80 dilution or higher with little or none of the granular type occurs rarely with sera from typhoid fever patients. This type of reaction is usually obtained with specimens from typhoid

---

* *Bile-Peptone Solution.*

| | | |
|---|---|---|
| Peptone | | 10 gm. |
| Ox-bile, dehydrated | | 5 gm. |
| (*or* fresh ox-bile | 30 gm.) | |
| Water, to make | | 1 kg. |

a. Combine the peptone and bile; add the water.
b. Boil the mixture vigorously for 3 to 5 minutes and dispense in bottles or flasks.
c. Place in a refrigerator overnight and filter through hard paper until clear.
d. Dispense in flasks or bottles, autoclave, and store in a refigerator.
e. When required for use, filter through hard filter paper until clear, dispense, and autoclave.

carriers or from persons who have received typhoid vaccine. Sometimes such a reaction is found in the serum of an individual who has had typhoid fever in the past, although no evidence of a carrier condition can be demonstrated. Granular agglutination in a 1:80 or higher dilution, with little or none of the floccular type, has been observed in sera from typhoid fever patients, but this type of reaction most commonly occurs in infections with microörganisms antigenically related to *Salmonella typhosa*. Unless the blood is collected soon after onset, only a very small percentage of sera from typhoid fever patients fails to react in dilutions of diagnostic significance.

### 2. *Test for Vi agglutinative properties* [1a, 37]

Employ a living suspension (see "Agglutinating Suspensions") of a pure Vi strain of *Salmonella typhosa*. Prepare serum dilutions of 1:5, 1:10, 1:20, 1:40, and higher if desired and combine with the bacterial suspension as directed under "Agglutinating Sera." For purposes of control, combine the suspension with (1) salt solution, (2) known Vi serum, and (3) typhoid serum containing floccular and granular ("O"), but no Vi agglutinative properties. Incubate the tests for 2 hours at 35°–37° C. and then leave in a refrigerator overnight. Record the reactions before and after centrifuging for 10 minutes at approximately 2,000 r.p.m. After centrifugation, read all reactions after shaking. Comparison with the tests included for purposes of control is very important. Record as follows:

| | |
|---|---|
| Definite complete clumping in perfectly clear fluid | 4+ |
| Definite clumping in clear or nearly clear fluid | 3+ |
| Definite clumping in slightly turbid fluid | 2+ |
| Slight or indefinite clumping in turbid fluid | + |
| No clumping, uniform turbidity | — |

Perform tests for Vi agglutination at least twice, preferably with not less than 3 or 4 days intervening.

Reports on the results of tests for Vi agglutination should always be accompanied by an explanation of the limitations of this examination.

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# Brucellosis (Undulant Fever)

*(Brucella abortus, Brucella suis, and Brucella melitensis)*

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## I. INTRODUCTION

BRUCELLOSIS is currently a widespread disease in both man and animals. As a consequence, laboratory aids to its diagnosis are among the most important procedures of medical and public health laboratories. No other infectious disease of man surpasses brucellosis in presenting baffling puzzles to the clinician; hence, laboratory aids, to be effective, must yield informative, if not conclusive, results. Unfortunately, the immunologic responses of infected persons show tremendous variability and the organism too often hides itself success-

fully within the host so that both skill and patience as well as sound interpretation of results on the part of both the clinician and the bacteriologist frequently must be exercised to the utmost before a diagnosis can be established beyond a reasonable doubt. It is regrettable but true that all efforts to obtain unequivocal laboratory confirmation of infection with *Brucella* may prove unavailing in some cases.

The more definitive laboratory procedures are: (1) Agglutination tests and (2) attempts to isolate the organism. A third laboratory procedure, the opsonocytophagic test, is of limited value for diagnostic purposes, useful in a few cases only when performed under ideal conditions. Another diagnostic aid, the allergic skin reaction is not within the scope of this chapter although it is a necessary adjunct to the interpretation of the opsonocytophagic test.

Under the circumstances, the laboratory should be prepared to offer to the physician intelligently planned and executed laboratory services for the diagnosis of brucellosis which are carried out as meticulously as though each suspected case were a research problem in itself. This is not to imply that these services are not routine in nature. It is, rather, an expression of the attitude which must be inculcated in the laboratory staff performing the tests in question or preparing materials for the tests. The methods described below are not to be regarded as standard or official but rather as examples of workable and reliable procedures prepared with due regard for minimum requirements.

No discussion of laboratory work on brucellosis would be complete without a statement that there is a not inconsiderable risk of laboratory infection. For that reason, stock cultures used for antigen preparation should be avirulent as recommended below. The danger of handling virulent cultures, particularly those freshly isolated, must be impressed upon laboratory assistants, and the manipulatory technics involved must be scrutinized by trained bacteriologists with the safety factor in mind. It is particularly important that non-technical workers who clean glassware be protected from exposure by meticulous sterilization of discarded materials emanating from the bacteriological laboratory.

## II. AGGLUTINATION TESTS

### A. Blood Serum

#### 1. *Collection of Specimen*

Preferably use specimen container furnished for this purpose by the laboratory. Obtain blood by venipuncture after cleansing and disinfecting the skin surrounding the point where the needle is to be inserted.

Use needle and syringe sterilized in the autoclave whenever feasible (otherwise by boiling) since the clot may be cultured. Obtain a minimum of 5 ml. of blood; more if other tests are to be made. (Culture of whole blood requires 10–20 ml.; opsonocytophagic test, 5 ml.) Transfer blood to a clean, sterile specimen tube or vial equipped with suitable leakproof closure. Do not remove closure longer than the minimum time required to place blood specimen in container; observe aseptic precautions throughout. Allow specimen to stand undisturbed until firm clot has formed. After taking steps for proper identification of the specimen transmit it to the laboratory. Unless requested to remove serum from clot by the laboratory, send the whole blood specimen.

### 2. *Separation of Serum*

Obtain clear serum by centrifugation of blood specimen. Use sterile tubes and aseptic precautions when handling any portion to be cultured.

### 3. *Rapid Plate (Slide) Method*[1,2]

a. Special apparatus and materials

(1) Glass plates (slides)—Use rectangular plates of double thickness window glass, cut to suitable size, preferably with polished edges. Before use these should be thoroughly clean and greaseless. Exact dimensions will vary from one laboratory to another depending upon size of viewing box if used and usual methods of procedure but it is recommended that the plate be no larger than is necessary to accommodate more than six rows of compartments for tests, 5–6 to the row.

Compartments may be ruled on the plate with a diamond point or wax pencil, may be deposited on the plate as wax rings or may be constructed in any convenient manner which permits thorough cleaning of the plate after use. Square compartments 1" on each side, or circular compartments 1" in diameter are suitable. Circular compartments are readily made with a ring-maker and a hot (130°–140° C.) mixture of 90 per cent paraffin (m.p. 48° C.) and 10 per cent petrolatum. When this mixture is used, the bulk of the wax is readily removed if the plates are immersed in cool water for a short time immediately after use. The ring-maker may be constructed by shaping a circle from 28 gauge wire around a 1" test tube leaving ¼–½" of both ends of the wire projecting side by side. The circle is then wound carefully with one layer of No. 12 linen thread which is secured by tying at each end. The projecting wires are then bent at an angle of 60°–75° to the plane of the circle and forced into any convenient handle. Multiple ring-makers may be constructed entirely of metal. Ordinary curtain rings about 1" diameter can be welded to strips of metal to make a multiple ring-maker so that an entire plate may be prepared at one time.

(2) Illuminating device—Although an illuminating device is not necessary, some workers find that it contributes markedly to the ease of reading reactions.

A simple wooden box, 14"–17" long x 9½"–11" wide x 5"–6" deep, with the top partly covered by a strip of wood 2½" wide running lengthwise, serves the purpose very nicely. A 10" showcase bulb mounted in a socket at one end of the box under the strip of wood, or two 50 watt electric light bulbs (one at each end), will supply sufficient illumination. The strip of wood on top of the box protects the eyes of the worker reading the test and provides for viewing the test by indirect lighting. Some prefer to insert a metal mirror behind the light source.

(3) Antigen dropper pipette—Prepare antigen bottles, 15–30 ml. capacity, fitted with dropper pipettes having an outside diameter at the extreme tip of 0.07" and an opening of 0.06". This size corresponds to gauge 15 by U. S. standard gauge No. 283. These dropper pipettes, if held vertically, will deliver 0.03 ml. of antigen in each drop.

(4) Antigen suspension—Use antigen prepared specifically for the rapid plate method, ordinarily known as "Antigen-Huddleson." Although this is available through supply houses, a method of preparation is described below.

b. Preparation of antigen

(1) Selection of *Brucella* culture—For routine use select an avirulent strain of *Brucella abortus* such as N. I. H. 456 obtainable from the National Institutes of Health, Bethesda, Md. Streak a series of tryptose agar (C.M. No. 13b) or liver infusion agar (C.M. No. 7a) plates with a single light inoculum of the culture, and incubate in air at 35°–37° C. for 3–4 days until one of the plates shows well isolated colonies 2–7 mm. in diameter. Examine colonies by both reflected and transmitted light. Typical smooth colonies are distinctly spheroidal in shape, slightly opalescent in color and translucent. When viewed from above by reflected light the colonies have a moist, almost greasy appearance. Using a small lens (2½–5X), scan the isolated colonies and select for use only those with the above characteristics which have circular edges and are not mucoid, opaque or wrinkled on the surface. If evidence of dissociation from the smooth state is apparent, obtain another culture.

(2) Seed cultures—From a smooth colony prepare one heavily seeded tryptose agar (C.M. No. 13b) or liver infusion agar (C.M. No. 7a) slant in ¾" diameter test tube for each 6 Blake bottles to be used for antigen production. Incubate in air at 35°–37° C. for 24 hr. At this point make a Gram stain of each culture and test the growth

for uniform emulsifiability in isotonic salt solution and for spot slide agglutination in *Brucella* antiserum (See III; A;3;e, below). If pure and serologically typical, use these slants to seed Blake bottles for antigen production.

(3) Cultures for antigen suspension—Suspend the growth from each seed culture slant in 12–15 ml. of sterile isotonic salt solution. Using aseptic technique inoculate each of 6 Blake bottles containing either tryptose agar (C.M. No. 13b) or liver infusion agar (C.M. No. 7a) with 2–2.5 ml. of the resulting suspension and by tilting the bottles cause the suspension to flow over the entire agar surface. Incubate in air with the agar surface uppermost and on a slight slant (neck of bottle higher than bottom) for 48 hr. at 35°–37° C.

(4) Harvesting growth—Add to each bottle 15–20 ml. of sterile isotonic * salt solution containing 0.5 per cent phenol or 0.3 per cent formalin. Phenolized salt solution is preferred by most authorities. Place the bottles on a flat surface with the agar surface down so that the bacterial growth is moistened. Suspend the growth by tilting each bottle back and forth. This is readily accomplished without scraping. Stand each bottle on end when this operation has been completed. When all bottles have been so handled, pool the suspensions after filtration through a layer of absorbent cotton to remove particles of agar.†

(5) Preparation of crude antigen suspension—Sediment the organisms, preferably in an angle centrifuge, until firmly packed, and discard the supernatant. Take up the sediment in a minimal quantity of the phenolized or formalinized salt solution (about 5 ml. for the growth from 6 Blake bottles). Insure even suspension by mixing with a tongue depressor, wooden applicator, or similar instrument. Filter by suction through a thin layer of absorbent cotton on a Buchner type filter.

(6) Titration of antigen suspension—Prepare minimal quantities of several dilutions of the crude suspension in the phenolized or formalinized salt solution as follows: 1:2, 1:4, 1:6, and 1:8. Test each of these diluted antigens for sensitivity to an immune animal serum of known tube test titer (usually necessary to dilute antiserum so that an endpoint

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* Some prefer to use hypertonic salt solution (12%) for this and further steps involving dilution of the antigen suspension for the rapid plate method. A recommended formula is: NaCl (reagent grade) 12 gm., glycerol (neutral) 20 ml., phenol (U.S.P.) 0.5 gm., distilled water q. s. 100 ml.

† If forced to use a virulent culture for antigen, the suspension should be heated in a water bath for 1 hr. at 60° C. after filtration.

occurs at 1:160 or 1:320) according to the technique described under c, below. Select that dilution of antigen which gives results with 0.08, 0.04, 0.02, 0.01, and 0.005 ml. of serum that are nearest to those obtained respectively with 1:20, 1:40, 1:80, 1:160, and 1:320 final dilutions* in the tube test. Further test the selected dilution against two or more sera of varying titers from cases and against at least one known negative serum. If the selected dilution gives results comparable to those obtained with a previous satisfactory antigen, consider the titration adequate. In some cases it may be necessary to test interpolated dilutions such as 1:3 or 1:5. Those who use isotonic salt solution as a diluent will find on rare occasions that such a suspension is insensitive; sensitivity may be increased by using hypertonic salt solution (see footnote to (4) above).

(7) Final antigen for test—Dilute the unused crude antigen suspension with the phenolized or formalinized salt solution to the point indicated by the above titration. To each 20 ml. add 0.01 ml. of 1 per cent aqueous brilliant green and 0.005 ml. of 1 per cent aqueous crystal violet and mix. Test the bulk of standardized antigen to check accuracy of dilution against the patients' sera used above. If satisfactory, dispense in 15–20 ml. quantities in bottles with dropper pipettes and store, when not in use, in the refrigerator.

c. The Test

(1) Be sure samples of serum and antigen are at room temperature.

(2) Shake the antigen gently but thoroughly and repeat at each 2 hour interval when in use.

(3) Test not more than five or six sera at one time. When using other antigens such as typhoid O, typhoid H, etc., test one serum at a time against all antigens in multiple tests in preëstablished order on one plate. Place 0.08, 0.04, 0.02, 0.01, and 0.005 ml. amounts of each serum to be tested in each row of squares or circles.

(4) Place a drop of antigen on each drop of serum.

(5) Mix the serum and antigen in each row with a fresh toothpick or wooden applicator, working from the smallest quantity of serum to the largest.

(6) Lift the plate and tilt it back and forth for 2 to 3 minutes.

(7) Read in bright indirect light against a dark background, *e.g.*, place gooseneck desk lamp over a black background with shade slightly

* Some prefer to standardize plate test antigens in terms of a series of dilutions such as 1:25, 1:50, 1:100, etc. Commercial antigens may be so standardized.

tilted and read plate while held above the rim. When using an illuminating box, place the plate on the box; turn on the light; and read the results. Avoid excessive drying of tests. The results may be read and recorded in serum amounts or in serum dilutions.

Immediately after use, rinse plate with cold water or place in a tray containing cold water for subsequent thorough cleansing.

### 4. *Test Tube Method*

a. Preparation of antigen—Use antigen suspension prepared from a smooth, preferably avirulent *Brucella abortus* culture selected, grown in Blake bottles and harvested as directed under 3 above. The pooled suspension may be kept in the refrigerator and diluted as needed. Antigen for use in the test may be standardized by dilution of the crude suspension with phenolized or formalinized salt solution to a density corresponding to 0.04 per cent bacteria by the centrifuge method of Fitch, et al.,[3, 4] to 200 p.p.m. silica (fuller's earth) standard.[5] to tube No. 1 of the McFarland nephelometer[6] or to 7 cm. on the Gates apparatus.[7] After adjustment of density check each antigen by making comparative tests with it and with a control antigen of known satisfactory sensitivity against sera of different titers including negatives (see b below). Satisfactory antigens should give results identical with or very closely approximating the control antigen.

b. The test

(1) Prepare dilutions of the serum in isotonic salt solution, 0.5 ml. final volume in each tube of the series. Preferably use a series of dilutions which require only simple manipulation, such as 1:10, 1:20, 1:40, etc., or 1:25, 1:50, 1:100, 1:200, etc. Make the initial dilution in sufficient quantity to permit serial transfer of 0.5 ml. to the second and succeeding tubes, each containing 0.5 ml. isotonic salt solution. Mix the contents of each tube thoroughly by sucking back and forth in a pipette five times before removing portion to next tube. Avoid frothing. Routinely make dilutions at least to 1:320 (or 1:400); these dilutions are doubled when antigen is added.

(2) Prepare controls for each run of unknown sera. Include either a series of dilutions of a serum of known titer or a dilution of antiserum known to yield complete agglutination; include also a tube containing 0.5 ml. isotonic salt solution only.

(3) Add 0.5 ml. antigen to each tube including controls. Mix by shaking.

(4) Incubate in a water bath at 50°–55° C. for 3–4 hr.; place in refrigerator overnight. (Alternatively incubate at 37° C. for 40–48 hr.)

(5) Read and record reactions after examining controls. Results may be read either as complete agglutination (+), partial agglutination (P or ±) and negative (—), or as 4+, 3+, 2+, +, ± and —, indicating approximate percentage of complete agglutination. Do not overlook zone reactions, particularly those which show slight or no agglutination in the lower dilutions.

### 5. *Tests for "Blocking Antibodies"*

There is reason to believe that demonstrable antibodies may be present in sera which do not show agglutination. These so-called "blocking" or "univalent" antibodies are presumably similar to those which have been demonstrated in sensitization to the Rh blood factor. At this time, however, it would be premature to incorporate suggested methods for blocking antibodies in brucellosis; the reader is referred to a preliminary report by Griffitts.[8]

## B. MILK SERUM

### 1. *Preparation of Specimen*

Do not use soured or decomposed milk; colostrum is unsatisfactory. Boric acid (1 per cent) is a satisfactory preservative for milk for this purpose. Place milk sample in refrigerator overnight or for a minimum of 6–8 hr. Alternatively centrifuge to separate cream. Pipette off the cream. Place 10 ml. of separated milk in a large test tube, add 4 drops rennin (1 per cent solution) and mix thoroughly. Incubate tubes at 35°–37° C. in a slanting position for 2–3 hr. Remove serum (whey) and place in refrigerator to allow solid particles to settle out.

### 2. *The Test*

Use clear serum obtained as above. Test by rapid plate method in the same manner as blood serum (see A; 3; c, above).

## C. OTHER MATERIALS

Occasionally there is some justification for the making of agglutination tests for *Brucella* on cerebrospinal fluid or other fluids derived from the body. Technics described for blood serum (see A, above) are suitable.

## III. CULTURAL ISOLATION OF THE ORGANISM

### A. BLOOD CULTURES

#### 1. *Collection Outfits*

a. For culture of whole blood—preferably use culture bottle or flask provided by the laboratory. The following types of outfits are suitable:

(1) Delayed culture (mailed specimens)—Into a narrow mouth bottle, 100 ml. or 4 fl. oz. capacity, equipped with diaphragm stopper, dispense 50 ml. of a suitable culture medium (see 3, a, below). After sterilization, using aseptic precautions replace about 10 per cent of the air above the medium with $CO_2$ in a suitable manner, *e.g.*, as follows: Sterilize the diaphragm with 70 per cent ethanol or with acetone; provide completely sterilized, a tuberculin syringe barrel containing absorbent cotton and fitted to a 23 gauge hypodermic needle; plunge needle through diaphragm; with rubber tubing attach open end of syringe barrel to one lead of a three-way stop-cock, the other leads of which are used for attachment respectively to a vacuum pump and to a vessel containing a measured amount of $CO_2$ collected over water by displacement; exhaust approximately 10 per cent of air above the medium; reverse stop-cock and allow $CO_2$ to replace it. A Y or a T tube with rubber tubing and pinch-cocks may be used instead of the three-way stop-cock. This is essentially the method and apparatus pictured by Huddleson.[2]

(2) Immediate culture only—Use any suitable flask with cotton plug containing 50 ml. of a suitable culture medium (see 3, a, below), sterilized after assembly. The use of this type of collection flask is predicated upon early incubation of the culture under suitable $CO_2$ tension.

(3) Emergency use only. (Outfit with culture medium not available.)—Use vial or tube (about 1 fl. oz. capacity) with suitable closure containing 1 ml. of 20 per cent sodium citrate, sterilized after assembly.

b. For clot culture—Use clean sterile tube or vial, preferably that provided by the laboratory for collection of blood for agglutination tests.

#### 2. *Procuring of Specimen*

Preferably take specimen during febrile episode. Procure blood by venipuncture aseptically (10–20 ml. for culture of whole blood; 5–10 ml. for clot culture), and transfer directly into the container provided, taking special care to avoid contamination. Insure thorough mixing if fluid is supplied in container, or allow to form firm clot undisturbed if clot culture is intended. Transport immediately to laboratory.

#### 3. *Culture of Whole Blood*

a. Primary culture medium — Use tryptose-dextrose-vitamin B broth (C.M. No. 13) or liver infusion broth (C.M. No. 7), either one pref-

erably modified by the addition of 0.5–1.0 gm. agar per liter prior to sterilization. When these media are used in field collection outfits for direct inoculation of fluid uncitrated blood, further modify by the addition of 10 gm. sodium citrate per liter prior to sterilization.

b. Incubation of primary culture—Incubate at 35°–37° C. under 2–10 per cent $CO_2$ tension. When a field outfit containing $CO_2$ similar to that described (see 1, a, above) has been used no additional $CO_2$ need be furnished but other cultures should be placed in sealed incubating chambers or jars. In the latter case, means should be provided for the introduction of $CO_2$, either by exhaust and replacement of air or by the burning of a candle in the sealed space. Shaughnessy[9] has described a method of generating $CO_2$ within the culture tube or flask itself. Exactly 10 per cent $CO_2$ tension need not be attained; atmospheres of 2–3 per cent have been shown to yield good results.[10] Incubate for 4–7 days before making first transfer described below; re-incubate primary culture for 7 days, renewing $CO_2$ if it has been dissipated, and make second transfer unless growth has been obtained on first; repeat for an additional 7 days and make third and final transfer at that time if growth has not been obtained. The high humidity which develops in sealed chambers or jars under these conditions promotes rapid growth of molds which will contaminate and render valueless many cultures if not controlled. A layer or tray of dry $CaCl_2$ (anhydrous, porous, 4 mesh) placed in the bottom of the chamber or jar will be found helpful in reducing the humidity.

c. Transfers to solid medium—Use freshly poured and solidified plates of tryptose-dextrose-vitamin B agar (C.M. No. 13a) or liver infusion agar (C.M. No. 7a). Mix primary culture, aseptically remove 0.5 ml. and streak over the entire surface of the medium in the plate. Incubate under 2–10 per cent $CO_2$ tension for 4 days at 35°–37° C. Methods of obtaining a suitable $CO_2$ tension are numerous. It is practicable to use large glass jars (of 1, 3, or 5 gal. capacity, gasketed screw caps) into which the inverted plates are placed. A lighted candle secured to a small tray is then placed in the jar near the top and the cap is screwed tightly in place. A candle allowed to burn undisturbed in the jar until it goes out will yield a $CO_2$ tension between 2 per cent and 3 per cent.[11] The development of a high humidity within the chamber or jar is not only favorable to rapid growth of contaminating molds but also constitutes a hazard to the worker since condensate on the plates may serve as a vehicle for the spread of *Brucella* to the hands of the

person examining plates for growth. Hence, a drying agent used as in b, above, is recommended.

d. Examination of plates—After the 4 day incubation period, examine plates for signs of growth. Characteristic *Brucella* colonies are 2–7 mm. in diameter, spheroidal in shape, moist and slightly opalescent in appearance, and translucent; these characteristics may vary somewhat with available moisture and with pH. Do not consider a blood culture negative for *Brucella* until the third streaking has failed to yield the organism.

e. Study of suspected colonies—Fish isolated suspected colonies to triple-sugar-iron agar slants (C.M. No. 20a) using a straight needle to stab the butt and streak the slant. Incubate under $CO_2$ tension at 35°–37° C. for 48 hr. and for an additional 24 hr. if insufficient growth has resulted. Most organisms other than *Brucella* including *Salmonella* as well as obvious contaminants may be spotted by heavy growth after the first 24 hr. of incubation. *Brucella* species yield a fine, streptococcus-like growth which does not produce any change in this medium. Enteric organisms which are not infrequently encountered present characteristic changes which are helpful as a screening procedure. Take necessary steps to identify the organism sufficiently to appraise its probable significance. In the case of organisms producing no change on triple sugar iron agar, make a Gram stain and examine for Gram-negative coccobacilli. Purify any mixed cultures obtained by plating on tryptose agar (C.M. No. 13b) or liver infusion agar (C.M. No. 7a) containing 1.4 ml. of 0.1 per cent aqueous crystal violet per liter with subsequent incubation under $CO_2$ tension at 35°–37° C. When a pure culture has been obtained, make a spot slide agglutination test with *Brucella* antiserum in suitable dilution (usually not less than 1:10); control for pseudo-agglutination by noting smoothness of emulsion of the growth in a drop of isotonic salt solution on a glass slide. Confirm positive slide agglutination tests by tube agglutination test. All *Brucella* types when first isolated will normally agglutinate to titer in stock *Brucella* antiserum. Identify species of *Brucella* as outlined in V, below.

### 4. *Clot Cultures*

a. Inoculation of primary medium—Subject clot to as little handling as practicable in separating and removing serum. Observe aseptic precautions, preferably leaving clot in original container. Macerate clot with the open end of a sterile straight-sided pipette about 9″ long

with a 2 ml. capacity rubber bulb attached. Such pipettes or " thieves " can be made from glass tubing, 7 mm. diam., with ends polished and slightly flattened by heating in a flame. When broken up, the clot is sucked into the pipette with the aid of the rubber bulb and transferred aseptically to tryptose-dextrose-vitamin B broth (C.M. No. 13) or to liver infusion broth (C.M. No. 7), dispensed in screw-capped vials or cotton-plugged test tubes in 8–10 ml. quantities before sterilization. The addition of 0.5–1.0 gm. of agar per liter to the broth before sterilization is recommended. In laboratories which handle specimens taken under varying conditions and shipped through the mail there will be many contaminated cultures which can be eliminated to some extent by adding 1.4 ml. of 0.1 per cent aqueous crystal violet (certified) per liter of broth before sterilization. However, according to Huddleson[12] this concentration of crystal violet may inhibit small numbers of *Brucella suis*.

b. Isolation of the organism—Proceed as directed above under 3, b, c, d, and e. When using screw-capped vials do not close cap tightly during incubation under $CO_2$ tension. Identify species isolated in pure culture as directed in V, below.

## B. MISCELLANEOUS CULTURES

### 1. *Body fluids and tissues other than blood*

Collect and handle aseptically. Inoculate primary medium, subculture and identify as for blood cultures.

### 2. *Urine*

Centrifuge 50 ml. urine at sedimenting force comparable to that produced by 3,000 r.p.m. in No. 2 centrifuge maintained for 1 hr. Spread sediment over the surface of two tryptose-dextrose-vitamin B agar plates (C.M. No. 13a), or two liver infusion agar plates (C.M. No. 7a), either media containing 1.4 ml. of 0.1 per cent aqueous crystal violet (certified) per liter added before sterilization. Incubate plates under 2–10 per cent $CO_2$ tension at 35°–37° C. for 48 hr.; re-incubate an additional 24 hr. if no growth. Identify suspected colonies as directed in section on blood cultures.

### 3. *Feces*

Ordinarily, attempts to isolate *Brucella* from human feces represent effort which might better be expended in making repeated blood cul-

tures. However, since occasional successes are reported, the following procedure is recommended, principally for those interested from the research rather than the routine diagnostic viewpoint:

Mix 1–2 gm. feces in 50 ml. sterile isotonic salt solution and agitate until a reasonably homogeneous suspension is obtained. Filter through four layers of hospital gauze to remove gross particles. Centrifuge filtrate at 500 r.p.m. for 3 min. Remove supernatant to sterile tube and add 0.5 ml. *Brucella* antiserum of at least 1:1,000 titer (tube test). Mix and place in 37° C. water bath for 2 hr. Centrifuge at 500 r.p.m. for 5 min. and discard supernatant. Resuspend precipitate by gentle agitation in sterile isotonic salt solution and centrifuge as before. Repeat this procedure twice more. Spread sediment over the surface of two tryptose-dextrose-vitamin B agar plates (C.M. No. 13a) containing 1.4 ml. of 0.1 per cent aqueous crystal violet (certified) per liter added before sterilization. Incubate under 2–10 per cent $CO_2$ at 35°–37° C. for 48–72 hr. Fish suspected colonies and identify organisms as directed in section on blood cultures.

### 4. *Dairy products*

The following technics are abstracted from *Standard Methods for the Examination of Dairy Products*[13]:

a. Preparation of sample

#### 1. *Milk or cream, bottled or dipped samples*

If milk, allow to stand 24 hr. in refrigerator and aseptically pipette off the entire cream layer to a sterile tube.

#### 2. *Milk from individual cows*

Collect milk at or near milking time after wiping teats with a clean damp cloth and discarding first 2 or 3 streams from each quarter of the udder. Use 2 sterile sampling tubes, 20–30 ml. capacity, for each cow. In one tube collect about 15 ml. milk from the front quarters, about 7.5 ml. from each. Similarly collect milk from hind quarters in the other tube. Refrigerate tubes in upright position for 24 hr. and aseptically pipette off cream layers to separate sterile tubes.

#### 3. *Cheese*

With a heavy, sterile glass rod emulsify 5 gm. sample in 50 ml. physiological salt solution warmed to 40° C. in a sterile 100 ml. centrifuge tube. Alternatively emulsify samples up to 25 gm. in a Waring Blendor.

b. Culturing—For each sample use at least 2 plates of tryptose agar (C.M. No. 13b) or liver infusion agar (C.M. No. 7a), either containing 1.4 ml. of 0.1 per cent aqueous crystal violet (certified) per liter added before sterilization. Inoculate plates as follows:

(1) Cream—Spread 0.1–0.2 ml. over the surface of at least two plates.

(2) Emulsion of cheese—Spread 0.1–0.2 ml. over the surface of at least two plates; similarly inoculate 2 plates each with 1:10 and 1:100 dilutions of the emulsion in sterile isotonic salt solution.

Incubate at 35°–37° C. under 2–10 per cent $CO_2$ tension for 48 hr. and examine plates; re-incubate and examine plates again after an additional 72 hr. Fish suspected colonies and identify organisms isolated as directed under section on blood cultures.

## IV. DEMONSTRATION OF BRUCELLA BY ANIMAL INOCULATION

Use healthy male guinea pigs, 300–600 gm. body weight. Secure 4–5 ml. blood from each prior to inoculation and test for *Brucella* agglutinins. Prepare specimens or samples in the manner suggested for cultural procedure (see III, above). If material is likely to contain many contaminating organisms, inoculate animals subcutaneously; otherwise, intraperitoneally. Inject about 2 ml. into each animal, at least 2 animals per specimen. Eight weeks after inoculation, procure 4–5 ml. blood from each animal and test for *Brucella* agglutinins. Kill and autopsy animals, examining each for characteristic lesions such as:

(1) Spleen—enlarged, sometimes 5–6X; usually with nodules that are at first hemorrhagic, later becoming encapsulated, gray, and discrete and occasionally having necrotic centers; occasionally abscesses (usually *Brucella suis*).

(2) Liver—small (0.5–2.0 mm. diam.), gray, glistening, discrete nodules just below surface on capsule; occasionally abscesses (usually *Brucella suis*).

(3) Genitalia—Sometimes abscesses in testes and epididymis (Sublumbar lymph nodes may also be involved).

Make cultures from suspected lesions by rubbing cut surface of tissue over the surface of crystal violet tryptose (or liver infusion) agar. Incubate, fish colonies, and identify organisms as directed under section on blood cultures.

## V. DIFFERENTIATION OF SPECIES

### A. METHODS OF CHOICE

For a complete discussion of methods available for differentiation of *Brucella* species see Huddleson.[2] Many such methods are not readily carried out in the routine examination of cultures. It is recommended that each culture isolated be identified by the dye-plate method of Huddleson [2] supplemented by tests for ability to grow in absence of $CO_2$ and by $H_2S$ production.

### B. PROCEDURE

#### 1. *Culture Media*

a. Tryptose agar slants (C.M. No. 13b).
b. Thionin tryptose agar—Melt previously sterilized tryptose agar (C.M. No. 13b). Heat small amount of 1 per cent aqueous thionin (certified) in a boiling water bath for 20 min. and then add 0.1 ml. while hot to each 100 ml. medium. Mix, pour plates and use within 48 hr.
c. Basic fuchsin tryptose agar—Melt previously sterilized tryptose agar (C.M. No. 13b). Heat small amount of 1 per cent aqueous basic fuchsin (certified) in a boiling water bath for 20 min. and then add 0.1 ml. while hot to each 100 ml. medium. Mix, pour plates and use within 48 hr. These plates should be dark rose red in color; 1 per cent basic fuchsin solution older than two months may deteriorate yielding lighter plates not suitable for use. Always store basic fuchsin solution in the dark.

#### 2. *Tests for $CO_2$ requirement and $H_2S$ production*

From each pure culture on triple sugar agar or other medium inoculate 2 tryptose agar slants. Suspend a lead acetate strip about 1″ below the cotton plug within one of the tubes for the detection of $H_2S$. Incubate the plain slant aerobically and the one for $H_2S$ production under 2–10 per cent $CO_2$ at 35°–37° C. for 4 days. Daily examine both for growth and record $H_2S$ production, replacing the lead acetate strip with a fresh one each day. Record $H_2S$ produced as " O " (none), " ± " (trace) and " + " (moderate to marked).

#### 3. *Dye-plate method*

From each pure culture streak one thionin-tryptose agar plate and one basic fuchsin-tryptose agar plate. Use moderately heavy inoculum

streaking one small area at top of each plate heavily and streaking off on rest of plate. Incubate plates under 2–10 per cent $CO_2$ at 35°–37° C. for 72 hr. Examine and record presence or absence of distinct growth; do not confuse heavy inoculum with growth but rely on distinct growth on portions of plates streaked after rubbing off the excess on the loop.

### C. Classification of Cultures

The dye-plate results constitute the most reliable criterion for species identification; results of other tests are informative. The following schema illustrates the manner of interpretation:

| *Dye-Plates* | | | | |
|---|---|---|---|---|
| *Thionin* | *Basic fuchsin* | *$CO_2$ Needed* | *$H_2S$ Production* | *Classification* |
| Growth | Growth | Yes * | — to ± throughout | *B. melitensis* |
| Growth | No growth | No | + throughout | *B. suis* |
| No growth | Growth | Yes * | + for first 2 da. only | *B. abortus* |

* Occasional strains are aerobic on first isolation.

## VI. OPSONOCYTOPHAGIC TEST

### A. Limitations

The opsonocytophagic test for brucellosis is of distinctly less importance in the public health laboratory than are agglutination tests, cultures, and animal inoculations. It can be used to advantage only in laboratories which serve small areas and, even then, is a procedure of doubtful value except when other measures fail. This condition arises from the need for prompt laboratory examination, preferably within 3 hr. and never longer than 6–8 hr. after collection of the specimen.

Rigid and nearly continuous bacteriological scrutiny of the culture utilized is essential for reliable results. The culture must be smooth and as free from variant cells as careful and repeated care can accomplish.

The test, by itself, yields little diagnostic information except when made in conjunction with the allergic skin test, a clinical procedure. Carefully controlled and interpreted, however, it does have value in some cases. Quite possibly the proper sphere for application of this test is the clinic, provided adequately trained bacteriologists are available. For discussions of this test see Huddleson,[2] Evans, *et al.*,[14] and Foshay and LeBlanc.[15]

## B. THE TEST

### 1. *Collection of Specimen*

Huddleson [3] recommends the collection of the specimen at the time the skin test is made or within 7 days thereafter. Aseptically collect blood by venipuncture and transfer exactly 5 ml. to a sterile vial containing exactly 0.04 gm. sodium citrate (0.2 ml. of 20 per cent aqueous solution). Mix and rush to laboratory preferably within 3 hr. and certainly within 6–8 hr.

### 2. *Bacterial antigen*

a. Selection of culture—Use avirulent culture tested for smoothness by selection of colonies from tryptose agar plates incubated for 72 hr. at 35°–37° C. (see Section II, A, 3, b, above). Do not use any culture more than 3 transfers removed from this process or more than 1 week old.
b. Preparation of suspension—Plant culture tested for smoothness on tryptose agar slant (C.M. No. 13b). Incubate 48 hr. at 35°–37° C. Suspend growth in sterile physiological salt solution. Adjust density to 1 cm. on the Gates' scale [7] or to tube 16 by the McFarland nephelometer.[6] Do not use suspension more than 12 hr. old.

### 3. *The test proper*

Into a clean test tube (75 mm. long x 7–8 mm. diam.) place 0.1 ml. of the whole citrated blood after thorough mixing. Add 0.1 ml. of the bacterial suspension, mix and incubate in 37° C. water bath for 30 min. without agitation. Remove tube, resuspend sedimented cells and make 2 or 3 spreads on clean, polished glass slides (3" x 1") as follows:

Remove small amount of reactive mixture with a finely drawn capillary pipette to which is attached a rubber bulb. Place a drop on one end of the slide and draw it across the slide with the edge of another slide as in the routine preparation of blood smears. Dry smears rapidly using an electric fan.

### 4. *Staining spreads*

Fix at least one spread from each specimen in chloroform (U. S. P. grade) for 5–15 sec., dry and stain for 1 min. with carbol toluidin blue.* Wash off excess stain quickly with distilled water and dry.

| * Toluidin blue | 0.5 gm. |
|---|---|
| Phenol (U.S.P.) | 3.0 gm. |
| Ethyl alcohol (95%) | 10 ml. |
| Distilled water q.s. | 100.0 ml. |

### 5. *Examining stained spreads*

Examine briefly for proper staining and lack of precipitate. Red cells are greenish; nuclei of leucocytes, bluish purple; bacteria, blue. Use freshly prepared stain on one of the reserve spreads if preparation is not suitable. Examine 25 polymorphonuclear leucocytes in different sections of the spread and record the number of cells showing each of the following degrees of phagocytosis:

None — no ingested organisms
Slight — 1–20 bacteria ingested
Moderate — 21–40 bacteria ingested
Marked — more than 40 bacteria ingested

### 6. *Reporting results*

Multiply number of cells in each category by 4 to obtain percentages and report as such. Evans [16] and Foshay and LeBlanc [15] have suggested other forms of reporting.

## VII. GENERAL COMMENTS ON INTERPRETATION OF RESULTS

### A. AGGLUTINATION TESTS

### 1. *Blood serum*

a. In *acute* brucellosis little or no titer will develop during the first 10 days of illness. Successive specimens taken as the disease progresses will ordinarily show a rise in titer to a peak sometime during the 3rd to 6th week which may afterward decline.

b. In *chronic* brucellosis there is no definite criterion whereby the significance of an agglutination test titer may be judged. No diagnostic question is likely to be raised about persons yielding high titers (complete agglutination at 1:160 and higher) with clinical findings compatible with modern knowledge of brucellosis and without symptoms of tularemia. So long as any degree of agglutination is obtained in dilution of 1:20, infection with *Brucella* cannot be ruled out on the basis of laboratory tests alone. As a matter of fact, some individuals apparently do not develop detectable agglutinins (or have lost them) although infected. This is small comfort to the clinician but nevertheless true in the light of present knowledge. The demonstration of "blocking antibodies" may prove useful in some questionable cases. Laboratories making clot cultures routinely will automatically resolve some of these problems by isolation of the organism from the non-reacting blood. Repeated blood cultures are helpful.

c. Cross-reactions with *Brucella* antigen are ordinarily found only in

sera from tularemia patients. The titer of the cross-reaction may be relatively high. Some cross-reactions may also be found in specimens from persons who have been immunized to cholera.

### 2. *Milk serum*

This test is a good, but not perfect, screening procedure for epidemiological purposes. It has been used effectively for many years in the city of Providence, R. I.,[17] and subsequently elsewhere, to spot infected herds by tests on market milk. When applied to individual cows it is less effective than blood serum agglutinations but very practicable for preliminary investigations in directing attention to probable sources of infection.

## B. CULTURES

The isolation of any species of *Brucella* from any fluid, tissue, secretion, or excretion derived from the human or animal body constitutes conclusive evidence of infection. Exceptions may occur only under very exceptional circumstances, *e.g.*, the isolation of *Brucella* from swabbings of tonsils after the patient has drunk raw milk from infected cattle. Negative cultures do not exclude brucellosis; repeated negative blood cultures combined with negative agglutination tests are convincing but not complete evidence for absence of brucellosis. Negative cultures from suspected lesions of an inoculated animal are evidence only that viable *Brucella* were not present in the portion of material injected.

## C. ANIMAL INOCULATION TEST

Results on animal inoculation tests are subject to the same limitations as those on cultures.

## D. OPSONOCYTOPHAGIC TEST

The opsonocytophagic test requires careful and expert interpretation. It is at best a test which can yield only supplementary information of limited diagnostic value and only after judicious weighing of all clinical and laboratory evidence. It should be reserved for use on difficult cases of suspected chronic brucellosis. The injection of *Brucella* vaccine or repeated skin tests can be expected to stimulate opsonins. Predicated upon performance of the test for diagnostic purposes under the rigid conditions imposed for collection of sample, promptness of examination, and meticulousness of testing procedure, Huddleson[2] has

suggested a schema for interpretation which is the basis for the considerably modified version given in Table 1.

TABLE 1

| *Agglutination Test* | *Allergic Skin Reaction* | *Range of Opsonocytophagic Activity* | *Probable Significance* 1. *As to infection* | 2. *As to immunity* |
|---|---|---|---|---|
| − | − | 100% none — 20% slight | Not brucellosis | Susceptible |
| + | − | 100% none — 20% slight | Inconclusive and anomolous; recent tularemia? | Susceptible |
| − | + | 100% none — 20% slight | Nonspecific sensitivity | Susceptible |
| − | + | 24% slight — 40% marked | Possible brucellosis; needs confirmation | Not immune |
| + | − | 24% slight — 40% marked | Very likely brucellosis; rule out tularemia | Not immune |
| + | + | 100% none — 40% marked | Brucellosis | Not immune |
| − or + | − or + | Over 40% marked | Brucellosis or recovered brucellosis | Immune defenses at high level, not necessarily permanent |

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# Tularemia

## *(Pasteurella tularensis)*

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## I. INTRODUCTION

FOR the laboratory diagnosis of tularemia, two methods are available. They are: (a) the agglutination test and (b) cultural examinations. Of these, the test for specific agglutinins in the blood serum, being practical and readily available, is the one which is most frequently used.

The extreme infectivity of *Pasteurella tularensis* for man renders inadvisable the employment of living virulent cultures in routine

examinations. Under the carefully controlled conditions of scientific research, and when exceptional circumstances demand their use in diagnosis, bacteriological culture methods will, of course, be adopted. Therefore the procedures likely then to be followed are included here. However, it seems advisable to state that 89 laboratory workers in 18 laboratories have become infected while studying cultures of *Pasteurella tularensis*, but more especially when autopsying infected animals. These accidents happened to them in spite of their broad experience in dealing with other pathogenic bacteria, and thus emphasize the danger of handling this organism to the laboratory technician.

## II. THE AGGLUTINATION TEST

### A. Selection of Strain for Antigen Production

The National Institutes of Health are distributing an avirulent strain of *Pasteurella tularensis* which has lost its virulence after years of cultivation on artificial medium. Strain No. 38 has proved to be very satisfactory for routine tests; it has the characteristics of a smooth strain, being easily cultivated, making homogeneous suspensions without difficulty, is highly agglutinable, and generally specific in its reactions.

### B. Culture Media

Blood-glucose-cystine agar (C.M. No. 35) (Francis[1] 1923) employed for stock cultures and the production of antigen is made as follows:

A beef or horse infusion agar (C.M. No. 5a) containing 0.5 per cent sodium chloride and adjusted to pH 7.3 is maintained in stock. When needed, 0.1 per cent cystine* and 1.0 per cent glucose are added. Merck or Pfanstiehl cystine has been found satisfactory. This mixture is heated in streaming steam until the agar has melted and sufficient time has elapsed for the sterilization of the cystine and glucose. Cool to 50° C., add 8 per cent defibrinated or whole rabbit blood. Human blood may be employed if rabbit blood is not available.

Since cystine is not very soluble in beef infusion, it should be pulverized before it is added. In order to secure complete solution the flask should be shaken frequently while in the streaming steam. Sometimes it is necessary to allow the melted cystine agar to stand

* It appears well to mention that some growth of *Pasteurella tularensis* will take place when glutathione or thioglycollic acid is employed in place of cystine. However, they cannot replace the essential cystine for adequate maintenance of growth.

overnight in a water bath at 56° C. to insure the solution of all visible particles. When cystine is added, the pH of the medium remains unchanged. On the other hand, many workers have found cystine hydrochloride the salt of choice because it is readily soluble and reduces the preparation time. If it is employed, a correction in pH may be necessary on account of the acidity.

The flasks of blood-glucose-cystine agar are allowed to remain in a water bath for 2 hours at 60° C. for the promotion of sterility. The medium is then tubed, using either a sterile pipette or sterile funnel. The tubes of medium are slanted and incubated 24 hours to test their sterility.

Although the organism has been found to multiply in liquid media,[2-4] the removal of the water of condensation still is advisable. In order to accomplish this, freshly prepared tubes which have a moist surface and abundant water of condensation should be placed with only the cotton stopper inserted in a slanted position in the incubator at 37° C. for about 1 week, or until they are free of the water of condensation. In order to hasten the drying, the water of condensation may be removed by any suitable means. When dry, the tubes of the medium are placed upright and plugged with cotton or cork stoppers which have been soaked in a very hot mixture of equal parts of vaseline and paraffin, thus preventing further evaporation. A newer and easier method is to employ parafilm. They are then stored in the cold room.

In cases where the above medium cannot be readily prepared, the (Difco) dehydrated cystine containing medium plus dehydrated hemoglobin has been employed successfully in all laboratory procedures in connection with *Pasteurella tularensis*. If desired, 8-10 per cent human or animal blood may be substituted for the hemoglobin.

*Coagulated Egg-Yolk Medium* (C.M. No. 54)—This medium was described by McCoy and Chapin. According to Francis[5] (1938), "The simplicity of composition of the egg-yolk medium accounts for the first isolation of *Bacterium tularense* in the United States, Japan, Norway, Russia, and Austria. We have not used it for 10 years, but beginners in different countries have success with it. It is efficient for growth but is not very suitable for agglutination antigen production because the growth, when washed off, is opalescent and cannot be cleared."

Francis[6] (1922) describes its composition as follows:

"Fresh eggs are scrubbed with a brush in soap and water, if fecal matter is present on the shells, and then placed in a wire basket. The basket containing

the eggs is dipped into 95 per cent alcohol for a few seconds, after which time it is withdrawn and the small amount of alcohol which still remains on the basket and eggs is ignited in order to remove the alcohol and help sterilize the shells.

"While one person with clean hands holds an egg, grasping it at each end, an assistant strikes the shell in its middle with a sterile knife with sufficient force to crack the shell. The whites are separated from the yolks by decanting from one-half of the shell to the other, thus allowing the whites to drain away while the yolks are saved and collected in a sterile beaker.

"The volume of yolks is measured in a sterile graduate and to this is added sterile saline solution in the proportion of 40 per cent saline solution to 60 per cent egg yolk. Mix thoroughly. Tube in sterile test tubes, using a sterile funnel or a pipette.

"Place the tubes in metal racks constructed so as to allow one-half inch space between the tubes for circulation. Heat the racked tubes, in a slanting position for the first half hour at 70° C. and for the second half hour at 72° C. A uniform temperature is best maintained for this purpose in a water-jacketed chamber. The chamber should contain about a half inch of water above which the racks of tubes are exposed in the moist, heated air. After coagulation, paraffined sterile cork stoppers are substituted for the cotton plugs and the tubes are incubated upright for three or four days to insure against a slow-growing contamination.

"Instead of the jacketed chamber one may, with patience, use an Arnold steam sterilizer, a board having been placed at the bottom to protect the tubes from the direct steam.

"The finished medium should be soft; that is, the surface of the slant should yield slightly when pressed with a platinum loop, and to that end the medium should not be overheated. A glazed surface results from overheating. The tubes should be stored in the cold room, unexposed to the light. The water of condensation in a batch of medium which grew the organism very well showed a reaction having a pH of 6.8. No titration or adjustment of reaction has been done on batches of this medium used for routine cultivation of the organism in the laboratory."

## C. Maintenance of Stock Cultures

A large loopful of culture is carried over when making transfers, and this is spread over the surface of the slant. A control tube of plain agar should be inoculated at the same time. The inoculated tubes are incubated at 37° C. for 48 hours or until sufficient growth is present. The blood-glucose-cystine agar slants should show growth while the plain agar tube should remain sterile.

Stock cultures may be kept in a cold room at 2 to 10° C., preferably at 2° C., and may even be frozen. They should be transferred at intervals of 2 months. They can be stored in the dried state.[7, 8] The cells can be suspended in a protective solution such as 10 per cent Bacto skim milk or Bacto peptone, shell frozen, dried under vacuum,

and then stored at 10° C. Viable cultures have been recovered after several years' storage. However, the viable cells present have been relatively few as compared to the number initially dried.

It is believed that a reason for the reported loss of cultures of *Pasteurella tularensis* stored in the cold room is due to the failure to transfer a large amount of inoculum. The usual procedure of using a needle or small loopful of culture as employed by most technicians will not suffice with *Pasteurella tularensis* cultures. The older the culture the larger the inoculum necessary.

### D. Preparation of Antigen (Stock Suspension)

When only a small amount of antigen is desired, slants of blood-glucose-cystine agar (C.M. No. 35) are inoculated heavily from a 48 or 72 hour seed culture, using a platinum loop to make the transfer. After incubation for 48–72 hours, or when good growth is obtained, wash off and suspend the bacteria in physiological saline containing 0.5 per cent formalin. Standardize the suspension as directed below.

For the preparation of larger amounts of antigen, use Blake bottles, each containing 90 ml. of blood-glucose-cystine agar from which the water of condensation has been removed by pipetting and drying.

1. Plant one seed culture (inoculating it heavily from the well grown stock growth) for each Blake bottle to be used.

2. Incubate the seed cultures for 48–72 hours or longer if necessary to obtain abundant growth.

3. Add to each tube 1 ml. of sterile saline and suspend the growth in this fluid.

4. Transfer this suspension, using flame technic, to a Blake bottle. Each Blake bottle receives the abundant growth from one slant.

5. Spread the suspension over the surface of the medium in the Blake bottle, using a rocking motion. All excess moisture will be absorbed by the previously dried medium.

6. Incubate the Blake bottle cultures for 3 days at 37° C.

7. Examine and discard all contaminated cultures. To each of the uncontaminated bottles add 15 ml. physiological saline containing 0.5 per cent formalin (U.S.P. strength 37 per cent). Loosen the growth from the agar by employing a rocking movement, thus suspending it in this saline free from gross particles of culture medium.

8. Place the collected suspensions in the centrifuge and throw down the bacteria. Rewash twice if desired. Pour off the saline and re-

suspend the sedimented growth in the same amount of formalin-saline.

This constitutes the concentrated stock suspension; it will keep for at least 2 years in the cold and maintain its reliability for the agglutination test.

## E. TECHNIC OF THE AGGLUTINATION TEST

### 1. *Sera*

It is unnecessary to heat human sera which are to be tested for the presence of tularensis agglutinins.

The titer of agglutinating sera may be preserved and the serum itself protected from contamination by mixing it with an equal part of pure neutral glycerine. It is advisable thus to store the remnants of highly positive sera received for diagnostic tests.

For the preparation of specific agglutinating serum, the rabbit is used, since agglutinins are normally absent from the blood of this animal. The rabbit is injected intravenously with a living suspension of non-virulent bacilli, or with a suspension killed with 0.5 per cent formalin. A single injection of 2 ml. of a suspension having a turbidity of 2,000 by the silica standard or No. 4 by the McFarland nephelometer will as a rule give a good titer after 8–14 days. Sometimes two injections, 5 days apart, will be found necessary.

### 2. *Dilution of Antigen*

For agglutination tests the concentrated stock antigen is diluted with physiological saline solution to a turbidity of 500 by the silica standard (*Standard Methods for the Examination of Water and Sewage*[9]), or tube No. 4 of the McFarland nephelometer if the National Institutes of Health method is employed for setting up the test. On the other hand, if serum dilutions are made using the antigen suspension as diluent, a silica turbidity standard of 250 or nephelometer No. 2 should be used. A photoelectric colorimeter can be used to standardize the density of the antigen and will give a more accurate duplication.

### 3. *Serum dilutions*

#### a. *National Institutes of Health Method*

(1) Set in a row, for each serum, 8 agglutination tubes, 10 mm. in diameter and of 3 or 4 ml. capacity.

(2) Add to the first tube 0.8 ml. of physiological saline solution and to each of the others 0.5 ml.

(3) Measure 0.2 ml. of serum into the first tube only, giving a serum dilution of 1:5 and a total volume of 1 ml.

(4) After thorough mixing, pipette 0.5 ml. from the first and add it to the second tube; from the second, after mixing, 0.5 ml. is added to the third, and so on down the row.

(5) The 0.5 ml. from the 8th tube may be held in reserve for further dilution if the serum under test is greater than 1:1,280. The serum dilutions are then 1:5, 1:10, 1:20, etc.

(6) Add 0.5 ml. of the antigen, diluted to silica standard 500 or nephelometer No. 4, to each tube.

(7) Shake the rack of tubes thoroughly. The dilutions now, beginning with the first tube, are 1:10, 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, and 1:1,280.

b. *Dilutions made in the antigen*

(1) Set up a row of 8 tubes ½" by 4".

(2) Add to the first tube 1.9 ml. and to the other 7 tubes 1.0 ml. of the antigen diluted to silica standard 250 or nephelometer No. 2.

(3) To the first tube add 0.1 ml. of serum, thoroughly mix, withdraw 1.0 ml., and pipette to the second tube, and so on down the line. This gives dilutions of 1:20-1:2,560 and has the advantage of saving one procedure.

When serum has been preserved with an equal amount of glycerin, twice the amount of the mixture should be used; at the same time the amount of diluent should be correspondingly reduced. That is, at step a. (2) above, use 0.6 ml. of saline and 0.4 ml. of glycerine-serum; at step b. (2) use 1.8 ml. of diluted antigen and 0.2 ml. of glycerine-serum.

Time can be saved in making dilutions by using the syringe pipette (A. H. Thomas Co.).

## 4. *Incubation*

Place the tubes in a water bath at 37° C. or 50–52° C. for 2 hours and record the preliminary 2 hour reading, then transfer them to the cold room at 10° C. for overnight. Make final reading the following morning.

An alternate procedure giving excellent results is to hold at 37° C. overnight in an ordinary laboratory incubator and to make the readings the next morning.

5. *Reading the tests*

Complete agglutination is obtained when there is a compact sediment and a water clear supernatant. It is recommended that reports be made on a 4+ basis for all dilutions employed.

6. *Proagglutinoid zone*

This phenomenon appears occasionally, and sufficient dilutions should always be made so that its effect may not give incorrect results.

7. *Diagnostic titer*

No arbitrary statement can be made in answer to the question as to exactly what titer 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, and tularemia should not be ruled out on the basis of agglutinin tests, until negative results persist beyond the 14th day of illness. The importance of repeated tests, when there is a suspicion of tularemia, is entirely obvious.

With the more widespread use of vaccines for prophylaxis,[10] the diagnostic difficulties are increased. Vaccines[11, 12] have now been developed which have greater antigenicity than formerly reported so that agglutinin titers of 1:320 to 1:640 are obtained. However, these titers usually decline to 1:40 to 1:80 in a few months. If individuals with these vaccine titers become infected, it has been found that the agglutinin titer will drop, at least two tubes, coincident with or just after the first symptoms of illness. Then a gradual rise in titer to 1:1,280 or higher will occur, i.e., to a higher level than is obtained by the vaccine. These titers due to infection are maintained for a year with very little fall.

Immune individuals[11] may also show this phenomenon if they are vaccinated. These individuals will however have a severe systemic reaction which precludes their being vaccinated.

The use of antibiotics or chemotherapeutic substances has not been found to interfere with or obscure the diagnostic methods. Publications[11-16] on the use of streptomycin in the treatment of tularemia all report that the usual rise in agglutination titers has occurred.

### 8. *Cross-agglutination*

Francis and Evans[17] (1926) showed that the agglutination test in tularemia is one of the most reliable in the whole field of serology and that tularemia sera do not agglutinate *Salmonella typhosa,* A and B species of *Salmonella, Pasteurella pestis, Shigella dysenteriae,* meningococcus, pneumococcus, nor *Proteus vulgaris X* 19.*

They 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. It is important to watch the progress of the test in the water bath and to record the results at the end of 2½ hours at 37° C., before transferring the tubes to the icebox overnight. If the serum under test is from a case of tularemia, the *tularensis* antigen will be agglutinated earlier and in more highly diluted serum than will the *abortus* antigen—in fact, the *abortus* antigen may not show any agglutination until the next morning. If the serum is from a case of undulant fever the reverse may occur.

The conclusions reached by Francis and Evans[17] (1926) are:

"1. That on account of the frequent cross-agglutination between *tularensis,* on the one hand, and *abortus* and *melitensis,* on the other, sera from suspected cases of tularemia and undulant fever should be tested for agglutination of *tularensis* and either *abortus* or *melitensis,* unless the clinical history points definitely to a recognized source of infection for tularemia or undulant fever.

"2. 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.

"3. That a serum which agglutinates all three organisms to the same, or nearly the same titer, should be subjected to agglutinin absorption tests."

It appears appropriate to report that the rapid agglutination test, both tube[19, 20] and slide methods, has been employed in the diagnosis of tularemia. The rapid tube test has been done by vigorously shaking in a Kahn shaker for 4 to 5 minutes and read. The rapid slide method used has followed that outlined for the diagnosis of brucellosis. If such a test is needed in tularemia diagnosis, further comparative studies should be done.

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* However, reports of fairly high *Proteus vulgaris X* 19 titers have been recorded in cases of tularemia.[18]

## III. METHODS FOR THE ISOLATION OF *PASTEURELLA TULARENSIS*

While it is sometimes possible to isolate *Pasteurella tularensis* directly from man by planting infected tissues or fluids on a suitable culture medium, the best procedure is to inject the inoculum into guinea pigs, rabbits, or white mice—preferably guinea pigs. The culture medium can then be inoculated from these animals after killing with ether when death is impending.

### A. ANIMAL INOCULATION

The following material may be used for the inoculation of animals: secretion from the sites of fly or tick bites or finger lesion, from the conjunctiva, from pleuritic effusion, from peritoneal or spinal fluids, from pneumonic sputum,[24, 25] from the suppurating glands of a patient within the first to the fifth month, or from the spotted spleen or liver of wild rabbits or other infected animals. Any of this material should be ground in a mortar, suspended in saline solution, strained through coarse gauze, and inoculated subcutaneously into guinea pigs or rabbits. Parasites such as ticks, suspected of carrying the infection, should be triturated in sterile saline and the inoculations made as described. If it is desired to isolate the organism from the blood of a patient, at least 5 ml. of blood should be drawn in the first week of illness. After defibrination this is mixed with an equal amount of normal saline and injected intraperitoneally into guinea pigs, each animal receiving 4 ml.–8 ml. of the diluted blood. Simpson[23] (1929) states that since an early bacteremia occurs in man it is possible to isolate the organism from the blood of the patient as early as the 3rd day of the disease, but no positive results have been obtained after the 12th day.

The organism has been and can be recovered from naturally infected water by direct inoculation into guinea pigs (Parker, *et al.*,[21] 1940).

Animals injected as above should die within a week or 10 days if *Pasteurella tularensis* is present in the suspected material. At autopsy they should show lymph glands with gray granular caseation, and small white foci of necrosis in an enlarged dark spleen and liver. Lillie, Francis, and Parker[22] (1936) emphasize the fact that the gross pathologic evidence of the disease should be considered more reliable than smears from the organs because the smears are usually negative for bacteria. Inasmuch as the lesions may be minute, a hand lens may be necessary to detect them. When the animal dies within the specified

time, transfers should be made to other animals even though gross lesions are absent. Make cultures on blood-glucose-cystine agar (C.M. No. 35) from the lymph nodes, liver, spleen, and heart's blood—especially from the blood.

In some laboratories animals are inoculated in duplicate; one animal receives an intraperitoneal injection and the other has the suspected material rubbed upon the shaved, abraded skin of its abdomen. As a general rule both animals will die within a week or 10 days if *Pasteurella tularensis* is present in the inoculum. There will be no pus at the site of the inoculation, but a hemorrhagic edema will be noticeable.

If it is desired to ship infected material, it should be placed in pure glycerine. Spleens of infected animals are the best organs to use for this purpose. Liver is unfavorable to the living organism, and, therefore, should not be placed in the same container with other organs. Parasites (ticks, etc.) can be placed in a test tube which is corked, or, better, plugged with cotton over which gauze is tied. The usual double shipping container should, of course, be utilized in all cases.

## B. CHARACTERISTICS OF PASTEURELLA TULARENSIS

In order to make a definite diagnosis of tularemia the organism isolated from suspected material should conform to the following criteria:

### 1. *Morphology*

A short rod, 0.2 by 0.3–0.7 microns, which occurs singly and exhibits extreme pleomorphism, coccoid forms being always present. In old cultures it frequently becomes coccoid but returns to the rod shape again when transferred to fresh medium. Occasional bipolar forms are noted. The organism is non-motile, and non-spore forming.

### 2. *Staining*

Gram-negative. Stains readily with crystal violet or aniline methyl violet. In animal tissue sections Giemsa's stain is the most satisfactory, but it is doubtful whether the organism has been stained in sections of human tissues.

### 3. *Cultural characteristics*

It does not grow on plain agar. Grows best on coagulated hen's egg yolk (C.M. No. 54), or blood-glucose-cystine agar (C.M. No. 35).

Strict aerobe. Optimum pH for growth 6.8–7.3. Optimum temperature 37° C. On favorable media produces minute, viscous colonies which may attain a diameter of 4 mm. when they are separated. In his study of 60 cultures on a serum cystine agar medium plus the carbohydrates, Francis[27] (1942) observed fermentation with production of acid but no gas. Dextrose, maltose, and mannose were fermented by all; glycerine by 53; levulose by 17; and dextrine was fermented more or less by about half of the cultures.

*Pasteurella tularensis* has been successfully cultivated in the embryonated egg.[28-30]

## IV. THE BACTERIAL SKIN TEST

The intracutaneous test, which is an exceedingly interesting immunological reactor, is not a normal laboratory test. For those individuals who are interested in this bacterial skin test, it is suggested that they contact Dr. Lee Foshay, University of Cincinnati, College of Medicine, Cincinnati, Ohio. Dr. Foshay has kindly agreed to furnish this material or information on the test to individuals who will fill out, and return, a questionnaire giving the results of their tests.

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# The Cholera Vibrio

## (*Vibrio comma, Vibrio cholerae*)

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I. Introduction
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## I. INTRODUCTION

THE problem of isolating and identifying the cholera vibrio is presented very infrequently to laboratories in the continental United States. However, with increasing rapidity of transportation the possibility of the infection by-passing quarantine measures which have hitherto been successful should be considered, particularly in connection with modern air transport. The disease is constantly active in India and China; from these foci it is always capable of spread to the rest of the world. In 1947 the disease extended to Egypt and Syria. Although the commonest mode of spread is by contact with cases and convalescent carriers or their belongings, water supplies may furnish a means of transmission. The carrier state may also be established in individuals in contact with the disease and may be entirely symptomless.

The presentation to be given here is largely for the use of laboratories only rarely called upon for the isolation and identification of *Vibrio comma,* and which must act promptly with materials at hand.

More refined methods of enrichment and isolation will be given under section D of the description of laboratory procedure; they will not be stressed since considerable time is required for their preparation, and they would therefore not be immediately available.

## II. DESCRIPTION AND CLASSIFICATION

The classification of *V. comma* is based on morphological, biochemical, and serological criteria, all of which are of importance, with the biochemical criteria exhibiting anomalous behavior somewhat more frequently than the other attributes of the organism. The vibrios are Gram-negative slightly curved rods 2–4 micra in length and about 0.5 micra in width, roughly the same size range as the *Salmonella* group. Many forms will appear perfectly straight; occasionally two or more curved forms will be attached end-to-end to form an S or spiral shape. They are motile having a single polar flagellum. No spores are formed and capsules have not been described. *V. comma* is much more susceptible to chemical disinfectants than are the other enteric pathogens. Suspensions of the organism undergo lysis in the presence of phenol or chloroform, and as a result of heating or repeated freezing and thawing. However, growth occurs in the presence of an alkalinity which is inhibitory to most of the enteric organisms (pH 9–9.5).

*V. comma* is predominantly aerobic though a small amount of anaerobic proliferation occurs. On the surface of nutrient agar growth is luxuriant; in nutrient broth it is delicate and a pellicle is usually developed in 24 hours. With few exceptions dextrose, maltose, mannite, and sucrose are fermented without gas formation, and lactose, dulcitol, and arabinose are not acted upon. Gelatin is liquefied. Indol is formed and nitrate is reduced to nitrite, these two simultaneous activities being responsible for the "cholera red" reaction which is elicited by the addition of a small amount of concentrated sulfuric acid to a peptone water culture. The cholera red test is of limited diagnostic value since some non-pathogenic vibrios show it. Moreover certain batches of peptone are unsuitable and a negative reaction is therefore unreliable unless the particular medium gives a positive reaction with known strains of the organism.

Much attention has been paid to the hemolytic activity of *V. comma*. Most strains produce clearing of the medium around colonies on sheep or horse blood agar, but this is said to be due to hemo-digestion rather than to the effect of a soluble toxin. The Grieg test performed by

mixing equal volumes of a broth culture and a suspension of goat erythrocytes (5 per cent) is usually negative (no hemolysis after 2 to 4 hours at 37° C.) with the true cholera vibrios, but Grieg-positive strains have been noted which appear to be identical with true cholera vibrios in every other respect including antigenic composition and pathogenicity.

The serological classification of the *Vibrio* genus has been discussed recently by Linton (1940),[1] and Burrows (1946)[2] with extensive literature citations. Briefly, a heat labile H antigenic mosaic which has been broken down into at least 10 components is distributed over the whole genus appearing in both cholera and non-cholera vibrios. The heat-stable O antigenic complex is more specific. Six or more groups designated by Roman numerals have been determined in the genus, and O antigenic group I contains almost all the pathogenic or true cholera strains. The group I heat-stable O antigen has been analyzed by agglutinin absorption into 13 components. Burrows (1946)[2] has designated these by capital letters and, in agreement with earlier workers, has found that the various types in group I are due to variations in at least 3 of the O components. Antigenic formulae based on these factors might better be used in place of the Japanese names usually applied to the *V. comma* types; thus Ogawa (AB), Inaba (AC), Hikojima (by inference ABC), and a hitherto undescribed type (A). The average diagnostic laboratory will not be concerned with the typing of cholera vibrios. Diagnostic agglutinating sera, however, must be devoid of H antibody, and must be capable of agglutinating both AB and AC types.

## III. LABORATORY PROCEDURES

### A. ISOLATION AND IDENTIFICATION

In acute cases of cholera the direct microscopic examination of carbol-fuchsin stained films of the intestinal dejecta is of some value since the curved organisms may be seen in large numbers often lined up in parallel arrangement. Because of the delicacy of the organism cultivation procedures should be carried out as promptly as possible; if delay or shipment of the specimen is necessary see section C on storage of material. Although *V. comma* is lactose-negative, it is inhibited on most of the usual plating media used in the primary isolation of other enteric organisms from feces, but these other organisms are in turn inhibited by conditions of pH to which the cholera vibrio is indifferent, namely

pH 9–9.6. This fact is made use of in isolation techniques. The suspected fecal material is streaked on alkaline (pH 9–9.6) nutrient agar and inoculated heavily into a tube of peptone water (1.0 per cent peptone, 0.5 per cent NaCl) of the same pH. After about 6 hours incubation at 37° C. the vibrio growth will be concentrated at the surface of this alkaline fluid medium, and the material from the surface is then streaked on alkaline agar.

The agar may be adjusted to pH 9–9.6 either electrometrically or by means of thymol blue as an indicator. If neither method is available, melt 3 tubes of nutrient plating agar (about 15 ml. per tube). Make up a solution of 10 per cent NaOH which may be assumed to be sterile when solution is complete. Add 1 drop of the NaOH solution to the first tube of agar, 2 drops to the second, and 3 to the third, mix and pour plates. All three plates are streaked, and one will be at about the desired alkalinity. This procedure may not be applied to the preparation of the alkaline peptone water which must be adjusted more accurately.

Colonies of *V. comma* on nutrient agar resemble those of *Salmonella* or *Shigella* in form and size except that they are usually more transparent. Portions of suspected colonies are rubbed up on a slide in small pools of specific serum of the proper dilution (see section B) and agglutination demonstrated after rocking the slide. Colonies showing agglutination are examined microscopically for vibrio morphology and staining, transferred to nutrient agar slants, incubated for 24 hours at 37° C., and the resulting growth transferred to the proper carbohydrate media, namely, dextrose, mannite, maltose, sucrose, lactose, dulcitol, and arabinose. A tube of 1 per cent Bacto tryptone containing 0.001 per cent potassium nitrate is also inoculated if the particular batch of trypone in use is known to be suitable for the cholera red test. After 24 hours at 37° C., 2 to 3 drops of concentrated sulfuric acid are added; a deep rose pink color constitutes a positive reaction. After inoculating the carbohydrate and tryptone broths, the 24 hour agar slant culture is washed down with 4 to 5 ml. of saline; the resulting suspension is used to determine the highest reactive dilution of the specific agglutinating serum against this organism.

## B. Preparation of Diagnostic Agglutinating Serum

For the preparation of specific agglutinating serum to be used for identification, suspensions containing about 10 billion vibrios per ml.

of the Ogawa (AB) and the Inaba (AC) types are mixed in equal volume, sealed in an ampoule, and completely immersed in a boiling water bath for 2 hours. This is necessary to destroy the heat-labile H antigen which is not specific for the pathogenic vibrios. Burrows (1946)[2] prefers to boil the suspension under a reflux condenser for 2 hours claiming that the former procedure fails to destroy the H antigen completely. The suspension is then injected into rabbits at 4 to 6 day intervals, 4 to 5 doses of 1 ml. usually being sufficient. The first 2 doses are given intraperitoneally, the last 3 intravenously. The animals are bled one week after the last injection. Three serum end points should be determined and confirmed occasionally with known Inaba and Ogawa strains: (1) The serum dilution which gives a reasonably prompt slide agglutination with both types, for use in the picking of selected colonies from streak plates; (2) the tube agglutination titer for the Inaba type, and (3) the same for the Ogawa type.

Living vibrio suspensions from 18 to 24 hour agar cultures should be used in these titrations. The tube agglutinations are incubated at 37° C. for 2 hours and overnight in the refrigerator. The agglutination is fine and granular. Types other than Inaba and Ogawa will not be expected to conform exactly to the titers as determined above. Individuals carrying out these determinations must use reasonable precautions, pipetting vibrio suspensions with rubber tubing on the pipettes; they should also be vaccinated since laboratory infections are not uncommon.

## C. STORAGE OF SPECIMENS

The following procedure devised by Venkatraman and Ramakrishnan (1941)[3] permits delay in the cultivation of the organisms from feces. If more than 2–3 hours are to elapse, 1 to 3 gm. of feces or intestinal discharge are mixed with 10 ml. of the following buffered alkaline salt mixture:

| | |
|---|---|
| Boric acid | 3.1 gm. |
| Potassium chloride | 3.72 gm. |
| Hot distilled water | 250 ml. |

Dissolve, cool, and add 133.5 ml. of 0.2 M NaOH solution. Dilute to 1 liter, add and dissolve 20 gm. of NaCl, and if turbid, filter through paper. The pH of this mixture should be 9.2. Dispense in 10 ml. amounts in 1 oz. wide mouth screw-cap containers and autoclave.

## D. SPECIAL MEDIA

### 1. *Enrichment*

An enrichment medium which gives better results than the alkaline peptone water described in section A, is that described by Wilson and Reilly (1940).[4] Its use is the same as the alkaline peptone water.

*Solution A*

| | |
|---|---|
| Peptone | 10 gm. |
| NaCl | 20 gm. |
| Distilled water | 1 liter |

Adjust to pH 9.1 with 14% sodium carbonate solution, and autoclave in 100 ml. amounts

*Solution B*

| | |
|---|---|
| Anhydrous sodium sulfite | 20 gm. |
| Boiling water | 100 ml. |

When dissolved add

0.1 gm. bismuth ammonio citrate dissolved in 10 ml. boiling water.

Cool and add

100 ml. 20% glucose solution which has been boiled and cooled.

To each 100 ml. of solution A, add 10 ml. of solution B and 1 ml. of absolute alcohol just before inoculating.

### 2. *Plating*

Dieudonne's agar for streaking material suspected of containing *V. comma* has been used widely and is said to be favorable for the isolation of small numbers of vibrios.

| | |
|---|---|
| Beef or sheep blood, defibrinated | 15 ml. |
| 1 N KOH solution | 15 ml. |
| Nutrient 3% agar pH 7 | 70 ml. |

Combine the blood and KOH solution and heat in a boiling water bath or Arnold steamer for 30 minutes. Add the melted agar, pour plates and leave the lids ajar for about 15 minutes before covering. The plates are not ready to use until they have been incubated overnight at 37° C.

## E. ISOLATION FROM WATER

The same principles are used as in the isolation from feces. One hundred ml. of suspected water are added to 10 ml. of concentrated peptone water (peptone 10 per cent, NaCl 5 per cent) which has been adjusted to pH 9.5 and autoclaved. After about 6 hours' incubation at 37° C., and again after 24 hours', the surface growth is streaked on alkaline agar, with further treatment of these plates as in section A above.

## IV. IMMUNIZATION

Examination of the extensive literature dealing with immunization against cholera indicates that there is some disagreement as to its effectiveness. There are, however, enough well controlled experiments to justify the conclusion that vaccination against this disease is of definite value. One of the most recent of these well controlled experiments involved a large group of natives in India.[5] There were 1.33 million persons followed, of whom about 400,000 received a single dose of cholera vaccine. The incidence of the infection was 11.4 times as high in the unprotected as in the protected group. Immunity began to appear on the fourth day, and had reached its height on the tenth day after vaccination. Other studies have indicated that, although vibriocidal antibodies may persist for over a year in immunized persons, resistance declines after about 6 months, at which time a stimulating injection should be given to those individuals in endemic areas.

The vaccine in current use consists of an 18 hour growth on infusion agar emulsified in sterile salt solution, standardized to contain 8,000 million vibrios per ml., and killed by adding phenol to a final concentration of 0.5 per cent. No further preservative is used. Equal volumes of vaccine made from Inaba (AC) and Ogawa (AB) types are pooled before bottling, with the usual controls for sterility. The final vaccine thus consists of 4,000 million Inaba and 4,000 million Ogawa vibrios per ml. in 0.5 per cent phenol saline. Due to the rapid lysis of the organisms in phenol solution, all standardization, including turbidimetric methods, must be done prior to the addition of phenol, and within an hour or two after harvesting the growth. On storage the vaccine may become almost water-clear; this does not interfere with its activity. It is given subcutaneously in two doses, the first 0.5 ml., the second 1.0 ml., at 7 to 10 days' interval. Reactions are not more severe than to typhoid-paratyphoid vaccine. A stimulating dose of 1.0 ml. subcutaneously is given at 6 months intervals to individuals under constant risk.

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# The Laboratory Diagnosis of Anthrax

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I. Introduction
II. Morphological Identification of *Bacillus anthracis* in Tissues
III. Identification of *Bacillus anthracis* by Cultural Methods
IV. Animal Inoculation
V. Lesions of Anthrax in Experimental Animals
VI. Diagnosis of Anthrax by the Thermo-Precipitation Test of Ascoli
VII. References

## I. INTRODUCTION

ANTHRAX is an infectious disease to which practically all warm-blooded animals are susceptible. The herbivorous animals are most susceptible to natural infections, sheep being more highly susceptible than cattle, and cattle more than horses and mules. It is in these species that the disease is most often seen. Carnivorous animals and birds are quite resistant but cases occur now and then in dogs, foxes, and mink which have fed upon carcasses of animals dead of the disease. Man and swine, omnivorous types, have a degree of susceptibility intermediate between that of the herbivora and the carnivora.

In the more susceptible species, and sometimes in the others, the disease becomes generalized after developing as a localized infection. When generalization occurs the blood stream usually becomes choked with bacilli and death occurs shortly, usually within several hours thereafter. Death sometimes occurs when there are few organisms in the blood stream, especially in swine. The more resistant animals usually succeed in keeping the disease localized, in which case the bacilli are phagocytosed and destroyed, and the animal recovers.

In man anthrax may be localized or generalized. The localized cases usually are the result of wound infections, the so-called "malignant carbuncles." These are usually located on the hand or forearm, as a result of handling infected carcasses or other contaminated

materials; on the face, as a result of infection from shaving brushes made from bristles containing anthrax spores; or on the back, as a result of carrying infected materials, especially dried hides, in stevedore fashion on the bare back. The generalized infections may occur as extensions of the localized infections of the skin, as a result of inhalation of spores from dried hides, hair, or bristles, or as a result of massive infection of the digestive tract through the eating of raw or imperfectly cooked meat of animals dead of anthrax. Digestive tract infections seldom occur among civilized peoples, but are said to occur among savage tribes.

The diagnosis of anthrax in the laboratory may be made either (a) by recognizing the *Bacillus anthracis* in the material at hand or, (b) by recognizing the antigens of this organism. If the material is fresh, it is easiest to isolate and identify the causative organism. In old and contaminated material, the antigens may be identified when it is impossible to isolate the organism.

## II. MORPHOLOGICAL IDENTIFICATION OF *BACILLUS ANTHRACIS* IN TISSUES

The tentative identification of anthrax bacilli in films and sections made from fresh exudates and tissues usually is easy and quite reliable. If putrefactive changes have occurred, the situation is complicated by the fact that some of the anaerobic organisms likely to be present have a morphological resemblance to the bacillus of anthrax. If facilities for culturing the material and for the inoculation of experimental animals are available, it is best always to confirm the morphological findings even when the material appears to be in good condition.

In generalized anthrax the bacilli usually are numerous in the blood, and in all organs containing blood. It is characteristic of *Bacillus anthracis* that the bacilli are always found in the blood vessels, except in the case of hemorrhages, and except for the local phlegmons which mark the point of entry of the infection into the body. For diagnosis of generalized infections it is best to examine the spleen, which usually is greatly engorged with blood and in which unusually large numbers of bacilli are found. It should be borne in mind however that fatal cases may occur in which bacilli are not found, or at least not in abundance, in the general blood stream. If a localized edematous area is found, especially in the neck region of swine, this should be subjected to examination.

In films of spleen pulp, blood, or edema fluid, made on glass slides and stained with any of the ordinary dyes commonly used in bacteriology, *B. anthracis* appears as large, straight-sided rods, rather square-ended, and occurring in chains of from two to six elements, or as single cells. The bacilli are encapsulated, a single capsule enclosing as many organisms as constitute the chain. The capsular material is not well seen in preparations stained with ordinary dyes, but imperfectly stained granular material, haloes, and ragged edges are indications of its presence. If the films are stained with some of the polychrome stains commonly used in hematology, *i.e.*, Wright's, Giemsa's, etc., the capsules may be demonstrated as a pinkish substance surrounding the bluish-black bacterial cells. Thick films of blood or spleen pulp, imperfectly fixed by passing them through a flame several times after drying, and stained with Loeffler's methylene blue solution, usually show purplish stained granular debris around groups of the bacterial cells. McFadyean[2] regarded this reaction as practically specific for anthrax. Anthrax bacilli in tissues never show spores unless the tissue has been exposed to the air for some time.

When making a tentative diagnosis of anthrax on the basis of the morphological findings, one must be on his guard against interpreting certain anaerobic organisms as anthrax bacilli. These are found when the tissue has undergone putrefactive changes. These organisms may be present in large numbers, and while their true nature may be recognized as a rule, it is often impossible to know whether or not anthrax bacilli are among them. Most of the anthrax-like organisms found in tissues tend to form long chains, and this feature is helpful in differentiating them. In putrefied materials it is best not to attempt to make a diagnosis by microscopic examination, but to wait until other tests have been completed before making a decision.

In view of the fact, also, that anthrax bacilli may not always be numerous in the blood and spleen pulp, a negative diagnosis should be made cautiously.

There are many common aerobic spore-bearing bacilli that resemble *B. anthracis*, morphologically. These are never found in considerable numbers in blood or tissues recently removed from a dead animal, hence they are of no concern in direct microscopic examinations. They may be encountered in films made from open surfaces, and they are often found, culturally, in materials that have been packed and shipped in unsterile containers.

## III. IDENTIFICATION OF *BACILLUS ANTHRACIS* BY CULTURAL METHODS

Since *B. anthracis* will develop on the simplest of organic infusions, it is easily cultivated from infected tissues providing these tissues are not heavily contaminated by organisms which outgrow it. Plain extract (C.M. No. 4a) or infusion agar (C.M. No. 5a) slants are entirely satisfactory. These are streaked with the suspected materials and incubated for 24 hours at 37° C. If anthrax bacilli are present they should be evident as well developed colonies at that time. From reasonably fresh animal tissues pure or nearly pure, cultures usually are obtained.

The colonies on agar surfaces are characteristic, but not diagnostic, for *B. anthracis*. They are relatively flat, translucent, grayish, granular, and have an uneven margin. Often they are somewhat comet-shaped. If uncrowded on the medium they may attain a size of 3 to 5 mm.—rarely larger. When viewed by transmitted light, the characteristic "ground glass" appearance may be seen. Under low magnification the margins of the colonies exhibit the appearance of carefully combed coiffures, because of the long wavy filaments which lie in parallel formation like locks of hair. This is the "Medusa-head" appearance, so often described.

This description applies to virulent strains of *B. anthracis* which occur in the rough form. Avirulent and vaccine strains often appear in the smooth or intermediate form of which the colony characteristics may be very different, but these have no significance in diagnosis and will not be discussed here. Good descriptions of colony forms and other characteristics of anthrax and anthrax-like bacteria are given by Stein.[4]

Many of the nonpathogenic, aerobic spore-bearing bacilli, commonly found in dust, form colonies which resemble those of *B. anthracis*. Most of these can be readily differentiated by those who are thoroughly familiar with the appearance of the true anthrax bacillus, but in some instances this is not possible. Since the anthrax bacillus is never motile it is helpful to examine the periphery of young colonies microscopically to detect possible motility. This can be done by dropping a flamed cover glass on the area and examining with the 4 mm. objective. If motility is detected, the possibility of its being *B. anthracis* is eliminated. If no motility can be detected, it is helpful to note the arrangement of the filaments in the marginal area. True anthrax colonies will always exhibit the beautiful long wavy filaments previously mentioned

providing the colony is not crushed by the cover glass and the normal arrangement broken up. Colonies with short criss-crossing filaments are never *B. anthracis.*

In culturing doubtful or contaminated materials it is helpful to make pour instead of streak plates in order that deep colonies can be observed. The deep colonies of *B. anthracis* are brownish, ragged, and stringy, reminding one of small whisps of colored cotton. The deep colonies of most anthrax-like organisms are compact. Some fail to develop in the depth of the medium.

Whereas *B. anthracis* will grow on nearly all kinds of laboratory media, in general it does not develop so luxuriantly as most of the saprophytic spore-bearers which cause confusion in diagnosis. On agar the true anthrax colonies are usually smaller than the saprophytes, particularly after 36 or 48 hours of incubation. On fluid media, it does not form a pellicle whereas many of the saprophytes do. The growth in fluid media usually is rather meager unless the medium is in very shallow layers, or air is bubbled through the fluid during incubation. If tubes of fluid media are incubated without agitation, a fine cottony mass usually develops near the surface after a few hours, but this usually sinks to the bottom before the end of the first 24 hours incubation period. There is little change in the appearance of the culture thereafter except a small ring of whitish growth may be deposited on the wall of the tube at the margin of the surface of the medium.

Other cultural features that are of some value in differentiating *B. anthracis* from similar aerobic spore-bearing bacilli are as follows:

Hemolysis:

*B. anthracis* produces little or no lysis of blood cells; many but not all of the saprophytes are actively hemolytic because of alkali formation.

Reduction of Methylene Blue:

*B. anthracis* usually will not reduce methylene blue in broth within 48 hours, whereas many of the saprophytes will rapidly and completely reduce this dye.

Fermentation of Salicin:

Salicin is not attached by *B. anthracis.* Many saprophytes will produce marked acidity from this substance.

Stein[4] who made a comprehensive study on the differentiation of *B. anthracis* from other aerobic spore-bearing organisms arrived at the conclusion that until better criteria are discovered it is advisable to depend principally on pathogenicity for identifying *B. anthracis.* In this advice the author fully concurs.

## IV. ANIMAL INOCULATION

Inoculation of experimental animals constitutes the most reliable way of isolating and identifying anthrax organisms in infected tissues. This method always succeeds unless the tissues contain extraneous organisms which cause death of the experimental animals before anthrax infection has had time to develop.

White mice and guinea pigs are the best experimental animals. Rabbits may be used, but birds and rats are not suitable because of their comparatively high resistance to anthrax. The material should be inoculated subcutaneously in the abdominal region. Since fewer than 100 bacilli will ordinarily kill mice and guinea pigs, it is not necessary to administer large doses.

The inoculated animals usually show little evidence of the disease for 24 to 36 hours. If they are picked up and examined, however, it will be found that doughy swellings have appeared around the points of inoculation. Acute illness generally appears only 2 or 3 hours before death occurs. When large doses are given, deaths may occur in less than 48 hours, but more often they occur on the 3rd or 4th day. When the inoculum contains putrefying tissue, the experimental animals often are dead in less than 48 hours. In these instances, other animals may be inoculated by rubbing the inoculum into scarified areas of the skin. This superficial inoculation discourages the development of the anaerobes but is not unfavorable to the development of anthrax bacilli should they be present. When a great deal of putrefaction has occurred, the rather delicate vegetative forms of the anthrax bacillus usually are destroyed and no method of isolation can succeed. In these cases a laboratory diagnosis can be made only by the recognition of the specific antigens of the bacillus in the tissues.

Animal inoculation is the best method of detecting anthrax spores in samples of bristles from shaving brushes, in soil samples, and on contaminated objects of any kind. Since the spores are very resistant to drying, viable spores may be found on objects which have been contaminated long before.

## V. LESIONS OF ANTHRAX IN EXPERIMENTAL ANIMALS

The dead animal should be tied out on a tray and the abdomen washed with water or an antiseptic solution to lay the hair. The skin

is then incised down the median line and reflected from the entire abdominal floor. Near the point of inoculation a yellow, gelatinous exudate will be found. Often this has spread through the subcutaneous connective tissue over the greater part of the abdomen. The blood vessels in this tissue are engorged and often, near the inoculation point, there are hemorrhages. The tissues are not reddened, as in many of the phlegmons produced by anaerobic bacilli, and gas is never present.

The peritoneum is quite moist but there is not much free fluid as a rule. The mesenteric vessels usually are congested. The most typical change is seen in the spleen. This organ often is several times its normal size, its margins are rounded instead of sharp, and it is much darker than normal. Whereas the normal spleen of the guinea pig or mouse is much lighter in color than the liver, in anthrax it usually is much darker and often quite black. The liver, kidneys, heart, and lungs show no diagnostic changes.

Anthrax bacilli can be demonstrated very easily in films made from any of the tissues. The engorged spleen is always rich in organisms, and is preferred for this purpose. A bit of the organ is pinched off with a pair of fine thumb forceps, and the film made by rubbing the cut surface very gently along the surface of a well cleaned slide.

Confirmation of the identity of the organisms in the tissues of experimental animals is not necessary, since the appearance of the lesions and of the stained films is quite diagnostic. It is a simple matter, however, to isolate the organism and study its cultural features if desired.

## VI. DIAGNOSIS OF ANTHRAX BY THE THERMO-PRECIPITATION TEST OF ASCOLI

The only practicable serological test which has been used successfully for the diagnosis of anthrax is that developed by the Italian, Ascoli.[1] This test has been widely used in some European countries for detecting anthrax-infected dried hides. The test may be used for detecting anthrax-specific antigens in materials of any kind. The test is very simple and rapid. Its only disadvantage is the great difficulty in securing a suitable antiserum. Most anthrax sera are not suitable for this test.

In applying this test, the suspected materials are chopped up and

placed in a small amount of saline solution in tubes. These tubes are then placed in a boiling water bath for 5 to 10 minutes, during which time some of the anthrax-specific antigens are dissolved out if the tissue contains living or dead anthrax organisms. The material is filtered until a clear filtrate is obtained. This is then layered, in narrow tubes, on undiluted anthrax-precipitating serum which has been proved by test. At the line of contact between the anti-serum and the tissue extract, a whitish ring will form after a few minutes if anthrax antigens are present in the extract.

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# The Laboratory Diagnosis of Glanders

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I. Introduction
II. Diagnosis
A. Microscopic examination
B. Cultures
C. Animal inoculation
D. Allergic tests
E. Serologic tests
III. References

## I. INTRODUCTION

IT is fortunate that the need for diagnosing glanders in North America has almost disappeared since the disease is now very rare. This situation is very different from that which prevailed at the beginning of the present century when it was a common disease in city horses, and from that reservoir was commonly carried to country districts through traffic in animals which had become too old or were otherwise unsuited for city work but could still be used on farms. It is not certain that glanders is wholly extinct, however, even though no cases have been reported for some years. When glanders was common in horses, occasional cases were recognized in human beings. Outbreaks also occurred now and then in zoölogical parks among members of the cat family which had been fed upon infected horse meat. Cases have been reported in goats, sheep, and dogs but these are rare. Cattle and pigs appear to be wholly immune.

The disease is manifested in members of the horse family in two ways, known, respectively, as *glanders* and *farcy*. In the form of glanders, the lesions generally are found in the lungs and upper respiratory tract with frequent localizations in other organs. In farcy, the lesions involve the lymphatic vessels and nodes of the subcutaneous tissue and the skin, secondarily, by ulceration. Sometimes both forms of the disease occur in the same animal.

In whatever form the disease develops it is characterized by the formation of nodules, or infectious granulomas, not greatly unlike those of tuberculosis. These nodules, when located under the mucous membrane of the upper air passages or under the skin, have a tendency to ulcerate to the surface. These discharge a glutinous pus which contains the causative organism. The disease is transmitted to other individuals largely through contamination with these discharges. Lung lesions occur very regularly in equine glanders and frequently in human glanders as well. These lesions may be nodular, or they may assume the form of a diffuse pneumonia.

Acute glanders in the horse may be diagnosed with considerable certainty by clinical examination alone, but the more chronic and latent forms cannot be recognized in the living animal except by use of the allergic test (mallein), or by one of the serological tests. It was by the use of these tests, singly or in combination, that infected animals were eliminated from our large city horse stables, and the main sources of infection were thus recognized and eliminated.

Most cases of human glanders have occurred in persons who have had much to do with horses—horse owners, stablemen, and veterinarians, but a considerable number have acquired their infections in the laboratory while working with cultures of the glanders bacillus. *Malleomyces mallei,* the causative organism, is one of the most dangerous organisms for laboratory workers that we have.

Human glanders is manifested in many different ways.[1,2] Most cases are acute and result in death in about one week. A few cases are chronic and may run a course of several years with acute exacerbations from time to time.[3,4] The lesions in man are similar to those in horses. In the acute cases, the patients are prostrated, there is likely to be a mucopurulent nasal discharge, and pustular eruptions may occur over most of the body surface. In the more chronic forms there are nodules which break down forming abscesses in the subcutaneous tissue, among the muscles, in the joints, and in the internal organs. Lung lesions, and lesions in the respiratory mucosa similar to those of horses, are frequent.

## II. DIAGNOSIS

### A. Microscopic Examination

In suspected glanders, microscopic examination should be made of the pus from one of the discharging lesions. In the more acute

processes the causative organism can usually be found, though not in great numbers, as rather long, slender rods which sometimes have clubbed ends, and are often beaded. The bacillus is not stained with the Gram technic, hence this stain should not be used to find the organism. It stains with most other stains but the characteristic beading is best seen in films stained with methylene blue. In the older lesions the organism often cannot be recognized in stained films, hence a negative microscopic finding is never conclusive.

### B. CULTURES

Cultures may be made from discharges or, better still, from incised, superficial nodules from which pure cultures often can be obtained. Since the glanders organism grows rather slowly it generally will be overgrown by contaminating bacteria when they are present. In such cases, pure cultures may sometimes be obtained by inoculating a guinea pig subcutaneously with the pus, destroying the animal after two or three days and making cultures from the nearest lymph node which drains the site of inoculation. Since this organism grows well on glycerinated potato, a medium not very favorable for many organisms which are apt to be present as contaminants, it is good for isolations. Glycerin agar * is also quite suitable.

Pure cultures of *Malleomyces mallei* on glycerin potato or glycerin agar slants develop rather slowly. The growth is mucoid and of a slightly yellowish color which darkens the longer the culture stands. After several days' incubation, the growth appears slimy but when touched with the inoculating loop it is found to be tough and rubbery. The growth usually spreads over the entire surface of the medium and thickens into a heavy mass of growth—smooth, shiny, and of a brownish color.

Cultural features, other than the appearance of the growth on solid media, are not particularly helpful in diagnosis. Litmus milk is slowly acidified and clotted after some days. Sugar-containing media usually show no acidity until after many days' incubation, when a little acid

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* *Agar, Veal Infusion Glycerin*

Infusion from 1 lb. of veal

| | |
|---|---|
| Peptone | 10 gm. |
| Sodium chloride | 5 gm |
| Glycerin | 50 ml. |
| Agar | 20 gm. |
| Water to make | 1 liter |

a. Mix all ingredients and boil to dissolve the agar.
b. Adjust to pH 6.0 to 6.4.
c. Dispense in tubes and sterilize 30 minutes at 15 lbs. pressure.

is developed from glucose. The growth in gelatin at 20° C. is poor, and the medium is not liquefied. In fluid media, growth develops very slowly as a slimy ring which, if the culture is not disturbed for a long time, often develops into a pellicle. From the under surface of this pellicle stringy stalactites form. The greater part of the medium remains fairly clear.

## C. ANIMAL INOCULATION

Guinea pig inoculation is often resorted to as a means of isolating the organism from infected tissues, and as a means of identifying pure cultures which have been isolated directly. The virulence of the organism of glanders fluctuates greatly, and in some cases bacilli may be isolated directly from equine lesions when inoculation of guinea pigs with the same material fails. These are from old chronic lesions in which the organisms have become greatly attenuated. From acute lesions, the discharges are usually highly pathogenic for guinea pigs.

If the inoculation is made subcutaneously, either with exudate or pure culture, the course of the disease varies according to the virulence. A local abscess first forms and this generally breaks down and discharges in 4 or 5 days. At the same time the neighboring lymph nodes swell and they, in turn, may ulcerate through the skin and discharge. Nodules commonly develop elsewhere, if the animal continues to live, in the subcutaneous tissue, in some of the muscles, in one or more joints, in some of the long bones, and in the internal organs. Death may occur at any time after a few days up until 6 months or more. Recoveries are uncommon.

The Straus reaction[5] has long been considered characteristic of glanders. This test is not diagnostic, however, since a number of other organisms will cause reactions which cannot be distinguished from the Straus reaction with certainty. This test is never complete, therefore, until the organism causing the reaction has been isolated and shown to possess the general characters of the glanders bacillus.

This test is done with male guinea pigs. The infective material is inoculated intraperitoneally, the dose being kept rather small. Usually it is best to inoculate several animals, using doses of increasing size, in order to be sure of getting at least one good reaction.

On the 2nd or 3rd day after inoculation, examination of the animals shows that the scrotal sacs are swollen, reddened, and unusually tender. The swelling usually increases for several days, and finally it is not

uncommon for ulceration to occur and a cheesy discharge to appear. The animals usually die in from 1 to 2 weeks. Autopsy shows testicles which are partly or wholly caseous and attached to the scrotal sac with a fibrino-purulent exudate. In this exudate it is usually not difficult to demonstrate the characteristic bacillus, microscopically. Cultures give pure growths of the glanders organism.

It must be borne in mind that some cultures, even when freshly isolated, are not sufficiently virulent to elicit a typical Straus reaction, and it cannot always be obtained when the inoculation is done with material from horse lesions. It is probable that it can always be obtained with cultures and tissues from human lesions, which commonly are more acute.

### D. Allergic Tests

The mallein test has been used in only a few cases of human glanders. Positive reactions have been reported.

### E. Serologic Tests

The complement-fixation test is the most reliable of the serological tests in horses, and it has been reported as successful in a few human cases. In the chronic forms of the disease, there is no obvious reason why it should not succeed in man, but in the acute forms the course of the disease is often too short to allow much antibody formation. Normal human sera often agglutinate the glanders organism in high dilution, hence this test is not of much service.

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# Serodiagnosis of Syphilis

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I. Introduction
II. Public Health Aspects of Syphilis
III. Biologic False-Positive Reactions
IV. Improvement in Antigen Composition
V. Evaluation of Performance of Serologic Tests
VI. Present Problems
VII. Future Problems

## I. INTRODUCTION

SINCE the development of the original Wassermann test the serum diagnosis of syphilis has been characterized by the appearance of a series of technical methods, each bearing the name of its originator. A limited number of these procedures employ the complement-fixation phenomenon of Bordet as an indicator, while a larger number are based upon the precipitation phenomenon originally described by Michaelis. All are empirical, employing as antigen an extractive of normal mammalian muscle which can have only an indirect relationship either to the disease itself or to the organisms responsible for the disease. A discussion of technical details will not be attempted.

The precise relationship between infection and the presence of a positive serologic reaction is not evident. Blood serum may continue to give a positive reaction long after adequate therapy presumably has destroyed the causative organism. This supports the belief that the reacting substance is not a direct result of spirochetal activity. It is now known that a patient may be reinfected with syphilis at any time following adequate therapy regardless of the presence of a high-titer serologic reaction. This seems to indicate that if the reacting substance is an expression of immune response the process is not sufficient to afford protection. There is always the possibility that the disease produces immune bodies which are distinct from the substance responsible for positive reactions in the usual serologic test.

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About all that can be said with certainty is that the large majority of individuals in the normal state of health are free from the presence of reacting substance in concentrations which can be detected by the usual test method. Following the invasion by the *Treponema pallidum* the concentration of reacting substance increases to a point which is demonstrable by practically all testing methods. In response to therapy the serologic titer has a tendency to recede steadily toward the negative phase. The time required for this recession differs with duration of the infection, titer at the time of treatment, and individual characteristics which cannot be defined or measured by available methods.

## II. PUBLIC HEALTH ASPECTS OF SYPHILIS

The clinical and public health aspects of syphilis and, by inference, the relationship between the disease and the science of serology, appear to be passing into a period of change. Several years probably will elapse before a rational appraisal can be made of the impact of forces now beginning to be effective. Because of this uncertainty, discussion of the general field of serology must be undertaken with the understanding that opinions expressed are for the moment only, and that an attempt to predict the structure of the serology of the future must be considered speculative.

That the public health aspects of syphilis may be transformed in the near future is well within the range of possibility. At present the spread of syphilis is attributed to transmission by the person with an early infection displaying either the lesions of early disease or those of infectious relapse. If latent or late clinical syphilis possesses an appreciable capacity for transmission the fact has escaped emphasis. If a significant proportion of patients with early syphilis are given adequate therapy a mathematical decrease in the opportunity for transmission probably will result. If such a comprehensive treatment program can be maintained for a sufficient period of time and over a sufficiently wide area the incidence of the disease can hardly fail to decline. This is predicated on the basis of the applicability of the usual epidemiological reasoning to syphilis and the correctness of the present concept of transmission.

In addition to a decline in incidence, following the general employment of antibiotic therapy in early disease, there may be a concomitant decline in the frequency with which the destructive lesions of late disease are encountered. Preventing the disease from seriously injur-

ing the human being would lead to the reclassification of the infection from one of great chronicity, with a potentiality for delayed damage of vital organs, to one of lessened importance to the individual and of minor public health significance.

## III. BIOLOGIC FALSE-POSITIVE REACTIONS

The relationship between serology and syphilis is changing in another important respect. During the years immediately following the development of the Wassermann test the medical profession attached to a positive reaction a degree of diagnostic infallibility which is now considered to be unwarranted. Without doubt many patients were subjected to antisyphilitic treatment on the basis of serologic findings alone when, in reality, they were not infected.

Malaria and leprosy were among the first diseases which were found to react with lipoidal antigens. As new testing methods were developed, as the sensitivity of the methods was increased, and as the use of blood tests became general, the frequency of nonspecific positive reactions also increased. Mononucleosis, vaccinia, upper respiratory infections (even of a relatively minor and transient character) have been incriminated. Further, a limited number of apparently normal individuals are found who display low-reading positive or doubtful reactions with some of the testing procedures. This condition may be permanent or transient but cannot be ascribed to the presence of intercurrent illness.

In untreated early syphilis it may be anticipated that all test procedures will be strongly and persistently positive. If the history of previous treatment is negative (or withheld), and if the serologic findings include low-reading positive or doubtful reactions in some tests and negative reactions in others, it may be well to place the patient in a category requiring continued observation with postponement of therapy during this period. For individuals who have no clinical evidence or history of infection and who give strongly positive reactions in all tests, an abstract diagnosis of syphilis on the basis of serology alone is not justified without pretreatment observation. If the positive reaction is due to causes other than syphilis a change in the pattern may be observed in the span of several months. Syphilis in pregnancy may be a possible exception to this type of management since the withholding of treatment for any protracted period may involve unwarranted risk to the child.

Mention has been made of some of the diseases other than syphilis which may give rise to nonspecific serologic reaction. Evidence is accumulating which points to the possibility of the presence of factors of an environmental character which may distort the serologic picture in certain population groups.

In some tropical areas an extraordinarily high incidence of positive findings has been observed. In a study recently conducted in one of the Central American countries, rates of positive reactors by some methods were as high as 60 per cent in population groups in which the presence of clinical syphilis was at a minimum. The prevalence of nonspecific reactions ranged from 12 per cent to more than 50 per cent. A reasonably complete medical survey failed to disclose the presence of an infectious disease or pathologic condition sufficiently common to account for the prevalence of the positive findings. The inference appears justified that an unrecognized environmental factor may be responsible for a serologic picture entirely at variance with the frequency with which syphilis is encountered.

A full scale study of this phenomenon would be helpful in delineating the extent of the syphilis problem on a world-wide basis. The study would require a medical and serologic survey of an adequate number of inhabitants of various geographic areas, with careful attention being given to study of environmental conditions, of racial distribution, and of seasonal changes in serologic patterns. A study of this type could form the groundwork upon which an accurate appraisal of the value of serologic surveys in tropical areas could be based.

The majority of false-positive reactions are of the mimimal or low-reading type; also, the reaction may be present in some tests of a battery but not in others. Because of this, the use of multiple tests appreciably contributes to the solution of the problem presented by the serum reaction in individuals in whom the probability of an existing syphilitic infection is reasonably remote. However, it is difficult to visualize just how a battery of five or more testing procedures could be operated in laboratories where the testing volume is so great that extreme difficulty is experienced in maintaining a simple routine involving, in many instances, only one test method.

As an aid in differentiating true- from false-positive reactions a group of so-called verification tests have been developed. No one of these methods has proved to be sufficiently reliable to bear the burden of differentiating between specific and nonspecific reactions. At the

present stage, the verification procedures contribute only additional confusion to an already complicated picture.

## IV. IMPROVEMENT IN ANTIGEN COMPOSITION

The serology of syphilis must remain completely empirical until one of the test components has been identified and its role in the reaction described. The work of Pangborn, which led to the identification of cardiolipin and, subsequently, to the use of cardiolipin-lecithin mixtures as antigens, constitutes a forward step in the serology of syphilis. The discovery offered the early hope of preparing antigens through a chemical formula, of eliminating impurities which contribute to nonspecific reactions, and of accurately fixing the level of reactivity of testing methods.

At present, purified phospholipin (cardiolipin) in mixtures with purified lecithins offers the most nearly identifiable component, but there is still no clear understanding as to how these mixtures function as antigens.

Although the exact chemical structure (even the elementary composition) of cardiolipin is yet to be determined, it is interesting that the preparation of the lecithins, whose structure and composition have been known for years, should be the greater hazard in the production of the antigens in a highly purified state. At least, it seems appropriate to suggest that chemists concentrate on preparing a constant product, on determining its composition, on synthesizing a lecithin or mixture of lecithins, or on locating a more practical source material to replace those now in use.

Once the problem of obtaining pure lecithins is solved, the interaction of them and cardiolopin can be studied. By suitable chemical research it may then be possible to determine which molecular groupings are essential for serologic activity and, perhaps, to synthesize a simpler molecule which will render antigens entirely specific.

Cardiolipin-lecithin antigens have been incorporated into several serologic testing procedures without change in test mechanics. In other instances, new methods for the preparation of antigen suspensions and for the reading and reporting of test results have been designed especially for the new antigen. These procedures have retained selected essentials of older methods and have not contributed major changes in the design of serologic tests for syphilis.

But in the adaptation of the basic serologic methods to the use of

cardiolipin-lecithin antigen no concerted effort has been made to adjust the various procedures to a uniform level of sensitivity or to establish reference standards for the guidance of individual laboratories or commercial concerns desiring to compound antigens.

In the test methods now being used the level of reactivity is a matter of arbitrary decision on the part of the originator. To a lesser degree, the level also becomes a matter of discretion on the part of the technical workers engaged in the conduct of the method.

With the methods of extraction employed in compounding lipoidial antigens, the reproducibility of a fixed level of sensitivity in repeated antigen preparations is relatively diffcult or impossible. This feature accounts for some of the discrepancies encountered when duplicate specimens are tested by the same method in different laboratories.

However, the use of cardiolipin-lecithin antigens gives promise of making an important contribution in this regard. It now appears possible, through varying the concentration of the several components, to adjust the level of reactivity of test methods to any desired point and to set up reference standards for the guidance of all future productive efforts. However, up to the present not any effort has been made to determine the level of sensitivity at which a technical procedure, if well carried out, would contribute the maximum of sound information in terms of clinical syphilis and would maintain the greatest freedom from the influence of reacting substances produced by diseases other than syphilis.

The confusion resulting from variations in reactivity levels probably will continue to harass the medical profession until such time as an official agency may be commissioned to develop standard technical methods for each of the three types of tests (complement-fixation, tube flocculation, and slide flocculation) each employing a cardiolipin-lecithin ratio capable of producing a level of reactivity considered to be optimal for clinical syphilis.

A study designed to portray the most advantageous level of sensitivity would require the cooperative effort of a group of clinicians having access to a wealth of clinical material and of several laboratories capable of assuming the large burden of serologic work. It is considered possible to arrive at a decision as to an optimal level by compounding a series of antigens for each testing method, with reactivity levels varying from extreme sensitivity to relative obtuseness. The development of reference standards adjusted to any determined point would not constitute a difficult technical task. With these stand-

ards available the adjustment of all serology of syphilis to a predetermined level could be accomplished. This step would contribute to the elimination of many of the inconsistencies resulting from the system now in use in which the author of a serologic method has arbitrarily established a level of reactivity for his particular method.

A further point in support of the development of standard methods is the fact that the present serology structure of the United States is based upon the decision and the guidance of a group of author serologists. In the event of the support and guidance of any, or all, of the group of originators being withdrawn, for any one of many reasons, the serum diagnosis of syphilis would be left without a foundation upon which could be based the changes, alterations, and advances needed to keep the science abreast of the clinical and public health patterns of syphilis. The creation of an official agency to guide the future evolution of the basic methods which have been created by the group of author serologists appears to be a logical step in maintaining the continuity of progress and scientific service that has characterized the methods under the personal guidance of their originators.

## V. EVALUATION OF PERFORMANCE OF SEROLOGIC TESTS

The initial effort in the United States toward the evaluation of serodiagnostic tests for syphilis was undertaken in 1934, sponsored jointly by the U. S. Public Health Service and the American Society of Clinical Pathologists. The aim of this study was to portray the ability of technical methods in general use, (1) to detect the disease, and (2) to avoid nonspecific reactions due to the presence of diseases other than syphilis. The technical work was under the immediate supervision of the originators of the various methods. It was felt that this would give reasonable assurance that the results would approximate the best obtainable with the procedures under evaluation. In 1941, for a similar study, author serologists were assembled and presented with a large number of test specimens, including sera and spinal fluids from syphilitic patients and normal individuals, and also pretitered pooled sera.

The findings in these two original-method studies indicated that all of the technical procedures were susceptible to the influence of reacting substances produced by diseases other than syphilis; also, that not any single test or group of tests completely covered the field of clinical syphilis.

With an appreciation of the demonstrated limitations of the most widely used methods, an annual evaluation study was organized in which an opportunity was extended to the state laboratories to compare their findings with those obtained in identical specimens by the originators of the several methods. A system of rating was employed based upon the ability of the field laboratory to produce findings in harmony with the clinical picture.

A general review of the surveys conducted during the past fourteen years appears to indicate that the initial six were instrumental in stimulating a general improvement in the caliber of serodiagnostic work. They also brought to the fore many of the shortcomings of the methods themselves and some of the dangers resulting from placing reliance upon inadequately trained personnel. In the more recent surveys there is an indication of only moderate benefit derived from the effort. As a rule, the majority of state laboratories have developed a performance which remains the same from year to year. If the annual survey is to retain its full usefulness a more critical analysis of findings will be necessary. Percentage ratings of sensitivity and specificity do not reflect the relative capacity of a laboratory to duplicate the findings of a control performance.

## VI. PRESENT SEROLOGIC PROBLEMS

There are several problems in the serology of syphilis which force the attention of all who perform the tests or use the results of them. Three of these are: trained personnel and technical errors; commercial sources of antigen; and the serology of spinal fluids.

In public health laboratories the testing of blood specimens for syphilis constitutes the major activity in terms of number of specimens. Hence the proper training of personnel becomes an important adjunct to the operation of the public health laboratory, for it is readily demonstrable that the well trained worker makes a real contribution toward the caliber of results produced. It is important in training that a spirit, or sense, of complete scientific honesty be instilled. This trait reduces to a minimum deviations from an accepted technical procedure and prevents the addition of personal modifications which, while they may be acceptable from the standpoint of the worker, inevitably lead to deterioration of the efficiency of the testing method.

Technical errors may embrace the use of improperly prepared or poorly standardized ingredients; improperly cleaned glassware; mal-

adjusted inactivating and incubating bath, shaking machine, pipetting machine, etc.; as well as the actual conduct of tests by improperly trained workers. Any one or any combination of these factors may be responsible for a substandard caliber of results.

A knowledge of the possibilities for error which are inherent in the present methods, in addition to their known scientific shortcomings, should have the effect of stimulating a healthy skepticism on the part of the medical profession in all instances in which the laboratory findings are not in accord with the history and clinical findings of the individual patient.

There are several important points to be considered in regard to the employment of commercially prepared antigens. In favor of the commercial product is its ready availability, which relieves the individual laboratory of the burdensome task of preparing and standardizing individual lots of material. However, it should be remembered that the standardization of antigen requires critical assay by trained serologists having access to clinical facilities; very few of the companies engaged in marketing these antigens are able to have such a staff or have access to the necessary clinical material. Manufacturers usually have an arrangement with the originators of the test methods whereby supervision over the quality and reactivity of the product is attempted; but the character of the product may change in the period between the approval of the sample by the originator and the use of the antigen in the serologic laboratory. Improper handling, incorrect storage, defective packaging, all may cause deterioration of quality and alteration of reactivity.

The weaknesses and shortcomings of the methods which are employed in the serum diagnosis of syphilis are present and somewhat magnified in spinal fluid work. A positive serologic reaction may be dependent upon the level of sensitivity of the method employed. If two methods of different sensitivities are employed discrepant findings may result. This occurrence is of major importance because it is difficult to secure a repeat specimen for study.

Many specimens reach the laboratory showing the presence of red blood cells, with the result that the protein determination or cell count may become valueless as items from which sound information may be drawn. Bacterial contamination also may render the results of protein determination of dubious value.

The cell count, if carried out within 1 hour following the collection of the specimen, may be relied upon as to accuracy of the actual count

only in the hands of careful workers. Total protein determinations cannot have any real significance unless the clinician is aware of the technical method employed and the level considered normal. A more precise definition of the normal range, based upon the study of a large number of non-syphilitic fluids and employing a scientifically accurate technic, would be helpful. Under the present system it becomes impossible to compare the findings of different clinics because of differences in the methods employed.

The diagnosis of asymptomatic neurosyphilis is hazardous when based upon deviations from the alleged normal which may only be the results of the shortcomings and weaknesses of the tests themselves.

## VII. FUTURE SEROLOGIC PROBLEMS

Many of the scientific advances needed for the improvement of serologic methods have long been known and much effort has been expended in their study; recent changes in therapy and laboratory practice have created or emphasized other problems. Foremost among them are:

1. The desirability of replacing the present technical methods, which are wholly empirical, by a procedure or procedures capable of accurately measuring the concentration of reacting substances.
2. Further exploration of the field of spirochetal antigens. From a theoretical viewpoint, extractives from closely allied spirochetal forms should be more nearly specific for the disease than are extractives from normal mammalian heart muscle. The cultivation of the *T. pallidum* would be of greatest importance in the development of spirochetal antigens.
3. A general departure from the use of highly sensitive test procedures and adoption of a more conservative and uniform level of reactivity.
4. The distortion of prevalence figures for syphilis in a population group by the use of sensitive procedures in the conduct of serum surveys.
5. The development of a central agency (preferably governmental) for clearing matters of general policy related to the serum diagnosis of syphilis.

# Chancroid and Granuloma Inguinale

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## I. INTRODUCTION

KNOWLEDGE concerning the etiology, methods of diagnosis and treatment of the venereal diseases of syphilis and gonorrhea have reached a relatively high state of accuracy. Until within the past few years, however, too little attention has been devoted to three other so called *Minor* venereal diseases: chancroid, granuloma inguinale, and lymphogranuloma venereum. Part of this defect undoubtedly stems from the peculiarities of the respective etiological agents, none of which as yet readily lends itself to what might be called routine laboratory procedures.

Lymphogranuloma venereum, because of its viral nature, has been included with other virus disease for a more complete discussion.* But

* *Diagnostic Procedures for Virus and Rickettsial Diseases*, 1948, p. 47.

from the standpoint of clinical differentiation, it should be pointed out that these three diseases in reality constitute a "triumvirate." Much confusion in diagnosis has been found to exist in the past because of certain similarities of clinical lesions. And much confusion will still exist in the future unless more attention is placed on these diseases as a group entity, and all available procedures for differentiation be utilized. This is especially true of chancroid and lymphogranuloma venereum.

## II. CHANCROID

The organism ascribed to this infection by Ducrey is now classified as *Hemophilus ducreyi*. Grave doubt is to be placed on the correctness of this classification. Aside from the fact that some constituents in blood are essential for its cultivation, it has nothing otherwise in common, clinically or morphologically, with other members of the Hemophilus group. This was pointed out in 1940,[1] and more recently has been elaborated on by Beeson.[2] There is still further need for study of this organism.

The organism is customarily referred to as a "strepto-bacillus." While it is true that long chains and filaments are characteristic of cultures, they are usually not observed in smears from genital sores or bubo pus, and one will be disappointed if he expects to find similarity to some of the textbook drawings.

### A. SMEARS

#### 1. *Primary lesions*

The ulcer should be carefully cleansed to remove accumulated debris, and material removed from its base or actively growing margin with wire loop or cotton swab, and placed onto a clean slide. Gram's stain is satisfactory. If the lesions are relatively clean, microscopic examination will reveal a small, Gram-negative bacillus, usually clear-cut and occurring singly, in pairs or short "chains" of three or four. Sometimes the bacilli occur in small clumps and clusters, and more rarely in "school of fish" formation—diagnostic when observed. Infrequently, the organisms are not numerous and careful search may be required to find them. In such cases, best success may be had by examining the shreds of mucus, for the bacilli may be observed lying in and among and parallel with them. On the other hand, dirty, secondarily infected ulcers occur with a concomitant multiferous flora, and an opinion based

on microscopic examination is of questionable value, except as it serves to exclude granuloma inguinale.

The polymorphonuclear is the typical exudative cell, and the presence of intracellular bacilli should be carefully studied. For this examination, fixed smears are stained 3–5 minutes, as Kornblith[3] recommends, by Unna-Pappenheim's stain. Pus cells are blue-green, and bacteria are red. The smears should be examined for organisms showing the morphology and characteristic arrangement of *Hemophilus ducreyi.*

### 2. *Buboes*

Inguinal buboes of varying size, unilateral or bilateral, may occur in syphilis, gonorrhea, granuloma inguinale and lymphogranuloma venereum, as well as in chancroid. Hence, the presence of a bubo is not of itself a criterion for diagnosis for any of these infections.

Pus from fluctuant buboes should be removed aseptically by aspiration, ***never incision,*** smeared onto a clean glass slide, and stained by Gram's method. Gram-negative bacilli, intra- and extra-cellular, from an unruptured bubo with accompanying primary genital sore may be considered to be the Ducrey bacillus. However, the organisms are never very numerous, and careful search is required. Failure to find them does not exclude chancroid disease. Ruptured or previously incised buboes are always secondarily infected with a variable flora, and smears are of doubtful value from the standpoint of recognizing Ducrey's bacillus.

## B. CULTURES

The paucity of references to the successful routine cultivation of *H. ducreyi* indicates that this organism does not respond readily to methods of cultivation yet devised. It grows slowly, and if mixed with an abundant contaminating flora, may not grow at all. Surface streaking methods have yielded little success in the writer's experience. Recently, Beeson and Heyman[4] have employed streaking of mixed cultures on blood agar plates and placing the plates in a jar under decreased oxygen tension by burning a candle. This method also serves to maintain moisture which is known to be essential to *H. ducreyi.*

### 1. *Method of Teague and Diebert*[5]

Rabbit blood is drawn by cardi-puncture, placed in small tubes in 1 ml. amounts, allowed to clot at room temperature, and then heated for 5 minutes at 55° C. These can be stored in the refrigerator or

used immediately. Equally good results may be obtained when the tubes are kept in the refrigerator for 3–4 days without heating.

Material for cultures is removed from the base of suspected ulcer with a sterile, stiff iron wire, gauge 18, and bent on itself at one end to about 1/8 inch. The inoculum is placed into the serum by passing wire around the clot. A second tube is made. Cultures are incubated at 37° C. and stained smears made and examined microscopically in 24 hours for characteristic growth of *H. ducreyi.*

### 2. *Method of Sanderson and Greenblatt* [6]

Agar slants in 12 mm. size culture tubes, with extract (C.M. No. 4a) or infusion broth (C.M. No. 5a) pH 7.4–7.6 as a base, are used. Before inoculation, 1 ml. of sterile, defibrinated rabbit or human blood—preferably the former—is added *onto* the slant. Swabbings or loop scrapings from a well cleansed ulcer are inoculated directly into the blood. Incubation is at 37° C. and cultures should not be discarded as negative for 4 days.

### 3. *Method of Beeson and Heyman* [4]

Rabbit blood is defibrinated by use of glass beads and dispensed in 1 ml. quantities in sterile 13 x 100 mm. Pyrex tubes and stored in refrigerator at 4° C. until used. Genital lesions and buboes can be cultured on this medium, using a small amount of inoculum. Although cultures are rarely pure, the characteristic morphology of *H. ducreyi* serves to identify it when smears are stained with Gram's stain.

## C. SKIN TEST (ITO-REENSTIERNA REACTION) [7, 8]

The intradermal inoculation of killed suspensions of Ducrey's bacillus was shown to give a focal reaction of induration and erythema in patients with chancroidal infection, and was therefore introduced as a method of diagnosis. Further study of the test indicated that a positive reaction could be interpreted as (1) indication of present infection, and (2) indication of a past infection. Hence, a combination of other tests, clinical history and clinical evaluation of the lesions themselves was necessary for establishing a diagnosis. A similar situation can be said to be true of lymphogranuloma venereum. Hence, if one is to employ a Ducrey skin test it should always be done *concomitantly* with a Frei test. Certain combinations of results may give valuable information, since it is quite well established that neither the

chancroid test nor the Frei test will yield positive skin reactions unless there is lymphatic involvement (buboes).[14] Hence, if a bubo is present and Frei test is positive and the Ducrey skin test is negative, one can be reasonably sure that the infection is lymphogranuloma venereum. Also, if there is a bubo, and the Frei test is negative and the chancroid test positive, one can be reasonably sure that the present infection is chancroid. As often happens, both tests may be positive; or one or the other skin test may be positive in the absence of bubo formation. In such cases other methods will have to be resorted to in establishing a correct diagnosis. Of all the venereal diseases, differentiation of chancroid and lymphogranuloma venereum entails the most difficulties. Hence, it may be necessary to employ multiple methods described: smears, cultures, skin tests.

Some pathologists claim that if only ONE method is to be employed, the histo-pathological diagnosis of chancroid lesion is the most satisfactory.

#### 1. *Procedure*

One-tenth ml. of bacillary suspension of killed Ducrey bacillus is injected intradermally on the cleansed flexor surface of the forearm, and the reaction read at 72 hours. A positive reaction will show an indurated zone of not less than 7 mm. in diameter and may be surrounded by an erythema of 14 mm. diameter. Central necrosis or bleb formation may occur in strong reactors. Even though infection may be chancroid, skin reactions are usually not elicited in the absence of bubo formation.

### D. AUTO-INOCULATION

This test involves the producing of a scarified area on the arm or leg, and the rubbing into it of material from a venereal lesion. A successful " take " being interpreted as chancroid infection.

This is the least useful of any of the diagnostic tests, and in fact may be even dangerous from the standpoint of the infection which may result from it, especially if the patient fails to return for an examination of the test. Aside from this, even in presence of Ducrey's bacillus, the inoculation may produce no " take " whatever. On the other hand, in the absence of Ducrey's bacillus successful " takes " may result from concomitant pyogenic flora. Hence, the interpretation of any successful " take " involves the identification of Ducrey's bacillus in the lesion either by smear or culture or both.

## III. GRANULOMA INGUINALE

Granuloma inguinale is usually considered to be of venereal origin, although in some cases evidence of sexual contact is not clear. Besides the external genitalia, lesions may occur in the anal region, inner aspects of the thigh, cervix, lower abdomen, side of neck, buccal cavity and even generalized lymphatic involvement of the pelvis.

The disease is quite general in members of the Negro race, and other dark-skinned people. In the United States, the disease is more commonly confined to Negroes, although a few authentic cases have been reported in whites.

As the name implies, the clinical appearance of the lesions is that of extensive granulomatous formation. It is very offensive to the smell, and can be very destructive if left untreated.

### A. "DONOVANIA GRANULOMATIS" (DONOVAN BODIES)

The organisms seen in direct smears from lesions by Donovan[9] are now generally accepted as the cause of this infection,[15, 16] and the name "Donovania granulomatis" suggested by Anderson, Goodpasture, and DeMonbreun[10] has come into use. Although the exact nature of these organisms was little understood, the consensus at present is that they are bacterial in nature. Anderson[11] has succeeded in cultivating them in yolk of developing chick embryos, and Dienst[12] has more recently cultivated several culture strains in fresh yolk medium. Anderson and associates[13] have established the immunological relationship of "Donovania granulomatis" to granuloma inguinale by using skin hypersensitivity employing as antigens the material from the cultivated organisms; also, similar antigens give positive complement-fixations with the sera from some of the infected patients.

#### 1. *Smears*

In the hands of those familiar with the appearance of Donovan bodies, the stained tissue smear is the simplest and most accurate method of diagnosis.[17] Stained biopsy sections are also of proven value. In suspected cases, scrapings, or preferably small fragments of tissue (not more than 1 to 2 mm. in size) are removed from the lesion, placed between two clean slides, crushed by pressing the slides together then separating them with a sliding motion. Thus, two smears result. When dry, they can be stained with Wright's stain. Better results are

to be obtained if the stain is left on for 1½ minutes, slightly longer than customary for the usual blood smears. Giemsa stain is also satisfactory.

The microscopic picture is highly characteristic, and cannot be mistaken for anything else in the hands of those familiar with this organism: the pathognomonic tissue cell is the large mononuclear (monocyte), which will be present in abundance. As a matter of fact, this cell when seen in large numbers in smears should immediately arouse suspicion of this infection. Within many of these cells will be found numerous small, straight or curved dumb-bell shaped rods stained purple, each in a "capsule," oval or round, and stained pink at the periphery. The organisms may occur distributed throughout the monocyte; or may be within one, two, or three well circumscribed vacuolar spaces, filling the spaces; or they may be peripherally arranged within the vacuoles. Sometimes the capsules are not manifest in all organisms. The organisms, with or without capsule, may be extracellular, but the unencapsulated forms are not easily identified because of similarity to miscellaneous flora frequently present as secondary invaders.

### 2. *Tissue sections*

Sections are made from pieces of granulomatous tissue,[18] 10 per cent formalin fixed tissue, or frozen sections stained with hematoxylin-eosin give a similar microscopic picture and are entirely satisfactory for histodiagnosis. The various silver stains may be used, but there is no apparent advantage over H & E. As a matter of fact, because of proved simplicity, economy of time, and accuracy, simple smears stained by Wright's method constitute the most satisfactory method of diagnosis.

It should be pointed out that careful selection of tissue to be examined is very important, and may be the difference between success and failure. Tissue should always be taken from the cleansed surface of the lesion, *at the active growing periphery of the lesion.* This avoids necrotic tissue, which stains poorly, and wherein Donovan bodies are few or cannot be found.

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# Infectious Mononucleosis

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## I. INTRODUCTION

OSLER has called syphilis "the great imitator" because its many clinical manifestations in various stages of the disease simulate the syndromes of many different and unrelated pathologic conditions. A somewhat similar status may be assigned to infectious mononucleosis (glandular fever) (monocytic angina). This disease may be confused readily with diphtheria,[1] typhoid,[2] myelogenous or lymphatic leukemia,[3] syphilis, scarlet fever, and serum sickness.[4] Skin eruptions which appear in about 15 per cent of infectious mononucleosis patients may closely simulate those of measles,[5] erythema nodosum,[6] typhus,[7] and other less important diseases. Moreover, infectious mononucleosis cases may yield false positive tests for syphilis, more or less transient in nature.[4]

Infectious mononucleosis is frequently a benign condition but it is evident from the foregoing facts that an accurate diagnosis allows

prompt reassurance to the patient and a fairly reliable prognosis as to duration and morbidity. Türck's attention was first directed toward this disease when a patient, diagnosed as hopeless acute lymphatic leukemia, recovered in two weeks. The blood picture is important in the diagnosis of infectious mononucleosis but not wholly reliable except perhaps in the hands of a few hematologists.

In acute infectious mononucleosis there is typically a leucocytosis with a preponderance of mononuclear cells. Leucocytosis is not as pronounced as in acute lymphatic leukemia although the count may be as high as 20,000 to 40,000 with 60 to 99 per cent mononuclear cells. The leucocytosis persists for a few days and the increased mononuclear cells gradually disappear. While a differential diagnosis is often made from hematological studies the more certain procedure is the heterophile agglutination test with sheep erythrocytes.

In 1917 Friedmann [8] demonstrated the presence of sheep erythrocyte antibodies (presumably Forssman heterophile antibodies) in normal human sera. Taniguchi [9] and others found that antibodies (presumably Forssman) for sheep erythrocytes were markedly increased in serum sickness. Paul and Bunnell [10] showed that a consistent and marked increase occurred in sheep cell antibodies in infectious mononucleosis. It was at first assumed that the sheep heterophile antibodies of infectious mononucleosis were Forssman in nature. Bailey and Raffel [11] and Stuart [12, 13] subsequently found that the sheep cell agglutinins of infectious mononucleosis could be differentiated easily from those of serum sickness and of normal serum. Sheep cell agglutinins of infectious mononucleosis are completely adsorbed by beef erythrocytes but are not reduced significantly by adsorption with guinea pig kidney; sheep cell agglutinins in serum sickness are completely adsorbed by both guinea pig kidney and beef erythrocytes; sheep cell agglutinins in normal human serum are not altered by adsorption with beef cells but are completely or almost completely adsorbed by guinea pig kidney. The antigenic fractions of beef cells and guinea pig kidney which adsorb their respective antibodies are thermostable so that boiled or even autoclaved cells and tissue can be used for differential adsorption tests.

An explanation for the presence of the particular type of heterophile antibodies in infectious mononucleosis must remain obscure until the etiological agent has been established. Although bacterial,[14-17] protozoal,[18, 19] and viral [20, 21] causes have been described, ample proof for any one agent is lacking. Sheep cell agglutinins in this disease, there-

fore must be considered as a specific or nonspecific (anamnestic) reaction of the host to the infecting agent.

## II. SEROLOGICAL PROCEDURES

### A. COLLECTION OF SPECIMEN

#### 1. *Outfit*

Clean, sterile tube or vial with tight stopper or cap, about 10 ml. capacity.

#### 2. *Specimen*

Five to 10 ml. of venous blood.

#### 3. *Time of taking specimen*

To establish a diagnosis 3 specimens may be necessary: The first, taken as soon as infectious mononucleosis is suspected, the second from 5 to 7 days after the first, and a third 7 to 9 days after the second. Increases in titer obtained with repeated specimens aid in the diagnosis.

#### 4. *Preparation of specimen*

Serum is separated from clot by centrifugation and inactivated at 56° C. for 30 minutes, since some normal sera possess relatively high lytic titers for sheep cells.

### B. AGGLUTINATION TEST FOR INFECTIOUS MONONUCLEOSIS

#### 1. *Antigen*

A 1 per cent suspension of sheep erythrocytes washed with physiological saline at least three times is used. In the final washing the volume of cells and saline should not be over 6 ml. and the cells should be packed at 2,500 r.p.m. for 6 minutes. The use of strictly fresh cells has been advocated[22] but it has been our experience that cells of defibrinated blood may be used for a week or even longer unless cell fragility is increased by bacterial contamination.

#### 2. *Technic of test*

To 0.5 ml. portions of progressively doubled dilutions (1:5 to 1:5,120) of the patient's serum add 0.5 ml. of the 1 per cent sheep

cell suspension. This results in final serum dilutions of 1:10 to 1:10,240 and all titers should be reported on this basis. For a control, 0.5 ml. of the cell suspension is added to 0.5 ml. of saline. The tests are shaken, incubated for 2 hours in a 37° C. water bath or for 3 hours in a 37° C. air incubator, then read and the titers recorded as in a bacterial agglutination test.

The following rapid method is quite satisfactory if all details are observed. The test is set up as described, shaken thoroughly and placed in a 37° C. water bath for 5 to 10 minutes. The tubes are then rapidly transferred to a centrifuge and the cells packed for a minimum of 1 minute or a maximum of 2 minutes at 2,500 r.p.m. To insure uniform results the rack, rather than the individual tubes, is shaken until the cells in the control are suspended when the titer is obtained by comparing each tube in the test with the control. By this method the titer is usually one dilution higher than after 2 hours in a 37° C. water bath. This should be considered in evaluating the results of the test.

### 3. *Interpretation of results*

Disagreement in the literature[23, 24] concerning presence and titers of sheep cell agglutinins in normal human sera can be explained for the most part, or perhaps entirely, by divergent technics.[25] With the procedure described above, titers of 1:10 to 1:40 should be considered as negative; titers of 1:80 or 1:160 as suspicious; titers of 1:320 or higher in the absence of serum therapy are indicative of infectious mononucleosis. It is essential that the tests be read after the specified incubation period at 37° C. since lower temperatures produce a marked increase in the titer of the normal sheep cell agglutinins.[26]

Serum from suspicious cases should be reëxamined after a 5 to 7 day period to determine rise in titer, or a confirmatory adsorption test can be made with the first specimen to demonstrate the presence of the infectious mononucleosis type of sheep cell agglutinin.

## C. ADSORPTION TEST TO DIFFERENTIATE TYPES OF HETEROPHILE AGGLUTININS

### 1. *Adsorbing agents*

By use of guinea pig kidney tissue and beef erythrocytes three types of sheep cell heterophile agglutinins can be identified in human sera. Usually adsorption with guinea pig kidney is sufficient for final diag-

nosis of infectious mononucleosis but, particularly when there is a question of lymphatic leukemia, a subsequent adsorption with beef cells definitely proves the agglutinins to be of the infectious mononucleosis type.

*a. Fresh guinea pig kidney*

A kidney or portion of kidney is finely minced and ground in a hand or mechanical mortar and the emulsion strained or rubbed through a small piece of 13xxx bolting cloth or its equivalent. Ten to 15 ml. of distilled water are added to the emulsion in a centrifuge tube. This is centrifuged at 3,000 r.p.m. for approximately 15 minutes and the supernatant fluid, particularly fat particles, discarded. This operation is repeated 3 or 4 times. The sediment is diluted with saline to a turbidity of at least twice that of the barium sulfate turbidity standard No. 10.

*b. Dried guinea pig kidney tissue*

The use of dried guinea pig kidney tissue is convenient and saves considerable time. It is prepared by grinding the kidneys in a fine meat chopper and washing in distilled water as described above. The washed macerate is dried in an evaporating dish at approximately 75° C. with frequent stirring to prevent caking. The tissue emulsion can also be dried by the lyophile process. The dried macerate is finely ground in a ball mill or by hand in a mortar, then sifted through 13xxx bolting cloth and stored in a dry place.

In using dried tissue an amount of the powder sufficient to give a turbidity at least twice that of the barium sulfate turbidity standard No. 10 is added to 1 ml. of saline and thoroughly wetted. This takes several minutes and should not be hastened.

*c. Beef erythrocytes*

Serum is removed from defibrinated beef blood by washing 3 or 4 times with saline. The packed cells are used in the adsorption test.

### 2. *Technic of adsorption test*

One ml. of the fresh or dried guinea pig kidney tissue suspension is added to 1 ml. of a 1:5 dilution of the serum to be tested. The mixture is incubated at 37° C. for 30 minutes in either a water bath or air incubator, centrifuged at high speed (4,000 r.p.m.) for 15 to 20 minutes,

and the supernatant fluid tested in serial dilutions for sheep cell agglutinins as previously described. It is essential that the supernatant fluid be clear after centrifugation since strong turbidity may inhibit agglutination of the sheep cells.[12] If agglutinins remain, further confirmation of infectious mononucleosis can be obtained by a second adsorption with beef cells. To 1 ml. of the serum dilution adsorbed with guinea pig kidney 0.5 ml. of packed beef cells is added, mixed, and incubated at 37° C. in either a water bath or air incubator for 30 minutes. After centrifugation the supernatant fluid is tested for sheep cell agglutinins as before.

### 3. *Interpretation of results*

The relationships of the different types of sheep cell agglutinins in human serum are shown in Table 1. Agglutinins which may be present in normal serum to a titer of 1:160, and in serum sickness, to a titer

TABLE 1

*Agglutinins for Sheep Erythrocytes in Human Serum*

| | *Agglutination of sheep erythrocytes* | | |
|---|---|---|---|
| *Type of serum* | *Unadsorbed* | *Adsorbed with guinea pig kidney* | *Adsorbed with beef erythrocytes* |
| Normal | – or + | – | unchanged |
| Infectious mononucleosis | + | + | – |
| Serum sickness | + | – | – |

of 1:20,480, are removed by adsorption with guinea pig kidney. Sheep cell titers of infectious mononucleosis, on the other hand, are not affected by the antigens of guinea pig kidney. When adsorptions are made with beef cells the sheep agglutinins from infectious mononucleosis and serum sickness are removed while those of normal serum are not. Adsorption with beef cells alone is not advised since this will not distinguish between infectious mononucleosis and serum sickness and, as Davidsohn[27] has shown, sheep cell agglutinins may persist in the blood for a year or more after treatment with horse serum. The failure of guinea pig kidney to affect the sheep cell agglutinin titer of the patient's serum is adequate evidence of infectious mononucleosis; however, removal of the agglutinins remaining after adsorption with guinea pig kidney by beef erythrocytes is a final confirmation seldom required.

When both infectious mononucleosis and serum sickness are present in the same patient the two types of antibodies frequently can be identified.[28] If upon adsorption with guinea pig kidney the sheep cell agglu-

tinin titer is only partially reduced, as from 1:5,120 to 1:640, both conditions are indicated since the guinea pig tissue adsorbed the agglutinins of serum sickness but not those of infectious mononucleosis.

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# Leptospira Infection

## (*Weil's Disease*)

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## I. INTRODUCTION

THIS chapter is written primarily for the bacteriologist, physician, or veterinarian who is interested in the laboratory diagnosis of leptospiral infections. Bacteriological and serological diagnostic methods, therefore, are described in detail. The technical section is preceded by a discussion of the various leptospiroses and the causative organisms,

as a basis for the discussion of laboratory methods, and as a foundation for a discussion of experimental studies with these organisms and of the diseases they produce. The chapter thus is practical in outlook rather than didactic. For the reader who may be interested in further details or in other aspects of the organisms or the infections they produce, references to general reviews on the subject are listed in the bibliography.[1–17]

Weil's disease, the most important member of this group of diseases, is an acute, febrile, septicemic, protean disease of man and certain lower animals, caused by a spirochete, *Leptospira icterohemorrhagiae,* and is associated, though not invariably, with jaundice, hemorrhages, nephritis, and a fairly high mortality. The natural reservoir of infection is usually the wild rat and dog, but other rodents and mammals are probably involved.

In 1886 Weil[18] described a new disease entity characterized by jaundice, hemorrhage, nephritis, chills, and splenomegaly. In recent years, however, chills and splenomegaly rarely have been associated with the disease bearing his name. In 1915 Inada and his associates[19] in Japan discovered the causative spirochete, and in succeeding years[20 a–g] studied the epidemiology, symptomatology, pathology, immunity, and serotherapy of the natural human disease and the experimental disease in the guinea pig. They proved the presence of virulent leptospira in apparently healthy wild rats and field voles.[21] In 1917 Noguchi[22] found in American wild rats a spirochete morphologically and immunologically identical with the Japanese strains. In 1918 he[23] further described the morphology of this organism and named it *Leptospira icterohemorrhagiae.* His erroneous belief in *Leptospira icteroides* as the causal agent of yellow fever[24] indicates that leptospirosis may simulate yellow fever clinically and that the two diseases may coexist in a given area. It was not until 1927 that Noguchi's concept was abandoned; the accummulated evidence[25] showed *L. icteroides* to be similar to if not identical with *L. icterohemorrhagiae.* Weil's disease is known to have occurred during World War I[17] and believed to have occurred in the American Civil War.[26]

Weil's and related diseases are world-wide in distribution, excluding areas where the temperature is consistently low. They are especially prevalent in Japan and Egypt, and are endemic along the North African coast, the shores of the Mediterranean, West Africa, and the Congo. They are known to occur commonly in Holland, France, Italy, Germany, Denmark, Austria, and parts of South America and Asia.[12] Epidemics

have been described in the Andaman Islands,[11] Dutch East Indies, and Malay States.[12]

In the United States, until 1941, there were only about 27 fully diagnosed and 20 presumptive human cases of the disease described in the literature.[27] However, a large number of cases have been reported from all parts of the country in more recent years.[27-35] It is difficult to estimate the total number of cases, as the disease is not reportable in all states, but there must be at least a total of 150. Surveys in representative sections of the United States[36-42] have revealed that 10 to 40 per cent of wild rats are carriers of virulent *Leptospira icterohemorrhagiae*; the same frequency of infection occurs in rats throughout the world.[12] Dogs have been found to be infected with this organism and the related canine strain, *L. canicola,* to such an extent that leptospirosis has become an important veterinary problem in the United States.[43] White rats,[7] gophers,[44] foxes,[45] guinea pigs,[7] and other animals[46, 47] have been experimentally or naturally infected. They must, therefore, be considered a potential source of infection.

In view of the above it is reasonable to assume that Weil's disease, if correctly sought for, recognized, and reported, will prove to be as common in this country as in Europe. Essentially the same animal reservoirs and opportunities for infection exist here as there, although generally sanitation is better here. Moreover, it seems improbable that North Americans as a group are naturally immune. Since the disease is transmitted readily through contact with materials contaminated by infected rat urine, workers in rat-infected environments would be particularly liable to infection.

That Weil's disease is an occupational hazard and hence a public health problem has been acknowledged in England, Germany, and the United States.[48] Laborers have been legally compensated as a result of disabling or fatal leptospirosis incurred while at work in occupations involving risk of exposure to leptospirosis.[46]

## II. THE GENUS LEPTOSPIRA

*Leptospira icterohemorrhagiae,* the spirochete causing Weil's disease, is the type species of the genus *Leptospira.* The following discussion will, therefore, be concerned mainly with this species and only secondarily with other species in the genus. However, an effort has been made to indicate the methods of differentiation between *icterohemorrhagiae* and *canicola,* the other important pathogenic species.

### A. SYNONYMS

*Leptospira icterohemorrhagiae* is also called *Spirocheta interrogans*,[49] *Leptospira icteroides*,[24] *Spirocheta icterohemorrhagiae*,[19] *Spirocheta icterogenes*,[50] *Spirocheta icterohemorrhagica*,[4] and *Spirocheta nodosa*.[51] However, the first two synonyms are probably erroneously applied to the etiological agent of Weil's disease, as they were first applied to organisms seen in,[49] or isolated from [24] the tissues of yellow fever patients.

### B. "SPECIES" OF LEPTOSPIRA

*Saprophytic*—Organisms with typical leptospiral morphology can be isolated from pond water, slime from icebox drains, and similar materials.[17] They have been termed *Leptospira pseudo-icterogenes* or *pseudo-icterohemorrhagiae*.[52] It has been claimed that they have been made virulent under certain conditions,[7] but this transformation is generally doubted. Some of the confusion is probably attributable to the heterogeneity of these supposedly saprophytic strains, the majority being true saprophytes and the remainder true parasites in the attenuated state which may be restored to full virulence.

*Parasitic*—Parasitic *Leptospira* other than *L. icterohemorrhagiae* include *L. canicola, L. grippo-typhosa, L. hebdomadis, L. autumnalis, L. pyrogenes, L. sejroe, L. bataviae, L. australis A* and *B, L. pomona,* and *L. icterohemoglobinuriae vitulorum.* The diseases they cause are discussed in Sections III and IV.

### C. BIOLOGY OF LEPTOSPIRA

*Morphology*—The morphological characteristics of the genus *Leptospira* provide a useful basis for differentiation of these organisms from other genera of spirochetes and from other microörganisms in general. Within the genus, however, both saprophytic and parasitic organisms present essentially the same morphology.

Only by means of the dark-field microscope can the morphology of the *Leptospira* be studied satisfactorily (See Figure 1). In this way the organism is seen to contain many regular, fine, closely wound spirals in contrast to the long undulating spirals of the relapsing fever spirochetes, the *Borrelias,* or to the coarser spirals of the syphilis spirochete, *Treponema pallidum.* Because or their fineness, the spirals usually appear as a string of beads under dark-field illumniation and are not visible in stained preparations (See Figure 2). Apart from the fine spirals the most characteristic morphological feature of the *Leptospira* is the sharp, tapering, hooked ends. The usual rapid spinning motion

FIGURE 1—Somewhat diagrammatic drawing of *Leptospira* as seen in dark-field preparation. Note beaded appearance, primary and secondary spirals, hooked ends, and button-hole appearance of ends of organism in motion at about 7 o'clock in figure.

FIGURE 2—Photomicrograph. Approximately 1000x. *Leptospira icterohemorrhagiae* in liver of experimentally infected guinea pig stained with Levaditi stain. Note absence of spirals and presence of hooked ends. Organism has a similar appearance in silver-impregnated or Giemsa-stained smears of cultures or body fluids.

of the organism about its long axis causes the hooked ends to appear as loops. Progression is in the direction of the long axis by corkscrew motion as though propelled from the rear by the rotating hook. Its boring through agar particles strikingly has been compared by one writer to the "gyring and gimbling of Lewis Carrol's slithy toves." During rotation secondary curves in the body of the spirochete may suddenly appear and just as rapidly disappear, resembling a C, O, S, or some other curved letter. The primary spirals remain intact throughout the various writhing movements of the organism. In fluid media, the organisms may become entangled with each other, assuming the appearance of the characteristic "nest." *Leptospira* is usually from 6 to 12 microns long and 0.1 to 0.15 μ wide; however, the dimensions are variable, being dependent on the medium, and temperature and rate of growth.[6] In serum-peptone media short forms predominate while at higher temperatures, above 30° C., long thread-like forms predominate, apparently due to an increased rate of growth.

Multiplication of *Leptospira* occurs by transverse fission. As the organism grows the middle portion elongates; the hooked ends always remain about the same size. When fully grown the organism begins to bend in hinge-like fashion in the center, at the point of future separation. The hooked ends twist about each other, then unwind, straighten out, bend and catch again. As a rule this process is repeated often during several hours. Finally, after violent tugging at the ends the hinge separates into the daughter organisms.

*Leptospira* may degenerate in older cultures.[17] It loses its motility, tends to agglutinate into granular, amorphous clumps on the periphery of which motile spirochetal fragments are often seen. The granules show up as bright, glistening points under dark-field. These coccoid bodies are generally considered degeneration products,[17] although some observers[14] consider them as analogous to bacterial spores, affording a means of maintaining life under adverse environmental conditions.

Staining of these organisms by ordinary bacteriological strains is not successful. They may be impregnated with silver by reduction of silver nitrate, and then appear black. This is discussed in some detail in Section VI.

*Counting of Leptospiras*—A prerequisite for *in vitro* studies of the susceptibility of these organisms to chemotherapeutic agents is a reliable method for counting them since they cannot be counted as colonies on solid media. They must, therefore, be enumerated by direct microscopic methods. These include the modified Wright[112] and Breed[132]

methods and the use of the Petroff-Hauser bacterial counting chamber.[55, 56] These methods are carried out as originally described except that in the Breed procedure the organisms are stained by negative stains, and in the Wright method the mixture of spirochetes and red cells is examined under a dark-field.

*Cultural characteristics* (See Addendum)—*L. icterohemorrhagiae* and *canicola* are the most readily cultivated of the pathogenic spirochetes if certain optimal conditions are provided. The primary requirement is the presence in the medium of 10 per cent animal serum. Greene[56] has found that the serum in the Schuffner medium for cultivation of *L. canicola* cannot be replaced by any of the amino acids studied. If peptone is omitted from the medium, asparagine, aspartic acid, or leucine will substitute for it to some degree.

The optimum pH for multiplication of *Leptospira* is about 7.4, the upper and lower limits being about 6.9 and 8.4. The optimum temperature range is about 28–32° C. Growth is more rapid at 37° C., but degeneration of organisms is also more rapid. The salt content of the medium is critical; too high a concentration will kill the spirochetes. Growth proceeds only under aerobic conditions, the organisms usually growing in the narrow zone immediately beneath the surface of the medium. The heavier growth obtained in agitated Erlenmeyer flasks containing glass beads and in flat-sided bottles laid on the side is probably due to increased surface area and oxygenation of the medium.[53]

A number of media satisfying these growth requirements have been devised.[17, 57–59] The two media most commonly used at present for the cultivation of *L. icterohemorrhagiae* and *canicola* are the Verwoort-Schuffner medium[60] and Fletcher's medium.[61] The Schuffner medium, being free of the agar particles contained in Fletcher's medium, is ideal for growing organisms which can be used without further treatment as antigen in the agglutination-lysis test.

In view of its wide use in the cultivation of pathogenic *Leptospiras* the preparation of the Verwoort-Schuffner medium follows. The ingredients of the basal medium are:

1,500 ml. distilled or rain water
0.15 gm. Witte's peptone
300 ml. Ringer's solution
150 ml. Sorenson's double phosphate buffer, pH 7.2
(72 ml. m/15 $Na_2HPO_4$ plus 100 ml. m/15 $KH_2PO_4$)

The water is brought to the boiling point and the peptone and other components added, and boiling is continued. After boiling for about

30 minutes precipitation of salts is fairly complete. The mixture is then placed in the refrigerator to secure further precipitation; it is then filtered and the pH is checked. The pH is adjusted to 7.4–7.6 before autoclaving so as to assure a final pH of 7.2 after autoclaving. After sterilization, either in large volumes in Erlenmeyer flasks or flat-sided bottles, or in 3–5 ml. amounts in test tubes, sterile rabbit or horse serum is added to the basal medium to a final concentration of 10 per cent. The tubed or flasked medium is then heated at 56° C. for 30 minutes, unless the serum has been previously inactivated, and is then incubated at 37° C. for 48 hours to test for sterility. The serum makes the medium a rich one for bacterial growth. It is, therefore, essential to insure the sterility of the medium since bacterial contaminants are usually fatal to the spirochetes within 24 hours.

Fletcher's medium is prepared by heating a 12 per cent solution of rabbit serum in distilled water to 50° C., adding 6 ml. of 2½ per cent melted nutrient agar to every 100 ml. of serum-water mixture, adjusting the pH to 7.4, tubing in 3–5 ml. quantities, and sterilizing at 56° C. for 1 hour on two successive days.

On these and other media multiplication occurs rather slowly and may not be evident for a week or more, depending on the virulence of the organisms. Organisms well adapted to artificial media, and hence not very virulent, may grow out in several days. When the clear Schuffner medium is used there may be only a very faint turbidity about 2 cm. from the top of the medium. In Fletcher's medium more turbidity is visible. Generally, however, microscopical examination is necessary to determine whether organisms are present.

It is difficult to generalize as to when cultures should be subcultured. It is safest to check them microscopically once or twice a week and subculture all viable ones at that time. Living organisms seem to die suddenly *in vitro* for unknown reasons.

At present there is no really satisfactory differential medium for *Leptospira*. To cultivate these organisms from materials which are contaminated with other microörganisms, sulfanilamide in a final concentration of 400 mg. per 100 ml. of medium may be incorporated into the Schuffner medium. If there are comparatively few contaminating organisms present, despite the presence of serum or peptone in the medium, the sulfanilamide often inhibits the bacterial growth and permits isolation of the spirochetes which are not affected by this concentration of the drug.[58] Since English workers[62] have concluded from *in vitro* tests that *L. icterohemorrhagiae* is relatively insensitive to the

action of penicillin, this antibiotic may be used for the differential isolation of the spirochete. *Cultivation in chick embryo*—*L. icterohemorrhagiae* has been cultivated on the chorio-allantoic membrane of the chick embryo for a number of passages with no loss in virulence for guinea pigs.[63, 64]

*Resistance to physical and chemical agents and preservation*—The resistance of *L. icterohemorrhagiae* to physical and chemical agents approximates the resistance of the vegetative form of bacteria to these agents.[6–7, 17] Moist heat at 50°–55° C. kills in 30 minutes. Dry heat and desiccation are rapidly fatal.[65] The organism is supposed to withstand freezing but rapid degeneration of the spirochetes into granular bodies occurs at freezing temperatures.[53] It is very sensitive to acid, and is rendered motionless in 10 to 15 minutes by 1:2,000 bichloride of mercury. Bile and bile salts rapidly dissolve this spirochete.

Lyophilization cannot be used to preserve the *Leptospira,* since it causes rapid death.[65] They have, however, remained viable and virulent for up to 100 days in non-contaminated infected guinea pig tissues stored at 5° C.[65] At −78° C. they have retained their morphology and motility for 10 months, but unfortunately no test was made of their virulence.[66] In defibrinated blood kept at room temperature in the light, the organisms remained viable for a week.[67] Since contamination is rapidly fatal to the spirochetal cultures, sulfanilamide, penicillin, and possibly other substances, may be employed to protect the cultures from bacterial contamination during preservation. However, the safest and most certain method of maintaining the organisms, and particularly their pathogenicity, is through repeated animal passage.

*Biochemical characteristics*—Knowledge of the biochemical characteristics of the *Leptospira,* as well as of other pathogenic spirochetes, is pitifully meager, there is no chemical basis for identifying the various strains or species of the genus, distinguishing avirulent from virulent strains, or for developing a synthetic medium. Most of the available data in the literature on leptospiral growth factors are summarized in Table 1. Space is not available here for extensive discussion of the data. However, it is interesting to note that many of the vitamins which are growth-promoting factors for other microörganisms enhance the growth of the *Leptospira.* It may be that the bottom yeast which fosters leptospiral growth [69a] is furnishing one or more of these factors. In general the data on growth factors indicate that enzyme systems similar to those in other microörganisms exist in the leptospiras.

Supniewski and Hano [72] found that the metabolism of *L. icterohemo-*

*giae* differs in many respects from that of *Treponema pallidum*. The former utilizes 1-arabinose but no other pentoses; it hydrolyzes galactose and glucose and to a smaller extent fructose and mannose. It breaks down urea but not uric acid or lactic acid.

TABLE 1

***Summary of Growth-Promoting and Inhibiting Substances for Leptospira***

| *Organism* | *Substances* | *Effect on growth* | *Remarks* | *References* |
|---|---|---|---|---|
| ***L. icterohemorrhagiae*** | *Salts* mixtures of NaCl, $CaCl_2$ $NH_4Cl$ | Promote growth | | Ono (68) |
| | *Acids* formic, tartaric | " " | | Ono (68) |
| | *Carbohydrates* starch, glycerol | " " | | Ono (68) |
| | *Amino acids* asparagine, glutamic acid, lysine, arginine, tryptophane, cystine | " " | when small amount used | Ono (68) |
| | cysteine | " " | | Ward and Starbuck (69) |
| | *Vitamins* nicotinic acid | " " | | Ward and Starbuck (69) |
| | *Miscellaneous* bottom yeast | " " | | Zimmerman (69a) |
| | yeast extracts | " " | | Ward and Starbuck (69) |
| ***L. hebdomadis*** | nicotinic acid | " " | | Yoshida (70) |
| ***L. canicola*** | nicotinic acid and amide, thiamin, riboflavin | " " | | Rosenfeld and Greene (55) |
| ***L. icterohemorrhagiae*** | citric acid | Inhibit growth | | Ono (68) |
| ***L. canicola*** | *Amino acids* | | | Ono (68) |
| | leucine, phenylalynine, alanine, arginine, glutamic acid, glycine, lysine, methionine, tryptophane, tyrosine, valine | " " | significant decrease in growth when the supplement is 0.025 per cent or greater | Greene (56) |
| ***L. icterohemorrhagiae*** | *Acids* lactic, acetic, malic | No effect | | Ono (68) |
| | carbohydrates in general | " " | on morphology or pH of culture | Noguchi (71) |
| | ***Protein split products*** peptone, egg white egg yolk | " " | | Ono (68) |
| | *Amino acids* tyrosine, valine, glutamine, leucine, methionine | " " | | Ward and Starbuck (69) |
| ***L. canicola*** | vitamin $B_6$, ascorbic acid, milk peroxidase | " " | | Rosenfeld and Greene (55) |
| | *Amino acids* asparagine, aspartic acid, histidine, leucine, proline | | | Greene (56) |

## D. ANTIGENIC AND CHEMICAL STRUCTURE

Upon injection into an animal, particularly the rabbit, the *Leptospira* induces the production of agglutinins, spirocheticidins, or lysins, complement-fixing and protective antibodies. Opsonizing or precipitating antibodies have not been demonstrated conclusively.[7] The agglutinins cause clumping of the spirochetes, whereas the lysins bring about fragmentation of the organisms *in vivo* (Pfeiffer's phenomenon) and *in vitro*.

*Leptospiras*, such as *L. icterohemorrhagiae* and *canicola*, can apparently be differentiated by serological methods and cross-protection tests. However, most leptospiral strains are agglutinated by most leptospiral antisera, although in different titers, necessitating the use of agglutinin-absorption for clear-cut resolution of different strains. It is, therefore, considered that the various "strains" are composed of a number of partial antigens and that the distribution of these antigens differs in the various "strains."[17] According to Gispen and Schuffner,[73] there are two such partial antigens, A and B, in *icterohemorrhagiae*. These results in two biotypes of the organisms, a complete one containing both antigens, and an incomplete one which contains only the A antigen. Some investigators refuse to consider serologically different strains as distinct species because of the overlapping of serological reactions. This problem is particularly difficult to resolve for *Leptospira* since there is a lack of other satisfactory criteria for classification generally applied to other microörganisms such as biochemical reactions. It is important, however, to stress the existence of antigenic variations among *Leptospira*; failure to obtain a demonstrable serological reaction against one antigen is not conclusive evidence of the absence of a leptospiral infection. An organism containing another antigen may be the etiological agent.

Little is known of the chemical structure of the *Leptospira*. Lipoid stains have shown that the organism contains lipoid material.[74] Recently a specific substance, carbohydrate in nature, has been extracted from the saprophytic *L. biflexa*.[75] The inability of these organisms to stain with ordinary bacteriological stains is supposedly due to their lack of nucleoprotein,[17] but this has not been demonstrated conclusively by analytical chemical methods.

## E. PATHOGENICITY

As details of leptospiral infections are discussed later, the subject is summarized briefly here. *L. icterohemorrhagiae* is highly pathogenic

for young guinea pigs, hamsters,[76-78, 84] deer mice,[79, 53] and some strains of young white mice,[80] by practically all routes of inoculation. Most strains of white mice and rats are not subject to fatal infections but become carriers.[19, 81] Young rabbits are only slightly susceptible.[19] Young dogs[82] and foxes[45] may be susceptible to infection. Other species and older animals appear to be refractory. *L. canicola* can infect puppies[82] and hamsters[78] but is relatively avirulent for guinea pigs and other species.[8]

## III. LEPTOSPIRA INFECTIONS IN MAN

### A. *L. icterohemorrhagiae*—WEIL'S DISEASE

(Synonyms: Leptospirosis icterohemorrhagica, Spirochetosis icterohemorrhagica, Infectious Jaundice, Spirochetal Jaundice)

*Source of infection*[7, 10]—Twenty to 30 per cent of older wild rats the world over carry virulent *L. icterohemorrhagiae* in their kidneys, probably for life, and excrete them in the urine. This can be demonstrated simply by running agglutination tests with their sera or by demonstrating the organisms in silver-stained kidney section.[39] Infected rat urine is probably the major source of infection, but feces also must be considered potential fomites. Other species such as the mouse, dog, fox, gopher, swine, cow, and vole may act as chronic disseminators of the organism.[7] Man himself may discharge leptospiras in his urine, and in certain environments, such as the paddy fields of Japan and China, may be responsible for the maintenance and spread of infection.[7]

*Mode of transmission*[7, 10]—Weil's disease is usually contracted through contact of the apparently intact or abraded, skin and mucous membranes with materials contaminated by infected rat urine. These materials include soil and water. Contact with infected dogs and other animals may also cause infection. A number of laboratory infections have been reported. The disease has been transmitted by a rat bite.

*Population susceptibility and natural immunity*[7, 10]—Susceptibility to leptospirosis seems general. The disease is more frequently recognized in adults than in children, and in males more often than females. However, this is probably due to differences in exposure as cases have been reported in children and women. Because of a greater occupational risk, sewer workers, fish workers, canal workers, bathers in ponds or canals, workers in wet fields, and miners are particularly liable to infection. Natural immunity is not known to exist.

*Seasonal incidence*[7, 10]—In general, the majority of cases have been recognized in the late summer and fall, but cases have been reported in every month. These seasonal variations are probably reflections of occupational and recreational exposure to the reservoirs of infection. However, the fluctuation of the distribution of the reservoir of infection with the seasons must be taken into consideration.

*Pathogenesis and clinical features*—The incubation period of Weil's disease varies from 5 to 13 days. The first signs of illness are chills, fever, muscular pains, conjunctival congestion, gastrointestinal disturbances, meningeal irritation, and extreme prostration. During this period (about a week) called the first or febrile stage, spirochetes rapidly invade and multiply in the body and are most easily recovered from the blood. The second or icteric stage of about 10 days is characterized by jaundice of the skin, conjunctivae and other visible mucous membranes, hemorrhages, and pulmonary congestion. Antibodies often may be detected in the blood serum during this stage. Organisms often can be demonstrated in silver-stained sections of the kidneys and liver of patients dying in this stage. The urine or cerebrospinal fluid may occasionally contain spirochetes. The third or convalescent stage may be quite prolonged. Leptospiruria may persist for weeks. Specific antibodies may be demonstrated in the serum for months or even years. Immunity is usually lasting upon recovery. The mortality in untreated, jaundiced cases is about 30 per cent.

A number of cases of leptospirosis assume the form of a relatively benign meningitis,[4, 83] with none of the classical symptoms of Weil's disease other than a positive agglutination test. The most perplexing problem connected with the disease is the mechanism of production of the typical icteric and hemorrhagic lesions.[7]

*Morbid anatomy*—The chief pathological features of leptospirosis are the diffuse hemorrhages in the skin, mucous membranes, liver, lungs, and, in fact, almost every organ. Generalized jaundice is found only in about 50 per cent of cases.[7] Various degrees of nephritis are common. The liver is affected to some extent. Degenerative changes and cellular infiltrations in the muscle fibers of the gastrocnemius are utilized by some workers[16] as an aid to diagnosis.

*Nature of immunity*—The nature of the immunity in the leptospiroses is not completely understood. The organisms are widely distributed in the body during the first week of the disease. During the second week, apparently coincident with the appearance of spirocheticidal antibodies, the spirochetes tend to disappear from the blood and

from organs other than the kidney.[20g] The role of phagocytes in the immunity is not definitely known. They either aid in destroying the spirochetes by phagocytosis and intracellular digestion, or protect them by providing an intracellular haven from destruction by antibodies. Recent studies [84, 85] of experimental guinea pig and hamster infections indicate that the humoral antibody mechanism is of primary importance while phagocytosis is only a secondary phenomenon.

*Diagnosis*—Weil's disease may be suspected on clinical grounds when coupled with a history suggestive of exposure to rat infested material. However, the disease may be confused with many conditions, including catarrhal jaundice, yellow fever, acute yellow atrophy, typhoid fever, sewer gas poisoning, blackwater fever, relapsing fever, dengue, malaria, influenza, rat bite fever and acute aseptic meningitis. The differential diagnosis of these conditions on the basis of clinical symptomatology is discussed in a number of reviews given in the bibliography. However, it should be emphasized here that the most reliable diagnosis of the leptospiroses is based upon bacteriological and serological procedures.

*Prophylaxis and control*—Prophylaxis and control are directed at breaking the chain of infection in this disease. The source of infection may be suppressed by control of rats and drainage of infected swamps. Alkalinization of soil and water with calcium cyanamide destroys the spirochetes.[11] The excreta of man and animals should be sterilized. Scrupulous care should be taken to prevent contamination of food with infected discharges. Every effort should be made to avoid direct contact with infected materials. Feet should be protected while working in rat infested areas. Abrasions which permit entrance of the organisms should be avoided. Laboratory workers should handle infected material with gloves since the organisms can apparently invade intact skin.[7] The susceptible population may be protected by immunization with killed leptospiral vaccines. Such vaccines have been used on a large scale in Japan with promising results.[7]

*Serum therapy*—It has been demonstrated that antiserum is effective in lowering the mortality rate if given in adequate dosage during the first 6 days of infection. The most widely used sera are derived from horses whose blood has an agglutinin titer of 1:100,000 or higher against *L. icterohemorrhagiae*. Strong [86] recommends that 20 ml. of serum be given intravenously every 3 hours for at least 3 or 4 days. Some of the failures reported were possibly due to a lack of antibodies to the particular strain of *Leptospira* involved. When immune serum

is not available, convalescent serum may be used; but the results are not so consistently good.

*Chemotherapy*—Treatment of Weil's disease with arsenicals has been unsatisfactory.[17] Better results have been reported with soluble bismuth preparations.[87] There is considerable dispute as to whether the bismuth is spirocheticidal or spirochetistatic or whether it merely activates the normal body defense mechanism.[17] Sulfonamides have thus far been without effect on the disease.[29, 1] Although *L. icterohemorrhagiae* is relatively insensitive to the action of penicillin *in vitro*,[62] it has proved very susceptible *in vivo* in experimental guinea pig infections.[88–91] Reports on the treatment of the human disease have been favorable.[92–95] Streptomycin has also proved effective against the experimental infection in guinea pigs.[96] At the time of writing, the status of these drugs in the therapy of human leptospirosis is promising but not definitely established.

B. *L. canicola*—CANICOLA FEVER

*Leptospira canicola* (Canicola fever)—In 1933 Klarenbeek[97] and Schuffner[10] discovered that the canine strain, *Leptospira canicola*, may infect man. Since then 25 or more cases have been reported in Denmark, Holland, Austria, and California.[98] *L. canicola* may cause a highly fatal infection in dogs. Apparently healthy dogs may harbor the organisms in their kidneys and excrete them in the urine. Since *L. canicola* has not been isolated from the rat, it is concluded that this strain does not infect rodents. Transmission to man probably occurs through contact with infected canine urine or feces, or through the handling of infected tissues. Contact infections have occurred in veterinarians and other persons frequently handling dogs.[98] *L. canicola* infections reported in man have thus far been mild, leading some workers to suspect that this disease often goes unrecognized or is mistaken for some other syndrome. Jaundice and hemorrhagic nephritis have been reported, and the disease has often followed an atypical, chronic course complicated by meningitis. Clinically it may simulate influenza, undulant fever, or tuberculosis. Serological examination of the blood, and bacteriological examination of the urine may establish the correct diagnosis. Since penicillin is of value in the treatment of the canicola infection in the dog,[53] it may be of value in the human disease. For further information on this disease, the reader may consult the papers by Meyer, et. al.,[98, 8] Bloom,[99] Davis, et. al.,[100] Jungherr,[101] Monlux,[102] and Elander and Perry.[103]

## C. OTHER LEPTOSPIRA INFECTIONS

*Other Leptospira infections*—Inasmuch as these diseases apparently occur chiefly outside the continental United States, they will only be discussed briefly here. For a more complete treatment of the subject the interested reader is referred to the comprehensive review by Walch-Sorgdrager [12] and shorter discussions in Topley and Wilson [17] and Jordan and Burrows.[104]

"Swamp fever" clinically resembles an abortive or mild form of Weil's disease. It has been reported chiefly in Russia and Germany. The sources of infection are the field mouse, wood mouse, and bankvole; possibly the dog, horse, and ox also are involved. The etiological agent, *L. grippo-typhosa,* is immunologically distinct from *ictohemorrhagiae* and *canicola.*

"Seven-day fever" is a mild leptospirosis similar to "swamp fever." It occurs in many rural regions of Japan and apparently is spread by the infected urine of the field mouse and possibly of the dog. The causative agent, *L. hebdomadis,* may be differentiated from *icterohemorrhagiae* and *canicola* by serology and, less accurately, by its pathogenicity for the guinea pig. "Autumn fever" is a Japanese leptospiral infection similar to seven day fever caused by *L. autumnalis.* The carriers are species of field mice and rats.

The following organisms and diseases have only been touched on in the literature. *L. pyrogenes* produces a dengue-like infection in plantation areas in Java, associated with a mild rash and jaundice. The reservoirs of infection are species of wild rats and possibly the cat. *L. sejroe* causes a swamp fever-like syndrome in Italian rice fields and in Denmark. Rats and mice are again incriminated as carriers. *L. bataviae* is the etiological agent of a mild febrile disease, with jaundice a comparatively rare symptom. It is common in Java, Japan, and Italy. Rats, mice, and possibly cats are the source of infection. *L. australis A* and *B* cause the mild "Mossman fever" in Australia, *L. pomona* is the etiological agent of "Pomona" or "field fever," a type of "swamp fever," in Australia and Italy. The organisms are believed to be excreted in the urine of rats and pigs. *L. icterohemoglobinuriae vitulorum* produces a type of human leptospirosis in Russia.[105] The disease is apparently associated with infected cattle. A similar human disease has occurred in Palestine in association with bovine cases.[106] The true nature of the *Leptospira* in these infections is not known, but it is apparently closely related serologically to *L. icterohemorrhagiae.*

## IV. NATURAL LEPTOSPIRA INFECTIONS IN ANIMALS

*Natural Leptospira infections in animals*—With the exception of the dog, and possibly the fox, fatal leptospiral infections seem comparatively rare in animals. However, a wide variety of species harbor the organisms in their kidneys and shed them in the urine. These include, wild rats, field mice, dogs, voles, bandicoots, foxes, wolves, ferrets, and swine.[7] Only occasionally is a particularly virulent strain or a massive inoculum able to induce a fatal, or at least serious, disease in these species. This discussion will, therefore, be limited mainly to the dog which is especially susceptible to highly fatal leptospiral infections. The canine disease is important both as a veterinary problem and as a human public health problem since the dog may constitute a reservoir of infection for *L. icterohemorrhagiae* and *L. canicola* and possibly for other strains of *Leptospira.*

The relative incidence of the two infections in the dog is not known with certainty, but *L. canicola* seems to be the more common. *L. icterohemorrhagiae* has recently been isolated for the first time from a dog in the United States.[78] Leptospirosis is very common in dogs and is world-wide in distribution. The most characteristic difference between the two types of infection is the jaundice which is found frequently and almost exclusively in the *icterohemorrhagiae* type ("yellows"). In the *canicola* infection ("Stuttgart disease" or "canine typhus"), kidney damage is usually greater, and uremia may occur. *L. icterhemorrhagiae* is more common in young dogs, *canicola* in older dogs. The mortality is higher in the more acute *icterohemorrhagiae* infection. The type caused by *canicola* is probably spread by dog-to-dog contact, since no other animal has thus far been incriminated as a carrier. The rat probably is the carrier for cases in dogs due to *icterohemorrhagiae.* In the latter cases the mode of transmission is the same as in the human disease.

The disease may be divided into two stages, the febrile and the toxic. During the febrile stage the organisms are disseminated throughout the tissues, producing a high fever, lethargy, anorexia, and congestion of the mucous membranes. Symptoms of gastric irritation may occur, and vomiting is often the first symptom noted. During the toxic stage, the icteric or uremic syndrome may be observed, depending on whether the liver or kidney is more severely damaged. Primary liver involvement leads to jaundice, which is partly hemolytic and partly obstruc-

tive in origin. Hemorrhagic gastritis and enteritis, and hemorrhages in the tissues are often present.

Diagnosis of the disease is similar to that in the human infection. The one important point of difference is the relative insusceptibility of the guinea pig to *L. canicola.* Instead, the hamster is used as a test animal.[78] Leptospiras are found in the blood from the 3rd to 5th day, and in the urine from the 7th to 10th day of the disease. Penicillin is apparently of value in the treatment of this infection.[53]

A disease similar to the canine type has been described in the fox in Germany,[107] and England,[45] but not in the United States.[53] More detailed information on leptospirosis in the canine family may be found in the references.[98–103] Cats have leptospirosis caused by *L. bataviae,* and organisms found in Java, Japan, and Italy.[108]

## V. EXPERIMENTAL LEPTOSPIRA INFECTIONS

*Experimental leptospiral infections*—A number of factors are important in the selection and successful use of test animals for the isolation of *Leptospira:*

A. Individual differences in susceptibility—Individual guinea pigs injected with virulent *Leptospira* have been refractory while the majority of animals receiving the same inoculum by the same route have succumbed. In part, this may be due to the use of animals of different ages or genetic backgrounds. These variations in susceptibility suggest the use of several test animals and the close observation of these animals for evidence of infection.

B. Age of Animal—The increase in resistance to leptospirosis with increasing age has been demonstrated in the guinea pig,[37, 85] white mouse,[80] dog,[82] and rabbit.[109] This resistance is manifested by lower mortality, absence of fever, prolonged period of survival, and lack of typical postmortem findings. Young animals are, therefore, preferred for leptospiral diagnosis or experimental studies. The mechanism of this age resistance is unknown.[85]

C. Strain of *Leptospira*—Upon prolonged cultivation on artificial media leptospiras gradually lose their virulence. This virulence may be restored by repeated passage through susceptible animals.

D. Size of inoculum—The size of the inoculum certainly influences the results of injection; failure to infect a test animal may merely indicate that too few organisms were present in the inoculum.

E. Route of inoculation—Virulent strains of *L. icterohemorrhagiae* in adequate numbers can infect young guinea pigs and hamsters by intraperitoneal, subcutaneous, intramuscular, intradermal, intraocular. intracardial, subdural, intravaginal, intraanal, and intravenous injection.[84, 85] Only the intact skin and mucous membrane may at times afford an effective barrier to infection.[85] The oral and intracranial routes are not as efficient as the others.[84, 85] Intraocular injection of relatively small numbers of organisms usually causes the death of the guinea pig.[84] Intraperitoneal and intravenous inoculation may cause the disease to appear sooner and in more severe form than subcutaneous injection.[17] The inoculum is given intramuscularly, subcutaneously, or by application to slightly scarified skin. To effect separation of *Leptospira* from non-pathogenic contaminants, several milliliters of the contaminated spirochetal suspension are injected intraperitoneally into a young guinea pig and 10 to 15 minutes later blood is withdrawn from the heart and is cultured. The heart's blood should contain the spirochetes which very rapidly invade the blood stream.[110]

F. Species and strain differences in susceptibility—The most important factor influencing the success of animal inoculation is the species or strain of animal employed. The guinea pig was the first species found to be infected readily with *L. icterohemorrhagiae*.[20a-g] Recently, species of deer mice,[79, 53] the hamster,[76-78] and certain strains of young white mice [80] have been infected. Other strains of young white mice are refractory and become carriers of the spirochetes in their kidneys.[81] The susceptible species and strains react similarly to the infection and are, therefore, equally suitable for diagnostic or experimental studies. The mechanism of species resistance is unknown.[85] The typical experimental leptospiral infection is described in Section VI.

When refractory species are inoculated with *L. icterohemorrhagiae* they occasionally develop typical fatal infections, but more commonly exhibit no signs of infection other than a transient rise in temperature, a low agglutinin titer, or are found to excrete organisms in the urine.

Upon inoculation with *L. canicola* guinea pigs ordinarily do not develop any symptoms unless an unusually virulent strain [102] is used. The fatal *canicola* infection of the guinea pig and hamster resembles the infection of these species with the classical *icterohemorrhagiae* strain, but jaundice is less common, while kidney damage occurs more frequently.

# VI. DIAGNOSTIC PROCEDURES

## A. OUTLINE OF STAGES OF WEIL'S DISEASE AND DIAGNOSTIC METHODS APPLICABLE TO EACH STAGE

In order to provide a basis for the choice of diagnostic method the detailed discussion of these methods is prefaced by an outline of the stages of the disease and the procedures to apply in each stage:

1. Acute, febrile, septicemic stage—3rd to 7th day
   a. Demonstration of organism
      (1) Circulating blood
         (a) Culture and animal inoculation best
         (b) Examination of dark-field and stained preparations less satisfactory
      (2) Bone marrow
         (a) Culture and animal inoculation possibly positive
         (b) Direct examinations rarely positive
   b. Detection of antibodies—rarely positive
2. Hepatic or icteric stage—7th to 10th day
   a. Demonstration of organisms
      (1) Blood—less likely to succeed than in first stage
      (2) Urine—occasionally animal inoculation and culture positive; other procedures valueless
      (3) Cerebrospinal fluid—occasionally animal inoculation and culture positive; other procedures useless
      (4) Bone marrow—Culture and animal inoculation may be positive; other procedures are rarely positive
   b. Detection of antibodies
      (1) Blood—often positive findings
      (2) Urine—less often positive
3. Convalescent stage—quite prolonged
   a. Demonstration of organism
      (1) Blood—rarely positive by any method
      (2) Urine—usually positive by animal inoculation and culture
      (3) Cerebrospinal fluid—occasionally positive by animal inoculation and culture
      (4) Bone marrow—data insufficient for evaluation of the methods for demonstration of organism
   b. Detection of antibodies
      (1) Blood—usually positive findings
      (2) Urine—less often positive
4. Postmortem—organisms may be demonstrated by culture, animal inoculation, or silver impregnation of liver, kidney and adrenal tissue.

## B. DISCUSSION OF DIAGNOSTIC PROCEDURES

The basis for the application and performance of diagnostic procedures has been given in previous sections. In this section emphasis

is put on the special details of these procedures and the precautions which must be observed.

*Preparation of material for testing*—There are a number of relatively crude methods for preparing materials for testing. It is important to know these methods since they may increase the possibility for successful diagnosis.

In blood, the spirochetes are so light that it requires centrifugation at 3,500 r.p.m. for 5 hours to centrifuge them so completely that the supernatant is negative by dark-field.[6, 53] To remove obscuring red and other cells, light centrifugation or hemolysis by addition of distilled water may be employed. However, the latter procedure dilutes the specimen. Incubation of the blood for as long a time as is convenient at about 27° C. (in the vest pocket) may increase the number of organisms and facilitate their direct observation. Some workers believe that the spirochetes are concentrated in the fibrin clot or sedimented red cells and use these materials in the various procedures. Defibrinated blood has been used by others.[6]

When examining urine, about 80 ml. of urine are collected as aseptically as possible. If not already alkaline, the urine is neutralized by the addition of alkali. It is centrifuged as recommended for blood. In order to remove lytic antibodies which may be in the urine, it is desirable to wash the sediment several times in Schuffner's medium before use. As urine often contains other microörganisms an antibacterial agent should be incorporated in the culture medium.

For cerebrospinal fluid examination, asepsis is again essential. Centrifugation of the fluid may concentrate the organisms. Bone marrow may be removed by aspiration or post-mortem, aseptically if possible.

*Demonstration, isolation, and identification of organisms*—This is the most certain method of proving the etiology of the disease. Dark-field examination is subject to three difficulties which render it unreliable. The first difficulty is that the organisms are seldom numerous enough in any human tissues to be observed directly. They are occasionally demonstrable in guinea pig blood or urine and rat urine by this method. A second drawback is the presence in the dark-field of artefacts, such as fibrin strands ("pseudo-spirochetes").[111] Therefore, only one thoroughly acquainted with the characteristic morphology and motility of these organisms should undertake their microscopic identification. Third, in contaminated urine, it is impossible by this method to distinguish between pathogenic and saprophytic *Leptospira*. For a description of dark-field microscopic technic see references.[112–114]

For the staining of smears the author has tried a number of stains. The Fontana-Tribondeau silver impregnation method was found unsuitable for tissue and blood smears, since the silver is deposited on fibrin thread, connective tissue fibrils, and tissue debris which may be confused with the spirochetes. Dilute, alkaline Giemsa stain is satisfactory for the demonstration of *Leptospira* in any type of smear. The air-dried smear is treated as follows:

1. Absolute alcohol, 15 minutes
2. Ether, 5 minutes, to remove fat
3. Absolute alcohol, 15 minutes
4. Wash in several changes of distilled water
5. Stain for 8–12 hours in Giemsa solution; the time must be determined for each batch of stock Giemsa stain.

The dilute stain is prepared fresh each time as follows:

50 ml. distilled water
2 drops 0.5 per cent sodium bicarbonate
1.5 ml. methyl alcohol
1.25 ml. Giemsa stain (stock solution)

In a well stained smear the spirochetes appear reddish violet while the erythrocytes, leukocytes and other tissue cells are deep blue.

The negative stains, congo red and nigrosin are suitable for staining smears of spirochetal cultures and clear body fluids free of tissue and other debris. Usually equal volumes of 2 per cent congo red or nigrosin in distilled water and the fluid containing the spirochetes are mixed on a clean slide and allowed to air-dry. The nigrosin-stained slide is examined without further treatment while the congo-stained slide is flooded with 1 per cent hydrochloric acid in alcohol and is then permitted to dry in the air. In both cases the spirochetes appear as white, unstained, hooked threads against a pale blue background.

For staining *Leptospira* in tissues, the various Levaditi silver impregnation methods are satisfactory. However, in most of these methods the entire block of tissue is impregnated with silver, rendering it impossible to use any other stain to demonstrate histopathological changes in that block. The Warthin-Starry stain for *Treponema pallidum,* as modified by Kerr,[115] is used on tissue sections making it possible to use as many different stains as desired on one tissue block. Also with this stain, a great deal of histological detail can be seen. This makes it suitable for observations on phagocytosis of *Leptospira in vivo.* The stain may be used as originally described or with the following modifica-

tions: Mercury salt fixatives may be employed if they are carefully washed out in the usual manner with running tap water, Lugol's solution, and sodium thiosulfate prior to staining. Sections may be placed on slides instead of coverslips. Recently a new simple silver stain for spirochetes in sections has been described.[116] For further information on the staining of *Leptospira* see references.[3, 7, 8, 11, 14]

In culturing *L. icterohemorrhagiae*, *L. canicola*, and other members of the genus, the importance of media carefully prepared as to sterility, pH, inactivation of serum, and antibacterial agents to inhibit bacterial contaminants, should be reëmphasized. The choice of material, depending on the stage of infection, has been discussed previously. Usually about 0.1 ml. of blood or ground-up blood clot is cultured in 3–5 ml. of medium, but it is probably better to inoculate about 0.25 ml. of blood or minced tissue into 10–20 ml. of medium to dilute any antibodies present. A larger volume of inoculum may reduce the oxygen tension too low for growth of the obligate aerobic spirochete. Cultures which are positive on the first or second day after inoculation may subsequently become sterile, especially where large amounts of tissues were inoculated.[39] To prevent this, subcultures are made about 24 hours after inoculation.[39] Both cultures are then incubated and observed daily for growth. Bone marrow has been found to contain *Leptospira* in the experimental guinea pig disease.[117, 84]

For animal inoculation, young guinea pigs, weighing less than 150 gm. preferably white to facilitate observation of jaundice of the skin, young golden hamsters, [76–78] and species of deer mice [79] are the animals of choice for the diagnosis of leptospirosis. Certain strains of young white mice are also susceptible.[80] The offspring of animals which have been unsuccessfully inoculated with *Leptospira* are also refractory to infection and should not be used for diagnostic purposes.[37] As mentioned before, it is advantageous to give a large inoculum to several test animals by a route conducive to infection. The susceptible species are inoculated intracardially or intraperitoneally with the test material if it is known to be free of contaminants. If contamination is suspected the material is injected subcutaneously or intramuscularly. The intraocular or intracerebral routes permit detection of relatively small numbers of organisms.[84] However, contaminants are apt to produce infection by the latter routes. The amount to be inoculated is limited only by the supply of material and the size of the test animal.

Typical experimental leptospirosis lasts 5–12 days and terminates in death. Conspicuous symptoms during life are fever, jaundice, and

conjunctival congestion. Fever usually commences the day after injection, rises to a peak in several days, and descends just before death. Jaundice is first apparent when the temperature begins to fall and increases until death. It is most easily observed in the sclerae and inside of the ears. Conjunctival congestion is first visible about the 5th day. Loss of weight, weakness, drowsiness, inanition, and anorexia are commonly present. At necropsy the findings are emaciation, intense icterus of skin and all internal surfaces, multiple petechial hemorrhages in skin, subcutum, muscles, mucous membranes, and viscera. The hemorrhages in the lung give this organ the appearance of a butterfly, hence the term "butterfly lung." This is practically a pathognomonic lesion. At postmortem, the organisms are most numerous in liver, kidney, and adrenal tissue.

The test animals should be examined daily for jaundice. At the same time each day temperature and weight should be determined. Puncture of the peritoneum and examination of the exudate for spirochetes often yields positive results as early as the 3rd day, at the same time a sustained rise in temperature occurs (from a normal of 100° F. to 103° F. and a peak of 105.6° F.). At this time the animal is anesthetized or strapped to a board, and 2 or 3 ml. of blood are withdrawn from the heart into an anticoagulant, and the blood is examined by dark-field or cultured. If organisms are seen in the blood, this may be considered presumptive evidence of infection in the patient. If the animal is jaundiced 7 to 10 days after injection, or if there is no evidence of infection within 2 weeks after inoculation, it is killed by administration of illuminating gas or by a blow on the head. Chloroform is not used since it may produce pulmonary petechiae resembling "butterfly lung."[37] The tissues, particularly liver, kidney, and adrenal are examined by dark-field, cultured, sectioned or subinoculated into other animals. At autopsy the characteristic icteric and hemorrhagic lesions are sought for. Usually three passages at intervals of about 2 weeks are recommended before a negative report is justified. In occasional cases guinea pigs die with classical symptoms but no spirochetes. In these instances their presence in the liver and kidneys can sometimes be demonstrated by the silver stain. *L. icterohemorrhagiae* can be presumptively differentiated from *L. canicola* by the relative non-virulence of the latter for the guinea pig.

As in other infections the detection of anti-leptospiral antibodies does not in itself indicate active infection but may be simply a sign of past infection. Therefore serology must be supplemented by other tests.

However, repeated tests showing a rise in titer may be a sign of present infection.

*Serology: Agglutination test*—The microscopic agglutination-lysis test is the most reliable and sensitive of the agglutination procedures, but has the disadvantage of requiring dark-field illumination and being, therefore, relatively time-consuming to perform. Consequently, a number of macroscopic procedures have been developed.[118–121] Although these technics are claimed to be specific and sufficiently sensitive to be of value in the detection of this disease, it is the opinion of this author that in the present stage of their development they are not entirely reliable. Emphasis, will, therefore, be put on the microscopic test.

The agglutination-lysis test of Schuffner and Mochtar[60] is the most satisfactory of a number of microscopic tests which have been described.[122, 123] It is, therefore, given in detail. For antigen, a 5 to 7 day culture of *L. icterohemorrhagiae* or other *Leptospira* on Schuffner's medium (diluted with basal medium to contain 25 to 50 organisms in a high dry dark-field) is used. Clumps in the cultures are dispersed by light centrifugation. Live organisms are sometimes preferred, but Schuffner considers the formalinized antigen more specific.[10] Formalinized (3 drops formalin to 10 ml. culture) antigen is usually stable for from 4 to 6 months. However, heat and formalin-killed antigens may agglutinate spontaneously and suddenly, necessitating the inclusion of adequate controls in the performance of the test.

Control antisera may be produced in rabbits. An initial intraperitoneal injection of 0.5 ml. of a virulent culture is followed after a week by gradually increasing volumes of organisms intravenously every 3 or 4 days. The titer may be raised into the millions if immunization is continued for several months. A trial bleeding is performed a week after the last injection. The rabbit is retained as a stock source of serum, the antibody titer being raised by further injections as required.

Agglutination tubes or porcelain plates may be used as receptacles for the antigen-antibody mixtures. Sera are inactivated. Incubation is for 2 hours at 37° C. for live antigen, and 4 hours at this temperature for killed antigen, preferably in a water bath. The sera may be diluted to final dilutions of 1:10, 1:30, 1:100, 1:300, 1:1,000, 1:3,000, 1:10,000, and 1:30,000. Known positive and negative sera should be included in each series of tests. Usually 0.15 ml. of antigen are added to 0.15 ml. of serum. A drop of the antigen-antibody mixture is examined by dark-field microscope. In a typical positive test, living antigen is agglutinated in lower dilutions and lysed in higher dilutions, while killed

organisms usually are clumped in the highest dilutions compatible with the strength of the serum (see Figure 3).

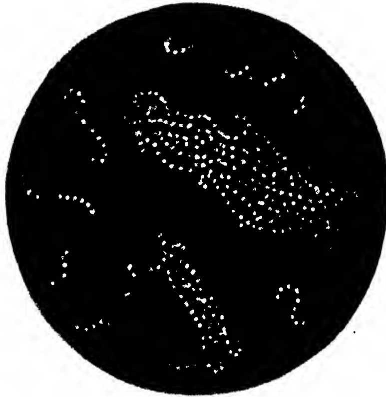

FIGURE 3—Drawing of *Leptospira* agglutinated and broken into fragments upon contact with a specific immune serum as in a positive microscopic agglutination test.

In the average case of Weil's disease, after 3 or 4 weeks, the patient's serum agglutinates avirulent *L. icterohemorrhagiae* in dilutions of 1:10,000 to 1:300,000, but will not agglutinate heterologous strains in dilution above 1:250.[1] A series of agglutination tests showing a rapidly increasing titer indicates present infection and excludes the possibility of being misled by an amnestic reaction in a patient. Strong false-positives are practically nonexistent. A negative reaction after the 30th day of illness rules out leptospirosis.

Pot[119] and Smith and Tulloch[118] have described macroscopic tests using formolized[119] and live[118] organisms. As the antigens are difficult to prepare and the tests difficult to read, for routine use they have little advantage over the microscopic test. Brown[120] has reported a rapid presumptive test employing a formolized culture in which the serum-antigen mixtures are rocked on a slide and observed with a hand lens against a dark background. Recently Fineman and Joyner[121] have modified Brown's test. A drop of antigen, which is now commercially available, (formolized, concentrated by *centrifugation, and gentian violet* added) and a drop of diluted serum are mixed on a clean glass plate ruled in squares. After gentle rocking for ten minutes the plate is examined over a white background by diffuse transmitted light. A positive reaction is indicated by light blue aggregates in the mixture. The

author has had some experience with these macroscopic tests and is somewhat wary of relying on their sensitivity or specificity.

Agglutinin-absorption is often necessary to provide a sharp differentiation between *icterohemorrhagiae* and *canicola* or other *Leptospiras*. The technic of Schuffner and Bohlander [124] is the best for this purpose. A well grown, thick culture, formolized to a final concentration of 0.5 per cent and concentrated by centrifugation is used for absorption. Absorption is allowed to proceed overnight.

Complement-fixation is usually as specific and sensitive as agglutination, but sometimes the results of the two tests do not correspond.[125–127] In these instances complement-fixation seems to display broader reactivity. Complement-fixation under carefully controlled conditions has the advantage of not requiring the skill and experience required for the agglutination test. The test may be run as for the diagnosis of syphilis. The antigen may be prepared by inoculating 5 oz. of medium in a nursing bottle and incubating at room temperature for a week.[126] The culture is heated at 100° C. in an Arnold sterilizer for 2 hours, centrifuged or filtered through a Seitz filter. This antigen does not show anticomplementary activity.[126] Cross-reactions with Wassermann-positive sera were obtained by Boerner [126] by incubation for 18 hours at 18° C., but were eliminated by incubation for 1 hour at 37° C. The cross-reaction suggests the existence of an antigen common to *Leptospira* and *Treponema pallidum*. There are few data available on the incidence of false-positive reactions with leptospiral antigen using the complement-fixation reaction.

The adhesion or thrombocytobarin reaction [128] is conducted by adding a mixture of spirochetes and specific serum to a suspension of blood platelets or bacteria, incubating for 20 minutes at 30° C., and observing the preparation by dark-field. A positive reaction is indicated by the platelets or bacteria adhering to the spirochetes forming small clumps. Living, motile spirochetes and fresh complement are required. By this test it is possible to distinguish between two closely allied forms like *L. icterohemorrhagiae* and *L. hebdomadis*. The usefulness of this method for diagnosis of the disease remains to be determined.

Thus far a satisfactory precipitation test has not been developed for the diagnosis of leptospirosis although flocculation may be obtained by mixing alcoholic extracts of leptospiras with anti-*Leptospira* serum.[129] These flocculation reactions are, however, nonspecific.

Protective or spirocheticidal antibodies may be detected by mixing 1–2 ml. of patient's serum with several lethal doses of the spirochete,

incubating the mixture at room temperature for 30 minutes, and then inoculating the whole into susceptible animals.[17, 37, 130] If protective bodies are present, the animals will survive while control animals die of leptospirosis. These antibodies are also demonstrated by giving immune serum intraperitoneally, waiting half an hour, and then injecting the organisms by the same route. In the presence of specific antibodies the spirochetes are agglutinated and degenerate into granular bodies (Pfeiffer's phenomenon).[17] In from 10 to 30 minutes no free or morphologically normal spirochetes can be found in fluid aspirated from the peritoneal cavity.

The protection test in mice is specific for *Leptospira,* no cross-reactions having been obtained with sera from patients suffering from the following diseases: influenza, malaria, poliomyelitis, Rocky Mountain Spotted Fever, typhus, Q fever, tularemia, rat-bite fever, relapsing fever, infectious jaundice, syphilis, and yellow fever.[130] Serum from rabbits immunized against *L. canicola* either failed entirely to protect mice against *L. icterohemorrhagiae* or yielded equivocal results.[130]

There is one report in the literature on the use of a skin reaction for the diagnosis of leptospirosis,[131] but it has never been confirmed.

## VII. SUMMARY AND OUTLOOK

There are a number of problems the solution of which undoubtedly will facilitate a more rapid, accurate, and easier diagnosis of leptospirosis. Dependable methods of early diagnosis are particularly needed. A medium which would promote faster and increased growth of the spirochetes than those now available might simplify isolation of the organisms and preparation of a stable antigen in large quantity for serology. Perhaps the refinement of the chick embryo method of cultivation of *Leptospira* will facilitate diagnosis. The development of differential media to prevent overgrowth of the organisms by contaminants would be helpful. Aspiration and culture of bone marrow may afford a method of earlier diagnosis. Further studies on the usefulness of intraocular injection of guinea pigs as a method of detecting small numbers of spirochetes are desirable. Perfection of a macroscopic agglutination test of greater sensitivity and reliability would encourage wider application of serology to diagnosis. The perfection of a skin test would also contribute to our understanding and diagnosis of leptospirosis. Increased knowledge of the biochemistry and metabolism

of these spirochetes and the pathogenesis and immunity of the disease may conduce to the development of better diagnostic methods.

Through the improvement of diagnostic methods or the wider and more intelligent application of existing procedure it is quite likely that leptospirosis in man and animals will be found to be more common in the United States and in the world than is now believed. Already the animal reservoir appears to be widening, and it is possible that few mammalian species will be found to be free of some type of leptospiral infection. As an occupational hazard, preventive measures must be instituted to curb its spread. In time it may be found to be a disease of more than sporadic or endemic occurrence. Indeed the leptospiroses are probably destined to grow into a considerable public health problem in the United States and elsewhere.

## VIII. ADDENDUM

Since this manuscript was completed a number of important data on the cultivation of *Leptospira* have become available. They are summarized below.

Rabbit serum is most commonly used in the cultivation of pathogenic leptospiras, but horse serum is equally suitable.[53] Human, bovine, sheep, guinea pig, mouse and rat sera support growth poorly if at all.[53] More rapid and abundant growth is obtained with rabbit serum tinged with hemoglobin,[53] but these cultures are not viable for as long a period as when unhemolyzed serum is used.[53]

As even a trace of soap is fatal to leptospiras, glassware must be scrupulously clean. Stuart[133] recommends, therefore, that the culture tubes be washed and rinsed in the usual way and then steeped in phosphate buffer at pH 7.6 for 24 hours before being finally rinsed and sterilized.

More recently, Stuart[133] has described a new and simple culture medium for isolation of *Leptospira,* for their maintenance in stock cultures, and for serology. Growth on the medium is claimed to be at least equal to that in any of the other commonly used media. Stuart also mentions the growth-promoting properties of glycerin in the medium. In addition glycerin delays for several minutes the drying of loopfuls of culture examined without cover glass, thus permitting more samples to be examined at one time and affording a longer period for examination. Chang[134] has developed a fluid and semisolid medium

for growing *Leptospira,* and a method for maintaining the virulence of the organism *in vitro.* The author has not had the opportunity to evaluate these media but feels that they merit careful trial. One particular advantage of the Stuart medium would seem to be the absence of Witte's peptone from the formula. This peptone is generally unavailable, and in the experience of the writer is not completely replaceable by any other commercial peptone.

Further data on spirochetal metabolism, including a description of a partially successful synthetic medium for *Leptospira* may be found in an article by Savino and Renella.[135]

Stuart [133] considers sulfaguanidine superior to either sulfanilamide or sulfadiazine for inhibiting the growth of contaminants. A mixture of 4 parts of a saturated solution of sulfadiazine in basic medium with 6 parts of basic medium is a satisfactory combination for this purpose.

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# Bacterial Food Poisoning*

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* This section is a revision of the one prepared by Stewart A. Koser, Ph.D., which appeared in the First Edition.

## I. INTRODUCTION

FOOD poisoning outbreaks may be caused by one of a variety of etiologic agents. Certain bacteria or their products are the most important of these agents.[1] A number of metallic compounds may produce poisoning. A few foods are inherently poisonous due to the presence of alkaloids or more obscure but perhaps related substances. The phenomenon of food allergy is also encountered. Finally, there are always a number of outbreaks in which no definite causative agent can be ascertained. At times the epidemiological evidence and clinical symptoms may afford a clue to the nature of the causative agent, but at other times this information is of little assistance.

Since the methods to be presented here deal only with bacteriological examination, no attempt will be made to outline procedures for those instances in which food has become poisonous due to other causes. There is ample evidence, however, that at times these other causes lead to very striking cases or outbreaks. In this category are the poisonings due to mushrooms or other fungi, the "milk sickness" following consumption of milk of cattle which have eaten white snakeroot or richweed, some forms of shellfish poisoning which have been traced to the presence of certain species of a dinoflagellate in the shellfish,[2] and other instances in which animal or plant poisons have been conveyed directly or indirectly through food.[1]

When there is reason to suspect that the illnesses are due to the presence of certain bacteria or their toxins, the laboratory worker must first have clearly in mind the microörganisms which experience has shown to be capable of causing food poisonings. At present several groups have been definitely implicated, with suspicion directed toward others. The groups for which definite evidence has been repeatedly obtained are: (1) *Clostridium botulinum*, the toxin of which gives rise to clinical symptoms distinct from those produced by the other food poisoning organisms, (2) certain members of the *Salmonella* group, (3) staphylococci, the enterotoxin of which causes symptoms, and (4) streptococci. In addition, there have been a number of instances in which a causative role has been claimed for other microörganisms.

Our chief concern must be the detection of those types which past experience has proved to be definitely implicated in outbreaks, though we should not overlook the possibility of finding some other organism which may be significant. The possibilities fall into many different bacterial groups, and the organisms concerned present widely different

characteristics. In attempting to outline laboratory procedures for examination of suspected foodstuffs, account must be taken of the greatly diversified natures of the causative bacterial agents, which will naturally complicate the procedure, while at the same time it is essential to formulate methods which are not impractical for the average diagnostic laboratory. The following outline has been revised from that originally published,[3] and is presented as a suggested procedure.

## II. COLLECTION OF SPECIMENS

Samples for laboratory examination must not be collected indiscriminately. Preliminary epidemiological investigation will reduce to a minimum the number of suspected foods in a given outbreak. Evidence concerning the particular foods most likely to be responsible should be sought by listing foods consumed by those made ill. While this evidence is not always clear-cut, at least a number of items can usually be eliminated. Some judgment may be called for in deciding what foods would be most likely to convey the various causative organisms or toxins. The time when symptoms first appeared and other pertinent items should also be recorded.

When food from sealed containers is under suspicion, the original container should be obtained wherever possible, together with the complete label of the product. A record should be made of code marks and any other identifying marks on the container.

When it is necessary to transfer a representative sample to a smaller container, sterilized sample bottles should be used. If at times the samples are of such a nature that they cannot be put into the common sizes of sterile containers, at least they should receive every possible protection from additional contamination in transit.

Fecal specimens are often of value if secured early in the acute stage of the disease. Blood samples are of less importance, but in cases of botulism or occasionally in *Salmonella* infections they may be useful, particularly where it has been impossible to obtain samples of the suspected foodstuffs. If necropsy material is available from fatal cases, portions of the colon, spleen, and mesenteric lymph nodes should be collected.

It is important that the samples be collected promptly and that the laboratory examination be started without delay. If specimens must be shipped to the laboratory, arrangements should be made for their refrigeration.

## III. LABORATORY PROCEDURE

An outline of the procedures is given in Figure 1. The initial steps should be started as soon as possible upon receipt of the food sample. These are A-1, 2, and 3, or A-4 and 5.

### A. FIRST EXAMINATION

#### 1. *Direct microscopic examination*

Make a Gram stain preparation directly from the liquid portion of the suspected foodstuff. With certain foods this may be impractical and can be omitted.

#### 2. *Plating*

Material for plating and for step C should be taken from the food in such a way as to secure a representative sample.* With a loopful streak in succession 2 agar plates, using preferably blood agar prepared from meat infusion (C.M. No. 33). If blood agar is not available, meat infusion agar without blood may be used (C.M. No. 5a).

A series of quantitative plates, using a meat infusion agar, is also desirable to give an estimate of the number of any predominating or significant bacterial types.

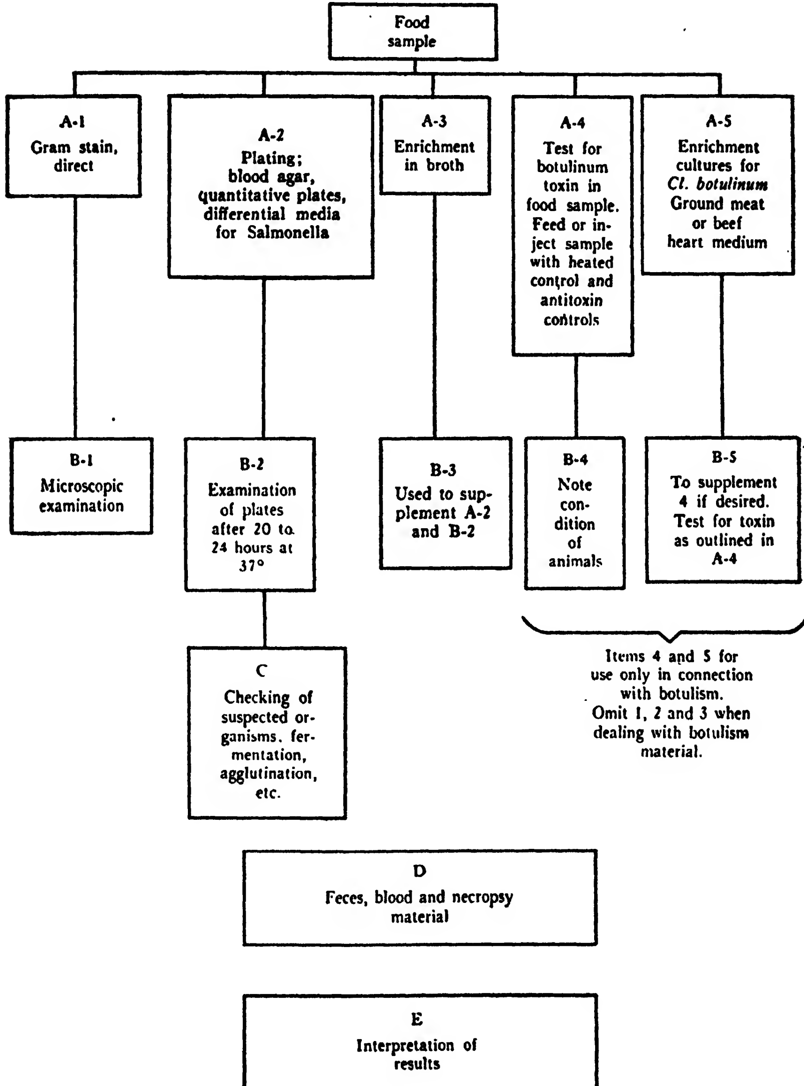
With another loopful streak in succession 3 plates of differential agar media. It is advisable to use two of the more selective media, such as bismuth-sulfite and bile salts citrate agar (C.M. Nos. 23 and 25), and one of the less inhibitory media such as MacConkey's bile salts agar (C.M. No. 26) or eosin-methylene blue agar (C.M. No. 21). Incubate all plates for 20 to 24 hours at 37° C. An additional incubation period of 24 hours will allow further development of certain of the slower growing *Salmonella* types, such as *S. pullorum*.

#### 3. *Enrichment in liquid medium*

*Isolating members of the Salmonella group*—Inoculate 25 or 50 ml. aliquots of filtered suspensions of the sample into an equal volume of freshly prepared "double strength" enrichment broth, such as tetrathionate broth (C.M. No. 28) or Selenite F broth.[4] (C.M. No. 29.) If

* If the only sample submitted is an empty jar or can, the interior should be thoroughly washed out with a few ml. of sterile salt solution or sterile broth. The washings can then be used for the examination.

FIGURE 1—Outline of Procedure



the sample is solid or semisolid, a portion may be placed in a wide mouth glass-stoppered bottle of appropriate size, containing the desired amount of sterile distilled water and glass beads. The bottle should be agitated for 3 to 5 minutes by hand or in a mechanical shaker. Portions are then inoculated as described above. Incubate the broth cultures for 20 to 24 hours at 37° C. Streak plates of differential media suggested in A-2.

*In the event of botulism*—If the illnesses are suspected or definitely known to be botulism, the following tests (A-4 and A-5) should be made. If not, they should be omitted.

### 4. *Test for botulinum toxin in food sample*

The liquor from the foodstuff or original container should be used if available.* If necessary, the food may be macerated in a little sterile salt solution, the larger particles allowed to settle out, and the supernatant used. If the supply of the food sample is ample, withdraw a portion for testing and hold the remainder in the icebox for additional confirmatory tests if needed.

Berkefeld filtration of the sample may be desirable at times to remove bacterial contamination. This can be done if a sufficient quantity of the sample and facilities for filtration are at hand. Instead of filtration, centrifuging of the material, and use of the supernatant for animal inoculation may be used to eliminate gross contamination and the loss of toxin which may be absorbed on the filter.

After preparing the materal for animal tests by filtration or centrifuging, remove a small portion to a sterile test tube and heat for 10 minutes in a boiling water bath. This serves as a control for heat-labile toxin.

Portions of the sample and heated control should now be administered to animals, injecting intraperitoneally guinea pigs or white mice. An alternative procedure is suitable for guinea pigs which can be fed 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 bacteria in badly spoiled samples which were not previously filtered.

The number of animals to be used will vary depending upon the

* See preceding footnote.

amount of the sample and whether *specific* antitoxins for types A and B are on hand.* A suggested procedure is outlined in Table 1.

If the quantity of sample or the supply of animals is limited, omit numbers 2, 5, and 7.

If specific antitoxic sera are not on hand, proceed as outlined, using numbers 1, 2, and 3. The controls with specific antitoxin can be included later when sera become available. If antitoxic sera cannot be obtained within a reasonable time, it is best to turn over the material

TABLE 1

| *Animal number* | *Food sample after filtration or centrifugation* | *Same after heating* | *Antitoxin A* | *Antitoxin B* |
|---|---|---|---|---|
| | *ml.* | *ml.* | *ml.* | *ml.* |
| 1 | 0.5 to 1.0 * | | | |
| 2 | 0.5 to 1.0 | | | |
| 3 | | 0.5 to 1.0 | | |
| 4 | 0.5 to 1.0 | | 1.0 | |
| 5 | 0.5 to 1.0 | | 1.0 | |
| 6 | 0.5 to 1.0 | | | 1.0 |
| 7 | 0.5 to 1.0 | | | 1.0 |

* Inject intraperitoneally, the amount depending upon the animal used. If guinea pigs are fed, larger amounts up to 2 or 3 ml. can be given. The amounts given each animal must be governed necessarily by the amount of sample available.

to a laboratory which has the required facilities. In the event of death of the control animals receiving Type A and B antitoxin a further protection test should be made using Type E antitoxin.

## 5. *Enrichment cultures for Clostridium botulinum*

Occasionally botulinum toxin may be missed in the foodstuff, especially if the sample submitted is scanty or otherwise unsatisfactory, but the presence of the organism can be detected by enrichment, in appropriate media, of the sample or washings from the container. Inoculate each of three tubes of ground meat or beef heart medium (C.M. No. 14) with approximately 1 gm. or 1 ml. of the food sample. In preparing this medium, ground lean beef, beef heart, or veal is tubed with the infusion from the meat so that the layer of ground meat occupies about half of the column of liquid. Addition of 0.1 per cent dextrose will facilitate growth of the anaerobe and does not interfere with subsequent toxin production by *Clostridium botulinum*. The final pH should be 7.2 to 7.6 after autoclaving.

* Types C and D may be disregarded since they are not found in outbreaks of human botulism.

Immediately after inoculation of the meat medium, two of the three tubes should be heated to 80° C. for 20 minutes to destroy vegetative cells. The third tube is left unheated. If an anaerobic jar is not available the three tubes should be layered with sterile vaseline or agar to form a seal. Incubate all three tubes at 37° C.

## B. SUBSEQUENT EXAMINATIONS

### 1. *Microscopic examination*

Microscopic examination of the stained smear will give an idea of the relative abundance of bacteria in the product and of the morphological types present. This may be of value when considered in connection with results from the cultures (B-2 and B-3 below) or again it may be found to have little significance.

### 2. *Streaked plates*

Examine the S.S., eosin-methylene blue, or other differential plating media for the presence of colonies resembling those formed by organisms of the *Salmonella* or *Shigella* groups. If suspicious colonies are found, proceed with identification of suspected intestinal pathogens according to additional procedures described in Section C. Plates showing absence of characteristic colonies may be discarded after 48 hours' incubation.

Examine the streaked blood agar plates and also the quantitative poured plates of infusion agar. Note whether one or more types of colonies predominate on these plates, and especially whether *staphylococcus* or *streptococcus* colonies are present in any considerable numbers. This item has considerable significance where the plates have been prepared from food materials secured promptly by the laboratory. Prepare Gram stains from several colonies of the predominating kind and make a record of morphology and Gram reaction of the cells. From the poured plates make counts of the predominating organism or any type considered to be significant. See Section E for significance of staphylococci or streptococci.

### 3. *Enrichment medium*

The use of specialized enrichment media should increase the probability of isolating members of the *Salmonella* group which might have been missed by direct plating. This procedure is to be preferred since materials which arrive at laboratories may have been overgrown by the contaminating flora, or the numbers of *Salmonella* organisms otherwise reduced. If the steps listed under A-2 and B-2 have yielded no

information of value, streak blood agar plates and the differential media listed under A-2 from this broth culture. Incubate at 37° C. for 24 hours. The examination of these plates should then proceed as given under item B-2.

4. *Results of test for toxin in food*

(For use only in connection with botulism)

The condition of the animals which were injected under item A-4 will afford evidence of the presence and type of botulinum toxin in the food. With a strong toxin the appropriate animals often succumb within a few hours. Weak toxin may cause flaccidity of the abdominal muscles. In such instances death may not occur for 3 or 4 days, or even longer. A typical result, if botulinum toxin were present in the foodstuffs, would be death of animals numbers 1 and 2, survival of 3, and death of either 4 and 5 or 6 and 7, depending upon the type of toxin present.

It must be borne in mind also that when animals have been injected with material from food samples not previously filtered, death may be caused by invasion of the animal body with contaminating bacteria and not by botulinum toxin. This is true especially of white mice, and hence the controls with heated material and with antitoxin are important.

5. *Enrichment cultures for Clostridium botulinum*

Examination of these cultures should supplement the foregoing direct test for toxin in the food sample. The meat medium tubes should be incubated for 3 or 4 days at 37° C. Note any macroscopic evidence of growth and prepare Gram stains from each tube. Note whether Gram-positive bacilli, with or without subterminal spores, are present. Select one or more tubes for a toxicity test similar to that carried out with the original sample under item A-4. The result of this test will serve to confirm that secured with the food sample and at times may be of value in affording information when toxin cannot be demonstrated directly in the foodstuff. In the event of a positive test, the meat medium cultures may be used for further purification and isolation of *Clostridium botulinum* if this is desired.

C. CHECKING OF SUSPECTED SALMONELLA

It should be noted at once that the final and complete identity of *Salmonella* types is a specialty of certain laboratories concerned chiefly with the study of salmonelloses. The procedure to be outlined may, therefore, appear to lack all the niceties of *Salmonella* work. It is

directed toward the majority of smaller laboratories which may be faced with food poisoning problems requiring rapid and relatively accurate solution. The ability to report the presence of a member of the *Salmonella* group, even though unidentified as to specific type, may, of itself, be highly significant and of importance to clinicians.

In items B-2 and B-3 it would be desirable to examine a large number of colonies suspected of being bacilli of the *Salmonella* group. The procedure should permit the screening of a maximum number of colonies in a minimum of time, employing a minimum of media. The tests should more firmly establish but not necessarily prove the identity of the suspected cultures.

From each plate of incubated differential agar medium a minimum of ten, distinctly isolated, characteristic colonies should be inoculated into Kligler's iron agar medium (C.M. No. 19) modified by the addition of 1 per cent sucrose,[5] or into the T. S. I. medium of Hajna.[6] (C.M. No. 20.) *Salmonella* types produce acid and, in most instances, gas and blackening in the butt of the medium, the slant remaining alkaline. (Most *Shigella* types produce an acid butt without gas or blackening.)

*Proteus* species have been found to be among the most troublesome of contaminating organisms. Therefore, it is of practical importance to eliminate this genus from consideration in the second step of this procedure. Consequently, subinoculations may be made into media for the detection of urea-splitting organisms.[7, 9] (C.M. Nos. 30 and 31.) This presumptive approach should eliminate entirely or limit considerably the numbers of suspected cultures. At this point it is desirable to determine whether one is dealing with a medium-size, Gram-negative, non-sporulating rod, and to replate to check the purity of the cultures.

Cultures which remain from the preliminary screening are now ready to be partially confirmed. Accordingly, the following practical procedure is suggested which may be found adaptable to the needs of most diagnostic laboratories. The partial confirmation pattern is based on a minimum number of media required to establish the presence of *Salmonella* members with a relative degree of certainty. The procedure is modified[10] after the work of other investigators.[5, 11] The pattern includes: Kligler's iron agar slant, semisolid mannitol-motility "deep," semisolid triple-sugar "deep." (See APPENDIX to this chapter: A.) Barritt's modification of the Voges-Proskauer test, tryptone broth and Kovac's reagent for the indol test, and Schneider and Gunderson's medium[8] for urea-splitting organisms. The media may be uniformly dispensed in Wassermann type test tubes. The biochemical pattern

may be read in 24 hours and a guarded interpretation made according to Table 2. The sugars should be incubated for longer intervals in order to rule out delayed fermentation which is not typical of the *Salmonella* group.

The complexity of this genus is such that a more detailed biochemical procedure of checking is necessary. Since many diagnostic laboratories will not find it feasible to maintain an unlimited assortment of media, it is suggested that cultures which are thus partially confirmed be forwarded to specialized laboratories equipped to complete the biochemical observations as well as conduct agglutination tests.

The Kauffmann-White diagnostic schema [12] emphasizes the antigenic complexity of the *Salmonella* group. It is, therefore, impractical for smaller laboratories to maintain complete stocks of specific agglutinating antisera. Since *S. typhimurium, S. enteritidis, S. cholerasuis* and certain other group C *Salmonella* organisms such as *S. montevideo* and *S. oranienburg* are included among the types more frequently implicated in food poisoning outbreaks,[1] it may be of some advantage to maintain polyvalent "O" agglutinating sera. Techniques for the preparation of the latter should be considered.[13-15] Obviously, only limited information may be added through use of the polyvalent agglutinating sera. Laboratories able to do so can prepare both somatic and flagellar antisera according to the methods adopted by Edwards and Bruner.[12] Final type identification of the cultures involved should be performed by laboratories which have specialized in the study of the *Salmonella* group.

Summaries of the characteristics and relationships of members of the *Salmonella* group should be consulted in connection with the identification.[16, 17]

### D. The Arizona Group of Paracolon Bacteria [18]

Edwards, West, and Bruner [19, 20] have characterized biochemically as well as serologically "a new group of bacteria pathogenic for animals and probably also for man. A general definition of the group is given as follows: ". . . motile coliform bacteria which produce abundant $H_2S$ but fail to form indol, are methyl red-positive and Voges-Proskauer-negative; produce acid and gas from glucose; do not utilize δ-tartrate or ferment sucrose, dulcitol, or salicin; ferment lactose with varying avidity and liquefy gelatin." The frequent isolations of these organisms from fowls (turkey, in particular) and egg powder is of considerable

interest from the public health standpoint. Therefore, primarily for evaluating their potentially pathogenic role, laboratories should be aware of their possible existence in suspected food poisoning. In certain instances the antigenic relationship of the Arizona group of paracolon bacteria to that of the *Salmonella* group implies that procedures for their isolation may not differ significantly from that employed in the

TABLE 2

*Differentiation of Enteric Bacilli*

(The partial confirmation pattern)

| *Organism* | *Kligler's iron agar* | | | *Mannitol-motility "deep"* | | *T-S "deep"* | | | |
|---|---|---|---|---|---|---|---|---|---|
| | *Slant* | *Butt* | *Medium blackened* | *Fermentation* | *Motility* | *Fermentation* | *Indole* | *Voges-Proskauer test* | *Urea split* |
| *Proteus sp.* | K | AG * | V | O * | † | V | V | V | † |
| *Salmonella sp.* | K | AG * | V | AG * | [1] † | O | O | O | O |
| *Shigella sp.* | K | A | O | V | O | Vs | V | O | O |
| *Paracolon sp.* | K | AG * | V | AG * | V | Vs | V | V | O |
| Arizona *paracolon sp.* | K | AG | † | AG | † | Vs | O | O | O |

K = alkaline
A = acid
O = no reaction
V = variable reaction
AG = acid and gas

* = usually
† = positive reaction
Vs = slow reaction when positive
[1] = except *S. pullorum*

case of the salmonellae. Therefore, Table 2 should prove of assistance in establishing their identity biochemically.

Serological identification as to types is as complex as that for the *Salmonella* group and should be made by laboratories specializing in this type of work.

## E. EXAMINATION OF FECES, BLOOD, AND NECROPSY MATERIAL

### 1. *Stool samples*

Stool samples are often of value if the illness happens to be due to *Salmonella*, particularly in those instances where the suspected foodstuff has been consumed or destroyed. While of course the causative agent is not known at the outset of the laboratory examination, cul-

tural examination of the stool specimens, if available, should be started as soon as possible. No detailed laboratory procedure need be given here, since the examination follows the usual steps employed for isolation of organisms of the enteric group from feces.

### 2. *Blood*

Blood cultures are of secondary importance in most food poisoning outbreaks. In those of *Salmonella* origin positive blood cultures are only rarely obtained, but where a definite *Salmonella* type has been isolated and identified, this item is of value when taken in connection with the other findings. In staphylococcus and streptococcus food poisonings blood cultures are of little or no value.

### 3. *Necropsy Material*

If such material is available following acute gastrointestinal illness which might have been caused by *Salmonella* organisms, cultures in appropriate media can be made from contents of the colon, spleen, and mesenteric lymph nodes. Positive cultures, definitely identified as *Salmonella* according to the procedures previously given, have some value in determining the cause of the illness.

### 4. *Blood and feces in botulism*

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 numerous contaminating bacteria which are present before using the specimen for toxin tests.

## F. INTERPRETATION AND REPORTING OF RESULTS

Since some outbreaks of food poisoning are due to other than bacteriological causes, perhaps the first duty of the bacteriologist is to realize that his part of the examination may at times prove to be fruitless and that he should not feel obligated to attach blame to any miscellaneous organism which happens to be conspicuous.

The laboratory is often asked for proof that the actual organism obtained is in reality the cause of the trouble. Unfortunately, symptoms of typical acute gastrointestinal illness can rarely, if ever, be reproduced in the ordinary laboratory animals by *feeding*. Results obtained by the

*injection* of the suspected cultures are open to question, and in general should not be advanced as the sole proof of an etiologic role of the organism in question. The following considerations apply to the different food poisoning types usually encountered.

### 1. *Salmonella group*

If a member of the *Salmonella* group has been isolated from the suspected food, this finding is highly significant. These organisms have been encountered in *foodstuffs* and there is good evidence that they produce the characteristic food poisoning symptoms. The incubation period is usually 15 to 24 hours, with occasionally a longer interval. In the event that it has been possible to isolate a similar *Salmonella* from specimens of feces or from necropsy material, additional confirmation is thereby obtained.

### 2. *Staphylococcus*

The finding of staphylococci, particularly large numbers of colonies on the plates made directly from the suspected foodstuff (A-2 and B-2), immediately raises the question of the causative role of this organism. However, considerable caution is necessary before assigning a definite etiologic role. Not all staphylococci are capable of forming the enterotoxin.* Since these organisms are ubiquitous, they may readily gain access to a foodstuff before or during the process of collection of food samples. Thus a correct interpretation of their presence is more difficult than in the case of the *Salmonella* group.

The question of distinguishing those strains of staphylococci which may be responsible for food poisoning outbreaks has been raised repeatedly. The ordinary biochemical and agglutination tests afford no satisfactory criteria for differentiation,[21, 22] and it has been questioned whether the power to provoke food poisoning is limited to any recognizable variety of staphylococcus. Feeding tests with the usual small laboratory animals give no definite information, and in routine laboratory work it is not advisable to attempt the feeding tests with human volunteers that were originally necessary to substantiate the causative role of this organism.[23, 24] The feeding of monkeys has also been used, but these animals are not entirely satisfactory since their suscepti-

* The staphylococcus food poisoning substance has been termed "enterotoxin" by some workers as a matter of convenience and to distinguish it from hemolytic, necrotizing, and associated substances.

bility to the staphylococcus enterotoxin is less than that of the human. In several instances staphylococcus filtrates known to be toxic to persons have elicited no symptoms in monkeys.[25, 26] However, a positive feeding test in the monkey is of value and obviates the errors inherent in the parenteral injection of test material into animals.

Several special procedures have been advocated for distinguishing those staphylococci which possess food poisoning capabilities. Dolman, Wilson, and Cockcroft[27] used the intraperitoneal injection of kittens with treated culture filtrates to detect the enterotoxin. This method is not entirely reliable since several investigators[28-31] have found that uninoculated medium alone, when injected intraperitoneally into kittens, may produce symptoms. However, Minett[32] feels that ample evidence has been obtained that at present this is the best means of detecting enterotoxin. The intravenous injection of kittens with culture filtrates offers certain advantages over other methods since nonspecific reactions are avoided,[31] smaller amounts of enterotoxin can be detected and susceptible animals may be used for 2 or 3 tests. Hammon[31] is enthusiastic about this method and recommends the use of a purified medium for enterotoxin production.

*Macaca mulatta* monkeys, where available, may be used for feeding tests. Cultures are prepared as outlined above and may be fed without heating or filtration. After centrifugalizing and removing the agar and many of the bacterial cells the supernatant fluid is ready for feeding. Fifty ml. is given by stomach tube to each of three monkeys. The animals are observed for vomiting over a period of 6 hours. If enterotoxin is present, usually vomiting occurs in 2½ to 3 hours. Monkeys may develop a tolerance upon repeated feeding, and it is necessary to test with known enterotoxin those animals which fail to react in order to eliminate from future tests naturally resistant animals or those that have developed a tolerance. The feeding test avoids nonspecific reactions caused by the parenteral injection of foreign substances. The details of Dolman's method are given below since his method of preparing enterotoxin is practical for the routine diagnostic laboratory. When it is used by the investigator of a food poisoning outbreak, it should be applied as soon as possible to cultures of staphylococci isolated from the plates of items A-2 and B-2. For laboratories where monkeys are not available the cat test is recommended. (See APPENDIX to this chapter: B. Tentative Procedure for Staphylococci —kitten injection method.)

Where staphylococci are suspected as the etiologic agent, an important

point in the decision of assigning to them a causative role rests upon the circumstantial evidence afforded by the finding of considerable numbers in the suspected foodstuff. This is especially significant where the food has been secured for laboratory examination shortly after the appearance of symptoms. With the lengthening of this interval the finding of staphylococci or any other ubiquitous organisms becomes less significant. This is particularly true if the temperature at which the food had been held subsequent to the outbreak has been such as to allow bacterial multiplication.

The incubation period is also important. In most cases of staphylococcus food poisoning the first symptoms have appeared 2½ to 3 hours after consumption of the toxic food.

It must be kept in mind that some strains of staphylococci from non-food poisoning sources are capable of producing an enterotoxin.[33] That is, although not originally obtained from a food poisoning outbreak they are *potentially* capable of causing one. Such staphylococci may gain access to a suspected food subsequent to an outbreak; for example, either just before or at the time of collection of the samples. Proper cooling, and relative numbers of the suspected organism, especially in the interior of the product, are highly important in evaluating the results of the laboratory findings.

### 3. *Streptococcus* *

In a number of recorded instances of food poisoning, streptococci have been implicated as the etiological agent.[34-37] Those cultures from such outbreaks which have been carefully studied have proved to be enterococci, specifically *Streptococcus faecalis*.[37-39]

The finding of very large numbers of cocci in pairs or short chains in smears made directly from the suspected foodstuff (A-1 and B-1), or large numbers of streptococcus-like colonies predominating on the dilution plates prepared directly from the material (A-2 and B-2) might be regarded as having some significance. However, considerable caution is necessary in interpreting such findings. If the food material had been subjected to conditions which would afford salivary contamination, one should expect to find alpha-type streptococci developing on the blood agar plates. In such cases little or no significance could be attached to these findings.

Since among the streptococci, only *Streptococcus faecalis* has been

* Revised by C. F. Niven, Jr., Ph.D.

associated with food poisoning, a selective medium for the quantitative detection of enterococci in food materials would be desirable. Such a medium, consisting of 0.5 per cent glucose, 0.5 per cent tryptone, 0.5 per cent yeast extract, 1.5 per cent agar, 0.03 per cent sodium azide, and penicillin in a concentration of 325 Oxford units per liter, has been reported by White and Sherman[40] for the enumeration of enterococci in milk. Most of the enterococci tested grew quantitatively on this medium, and practically all of the bacteria from milk which grew on it proved to be enterococci. The plates should be incubated at 37° C. for 48 hours before examining. This medium may find possible use in examining foods other than milk.

In cases where very large numbers of microörganisms are encountered that appear to be enterococci it would be desirable to isolate, and identify as such, several colonies from the food material. Their identity can be established by serological methods, if Lancefield group D serum of known potency and specificity is available. Also, by use of certain physiological and biochemical tests, members of this group are among the easiest of the streptococci to identify. They are able to grow at 10° C. (observe after 7 days), and at 45° C.; and in a glucose-tryptone-yeast extract medium containing 6.5 per cent sodium chloride. No other streptococcus possesses this combination of characteristics. These, and other tests that have proved their value as practical and convenient aids for identifying enterococcus strains have been summarized by Sherman.[41]

Since enterococci are normally found in large numbers in the intestine of men and animals it is indeed puzzling that they are incriminated in food poisoning cases at any time. It would appear that only rare strains, grown under certain conditions, possess this property. Thus far, no one has been able to detect any physiological, nor serological, difference between a known food poisoning and the so-called typical enterococcus strains. Therefore, one can rely only upon the circumstantial evidence afforded by the finding of considerable numbers of enterococci in foodstuffs and the relative absence of other food poisoning microorganisms. The laboratory findings may be of significance if they are in agreement with the epidemiological features of streptococcus food poisoning.[1]

The available evidence to date indicates that very large numbers of viable food poisoning enterococcus cells must be ingested (perhaps 10 billion, or more) before symptoms are apt to occur. In contrast to the staphylococci, culture filtrates do not appear to give rise to symp-

toms. Food poisoning due to *Streptococcus faecalis* results in symptoms very much like that of staphylococcus food poisoning, though usually less intense. The time for onset may vary from 2 to 18 hours. There appear to be no satisfactory test animals in which to determine whether a suspected strain may be a food poisoning type.

#### 4. *Botulism*

In cases of botulism, direct evidence is supplied by the injection and feeding of guinea pigs or mice and the protection afforded control animals by the heated control and by antitoxic serum. Where the clinical symptoms of the persons affected have been those of botulism, the demonstration of toxin or toxic cultures in a foodstuff serves, under all ordinary conditions, to designate the particular food responsible. Where type-specific antitoxic sera have been used (A-4) additional information on the type of the causative *Clostridium botulinum* is obtained.

In occasional cases cultures of *Clostridium botulinum* may be obtained from an empty discarded container while none of the suspected foodstuff remains for direct test of the presence of toxin. While such a finding possesses some significance it will be recalled that botulinum spores occur in soil and might conceivably have gained entrance to the empty container after it was discarded. Conclusions based on the finding of spores without toxin in an empty discarded container should be qualified and must take into account the conditions to which the container was exposed between the time of opening and the time of examination.

## IV. APPENDIX

### A. Preparation of Semisolid Agar Media To Assist in Establishing Identity of Salmonella Species

The semisolid media described below are modifications[10] of similar media reported elsewhere.[11] They are used unslanted in Wassermann type tubes, a stab inoculation being made. Observations are recorded after incubating for 24 and 48 hours at 37° C. Most *Salmonella* species produce an acid (yellow) reaction with visible gas formation (gas will be trapped in the agar "deep") in the mannitol-motility tube. Motility is detected by the ability of the growth to spread from the line of stab. *S. pullorum*, a non-motile species, is the only exception.

The triple-sugar " deep " contains three sugars important in the biochemical differentiation of *Salmonella* species from other organisms of the enteric group. *Salmonella* species do not attack lactose, sucrose, or salicin.

SEMISOLID MANNITOL-MOTILITY " DEEP "

Base medium:

| | |
|---|---|
| Bacto-Tryptose | 10 gm. |
| Sodium Chloride | 5 gm. |
| Bacto-Agar | 3 gm. |
| Distilled Water | 1,000 ml. |

Adjust the pH to 7.6–7.8 and add:

| | |
|---|---|
| Mannitol | 10 gm. |
| Brom Cresol Purple (1.6 per cent alcoholic solution) | 2 ml. |

Tube in 3–4 ml. amounts in 10 x 1.2 cm. test tubes. Sterilize in the autoclave for 25 minutes at 10–12 lbs. pressure.

SEMISOLID TRIPLE-SUGAR " DEEP " (T-S)

Base medium: Same as above.

Add

| | |
|---|---|
| Lactose | 10 gm. |
| Sucrose | 10 gm. |
| Salicin | 5 gm. |

Tube and sterilize as above.

## B. TENTATIVE PROCEDURE FOR STAPHYLOCOCCI

### *Kitten injection method*

Dolman, Wilson, and Cockcroft[27] suggested the intraperitoneal injection of kittens for detection of enterotoxin-producing strains of staphylococci.

*Preparation of filtrates*—Cultures to be tested are grown on the surface of the soft agar (C.M. No. 17) in Petri plates, Kolle flasks, or wide mouth bottles laid sidewise. Incubation is at 37° C. for 40 hours in an anaerobic jar into which has been introduced an atmosphere of 30 per cent of carbon dioxide.

The cultures are then squeezed through cheesecloth and centrifuged at high speed. The supernatant liquid is decanted and sterilized by passing through a Seitz filter. The $\alpha$ and $\beta$ hemolysins (rabbit and sheep-cell hemolysins) in this filtrate should be inactivated before use. This may be accomplished in several ways, the simplest of which is to heat in a bath of boiling water for 30 minutes. Other methods, in-

volving the use of formaldehyde or neutralization of the hemolysins by specific antiserum may also be employed.

*Injection of filtrate and symptoms*—The heated filtrate should be brought to body temperature and injected intraperitoneally into kittens, using preferably two or more animals—1.5 to 2.0 ml. are usually injected but up to 5 ml. of filtrate may be given by this method. As little as 0.5 ml. of a potent filtrate will cause a severe reaction. Kittens weighing 350 to 550 gm. and aged about 6 to 8 weeks are recommended. Intravenous inoculation may be made into adult cats using 3 to 5 ml. of the boiled filtrate.

A characteristic syndrome is produced. Marked lassitude and weakness appear and this is followed, often within 15 to 30 minutes, by a series of paroxysms of vomiting associated with diarrhea. The kitten may appear extremely ill for several hours and then rapidly improve, or, following larger and more potent doses, progressive weakness culminates in death in about 24 hours after the injection. The appearance of these symptoms is regarded as a positive test for the enterotoxin.

Post-mortem examination of the animals shows absence of petechial hemorrhages, and no gross inflammatory changes of the gastrointestinal tract are noticeable. Attempts to produce the same syndrome in rabbits, guinea pigs, and mice have been unsuccessful.[42] Kittens develop a marked resistance to the enterotoxin after receiving several spaced doses of filtrate and should not be used repeatedly.

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# The Malaria Parasites of Man

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## I. INTRODUCTION

DURING the past twelve years a very great recession in malaria has taken place in the United States. The return of World War II service men from highly malarious areas for a time increased the number of cases of malaria to be diagnosed by the average laboratory but did not cause the transfer of cases to the civilian population and the scattered epidemics that were expected. The relative scarcity of cases of malaria makes it no less important that the technician be prepared to recognize the parasite when it is present, however, and it may be a long while until malaria is eradicated from this country.

The parasites which cause malarial fevers in man all belong to the Genus Plasmodium and are generally classified in the Order Haemosporidia, of the Class Sporozoa and of the Phylum Protozoa. Four species of the Plasmodia are now accepted. These are ***Plasmodium vivax,*** the cause of benign tertian (tertian) malaria; ***P. malariae,*** the cause of quartan malaria; ***P. falciparum,*** the cause of estivo-autumnal (malignant tertian, subtertian) malaria, and ***P. ovale,*** a species which, so far as is known, has not yet been introduced into the United States.

The general course of the life history of human malaria parasites is presumably the same for all the species. There is a cycle of development passed in certain of the anopheline mosquitoes called the *sexual cycle,* the process of reproduction during this cycle being termed *sporogony.* There is also a process of development in man, now known to involve cycles in both the tissues and the blood cells. The process of development during these stages is termed *schizogony* and the cycle in the blood cells is termed the *asexual cycle.*

"The microscopical detection of malaria parasites in stained blood films is the most reliable and accurate method of laboratory diagnosis thus far devised."[1] This diagnosis should be based not only upon the demonstration of the plasmodia in the blood but upon identification of the species of plasmodia present. There are a number of reasons for this, most important of which is the propensity for *falciparum* infection to terminate rapidly in death.[2] Each of the species has distinctive differences in morphology, and only a careful study of this differential morphology of *P. vivax, P. malariae, P. falciparum,* and *P. ovale* makes this species identification possible. "For accurate results with either the thin or thick film techniques, special materials and procedures have been found highly desirable, since the best efforts of a qualified microscopist may be frustrated by poor condition of the specimen."[1]

Much of the material in this article is taken verbatim from the *Manual for the Microscopical Diagnosis of Malaria in Man*[3] by the author, published as *N.I.H. Bulletin, Number 180,* 1942.

## A. LIFE CYCLES

### 1. *Sexual Cycle (in the mosquito)*

If a person bitten by a female anopheline mosquito is an infective carrier of malaria, that is, if he has in his blood the necessary number of mature sexual forms (*gametocytes*), the mosquito will become infected.[4] When the blood, containing the malaria parasites, is taken into the mosquito's stomach, all the asexual forms and immature gametocytes are digested, but many of the mature sexual forms (called in man the *microgametocyte* in case of the male, and *macrogametocyte* in case of the female) not only survive but change form and continue their development. By movements of contraction and expansion they rupture the red blood cells which contain them and escape into the stomach cavity of the mosquito. The microgametocyte throws out from 4 to 8 flagellum-like structures called *microgametes,* which break

away from the microgametocyte, leaving a residual body in which the pigment granules remain. Meanwhile, the macrogametocyte undergoes a change. There is presumably an elimination of part of the chromatin and formation on the surface of the parasite of a cone of attraction sometimes called a " polar body."

This stage of the female parasite is called the *macrogamete* and it is now ready for fertilization, which takes place when one of the free microgametes comes in contact with a macrogamete, penetrates it, and the nuclei of the two become fused. This fertilized parasite is called the *zygote*. This soon elongates and becomes motile and is then called the *oökinete*. The oökinete forces its way into the wall of the mosquito's stomach and comes to rest between the outer membrane and the inner epithelial cells. Here it contracts to a small round body, becomes surrounded by a kind of cyst wall and is called an *oöcyst*. It has a single mass of chromatin and a mass of cytoplasm containing the pigment of the original macrogametocyte. The oöcyst grows enormously; the nucleus multiplies by repeated divisions; the cytoplasm develops vacuoles, forming a sponge-like arrangement of interconnecting protoplasmic masses. When vacuolation is complete the nuclei arrange themselves near the surface of the cytoplasm. Above each nucleus a finger-like process of cytoplasm protrudes carrying the nucleus with it, elongating, and finally breaking away as an individual from the unused cytoplasmic mass. Each such individual is called a *sporozoite*. The oöcyst when mature bursts and liberates the sporozoites, which number from several hundred to several thousand per oöcyst, into the body cavity of the mosquito. The sporozoites are thin, pointed at each end, have a nucleus, and are very motile. They migrate to practically all of the organs of the mosquito's body and many get into the salivary glands where they lose their motility. When a mosquito bites a man, these sporozoites are injected with the saliva, which is emitted at the time of biting, into the skin, and thus begins the development of the parasite in man.

### 2. *Pre-erythrocytic Cycle (in the tissues)*

Numbers of workers have reported or described tissue stages of the avian malaria parasites, and Huff and Coulston[5] traced the development of these cryptozoic forms from the injected sporozoites to the production of the parasites that invade the red blood cells. These have commonly been designated exoerythrocytic or EE stages and have long been suspected[38] in human malaria. Early in 1948 Shortt and Garn-

ham [34] proved their existence in human malaria. The forms were demonstrated by inoculating a volunteer with massive doses of sporozoites on two successive days and then biopsying the liver on the seventh day following the first inoculation. Non-pigmented dividing forms (schizonts) were found in the parenchyma cells of the liver and it is thought that they may exist in other organs as well. Garnham has called this stage of development of the parasite in man "pre-erythrocytic schizogony." The first form is presumably a small uninuclear parasite (formed from the sporozoite) which enlarges and shows repeated divisions of nuclear material until a schizont 40 μ or more in length is produced. Each schizont divides into individual small parasites, consisting of cytoplasm and nuclear material. An estimation of the number of divisions in one parasite was placed at 800. The cell finally breaks and the small parasites are liberated into the surrounding tissue. Many of the parasites are destroyed by invading phagocytic cells, but some enter red blood cells and produce the *asexual cycle.*

It is believed that others of the parasites may enter other tissue cells and continue an exoerythrocytic schizogony, which is very likely associated with relapses or recurrences of malaria.

### 3. *Asexual Cycle (in man)*

#### a. *Explanation of terms*

In this article the term "trophozoite" is used to include the asexual stages from the young form (ring) through the stage where the parasite has completed its vegetative growth but, as yet, has an undivided chromatin mass. The rings and young forms are called small trophozoites and the older, ameboid and more solid forms are termed, according to their age, growing and large trophozoites. "Schizont" (presegmenting schizont) is used to denote the asexual stages of the parasite in which the chromatin shows evidence of schizogonic division. There may be such forms with two, four, or more chromatin masses, but the merozoites are not yet differentiated. "Mature schizont" (segmented schizont) is used to denote a *fully developed* schizont in which the merozoites are differentiated.

#### b. *Trophozoites*

The parasites do not appear in the circulating blood until several days after the bite of the mosquito. Those of *P. falciparum* have been found as early as 6 days after inoculation, but those of *P. vivax* never earlier

than the 8th day.[6, 33] In *P. vivax* the pre-patent period is about 10 to 12 days; in *P. falciparum* about 8 to 12 days; and in *P. malariae* about 21 days.

The parasite, when it has entered or attached itself to the red blood cell, grows, using the substance of the cell as food. In about 5 to 6 hours after the cell is infected, fine yellow or brown granules of pigment begin to appear in the cytoplasm of the parasite. These pigment granules probably represent chiefly the by-products of the digestion of the hemoglobin and are frequently called hemozoin. In smears stained with Giemsa's or Wright's stains, the chromatin of malaria parasites stains dark or purplish red, the cytoplasm stains blue, and the pigment granules vary with the species from golden brown to almost black.

The youngest form of the parasite seen in the peripheral blood in all of the species is termed the "*signet ring*" from its appearance—a small dot of chromatin usually on the periphery of a circle of cytoplasm surrounding a central vacuole. In many cases, probably, the appearance is due to the fact that an optical section of a sphere is seen, the protoplasm enclosing the vacuole all around, although it is scarcely apparent on the upper and lower surfaces owing to its tenuity. Sometimes the chromatin dot is seen in the center of the circle of cytoplasm. Here again, however, it is probable that in many of these cases, the chromatin mass is really in the cytoplasm of the upper or under surface of the sphere. There may be more than one chromatin granule in the ring stage and this division is thought to be fragmentation rather than a division of the chromatin prior to schizogony.

The growth of the parasite is a gradual one, with some slight differences in the species, both in the parasite and in the effect on the cell. Malaria parasites in the trophozoite stage perform ameboid movements and ingest food supposedly by osmosis. After the parasite has used up the available food in the red cell and has thus reached maturity so far as vegetative growth is concerned, it prepares for asexual reproduction by gradually slowing and finally ceasing its ameboid movement, thus assuming a more compact form, often with an irregular outline.

c. *Schizonts*

After maturity is reached the chromatin of the trophozoite divides into two parts and there is progressive segmentation until from six to twenty-four or more chromatin masses are produced, the number depending on the species of the parasite. As this division takes place, the

cytoplasm also breaks up into parts, one part finally accompanying each small chromatin mass. These divisions, composed of chromatin and cytoplasm, are termed *merozoites*.

There is a tendency on the part of the pigment in *P. vivax* and *P. malariae* to begin to aggregate as the chromatin begins division and to continue collecting until this division is completed, when all the granules are clumped in one or two places. In *P. falciparum* the pigment clumps early—often before any division of the chromatin (this being a differential characteristic when these stages are found).

Shortly after division is completed, the schizont bursts the red blood corpuscle, the merozoites are liberated into the blood along with the pigment, a possible residue of cytoplasm, and possibly toxic materials produced by the parasite. It is just after this time that the person affected is likely to have the paroxysm or the chill, followed by fever, characteristic of malaria. The pigment and the residual cytoplasm are phagocytised in the peripheral blood by the leukocytes—usually the monocytes, though sometimes by the neutrophils also. It has been stated that the neutrophils of the Negro are phagocytic to this pigment more often than those of the white person. Not all the merozoites survive after the bursting of the cell, but those which do are found shortly thereafter in or on the red blood cells. Here the designation " trophozoites " is used again and here they start another asexual cycle. As the disease continues the number of parasites is increased by geometric progression until frequently enormous numbers develop. Sometimes more than one parasite attacks and enters a single cell.

d. *Gametocytes*

The general opinion through the years has been that gametocytes or sexual stages are formed from certain of the merozoites developed in the asexual cycle, though in the past few years some workers have entertained the idea that sexual forms may develop directly from exoerythrocytic or tissue stages.[7] We cannot say which of these theories is right. We do know that male and female forms, microgametocyte and macrogametocyte, are produced. After entering the red blood cell, the parasite which is to develop into a gametocyte becomes rounded off into a small, compact body which stains blue with a red granule of chromatin. The chromatin dot may be surrounded by a colorless halo which is presumably the unstained part of the nucleus; but the parasite does not develop a vacuole and so does not assume the signet ring form. For some reason not properly understood, the growth of the gametocytes of

all the species takes place almost entirely in the vessels of the spleen or bone marrow, but some immature gametocytes are at times seen in the peripheral blood. Growth of the gametocyte is much slower than that of the schizont, being thought to require nearly twice as long to reach maturity. In *P. vivax* and *P. malariae* the gametocyte grows steadily as a rounded or ovoid compact body; but in *P. falciparum* the young gametocyte often assumes a more or less angular shape and gradually takes on the characteristic sausage or *crescent* shape as it matures. The gametocyte remains within the membrane of the red cell for the period of its life in the blood of man, which is thought to be only a few days; it degenerates and dies unless taken up by the mosquito host.

## II. TYPES OF BLOOD FILMS AND ADVANTAGES OF EACH

### A. THE THIN FILM

"The thin film is ideal for the study of the morphology of the individual parasite, for the identification of stages and species and suffices for diagnosis if the infection is heavy. It also gives the accompanying blood picture. It dries quickly, can be stained in from 6 to 10 minutes, and is ready for immediate examination. However, it has the great disadvantage of failing to reveal parasites when density is low . . . A working knowledge of the morphology of the malarial parasites in the thin film is absolutely necessary before attempting their diagnosis in the thick film." [1]

### B. THE THICK FILM

The thick film is a method by which a relatively large quantity of blood is placed in a small area and stained so that the hemoglobin is dissolved from the red cells and the blood smear is rendered sufficiently transparent for examination by transmitted light. Knowledge of the procedure is indispensable to the person who is to examine the blood for malaria.[8] It reveals, comparatively quickly, sparse or scanty infections such as occur in new or chronic cases. There are occasions when immediate diagnosis is a matter of urgent necessity, and frequently in these cases no certain information can be deduced from a blood examination by the thin film method, whereas the thick film taken at the same time will reveal parasites. The number of cells per thick film field as compared to the thin film field has been estimated by various workers as from 10 to 50 times as great. The actual increase in effi-

ciency in discovering parasites is reported to be from 3 to 22 times as great—this depends, of course, on the thickness of the smear and density of parasites, as well as the accuracy of the technician. Because of its efficiency in picking up cases with rare parasites, it gives a much more accurate idea of the incidence of malaria in a survey, and because great numbers of slides can be dehemoglobinized and stained at one time, it is particularly well adapted to surveys on given populations and to the use of state laboratories where large numbers of slides for malaria examination are received daily during the summer and fall. The thick film gives an idea of the degree of infection and can be a great help, because of the increased density of parasites, in identifying the type of malaria in cases where only one or two young forms can be found in the thin film. It gives a fairly good idea of the number of leukocytes and shows pigmented white cells more readily than the thin film. It also makes it possible to compare the number of parasites with the number of leukocytes and, together with the white cell count, permits a rough estimation of the parasite density.

In so far as examination time is concerned, it has been estimated that 30 minutes is the minimum that should be spent on a thin film before calling it negative. This is based on the belief that fever is produced by one parasite per 100,000 red cells and it takes 30 minutes to scan 100,000 cells.[9] Since so much more blood can be scanned in a single field in the thick film, it is evident that the time is cut drastically. To quote Barber,[10] "The time spent varies with the examiner, however, for it is difficult to standardize skill, experience, and, above all, conscientiousness." In the Public Health Service laboratory we prefer to use as a criterion a certain number of microscopic fields covered. In surveys with fairly well trained technicians, we limit the examination to 100 fields; in clinical cases we advise a longer examination if anything suggestive of parasitism is seen. The trained technician can examine 100 fields on a good thick film in 3 to 5 minutes, and experience has shown that the first parasite is most often found in the first 20 or 30 fields.

Practice and experience are necessary to become proficient in the interpreting of thick films for they are different in appearance from thin films and, at first, may be confusing. However, the ease with which parasites can be found in the thick film, after a long unfruitful search by the thin-film method, is ample recompense for the time spent in learning the technique. It has been repeatedly observed that when

a person has learned this technique, he has no desire to return to the thin film for diagnosis.

In nearly all cases parasites will be found in thick films from patients who show active clinical symptoms of malaria. Parasites may be reduced, however, to a microscopically undetectable level by antimalarial drugs. Also, in persons with extreme susceptibility, symptoms may occur before parasites can be found. In these latter cases examination on subsequent smears should be made on successive days.

### C. Thick and Thin Smears on the Same Slide

"It is often desirable to have a thick and thin film on opposite ends of the same slide."[1] For beginners this gives an opportunity to check species identification in the thin film, after parasites are found in the thick. In study slides it gives an excellent contrast of number to be found in the same blood on thin and thick films. The thin film, in specimens of this sort, should be made first, using a very small drop of blood, taken near one end of the slide and smeared toward the other end, so that the thinner, better portion of the thin film comes very close to the spot where the thick film is made on the opposite end of the slide. This manner of making the smear facilitates staining (when slides are stained standing on end in a staining dish), and makes examinations easier by permitting the objective to slip quickly from the thick film to the better portion of the thin film (for staining, see IV,C,3).

## III. THE THIN FILM

### A. Making the Film

"The skin surface to be pricked, preferably of the finger, should be well cleansed by a pledget of cotton or small piece of gauze moistened with alcohol to remove grease, perspiration, or dirt, and then should be dried with a fresh piece of cotton or gauze so that residual alcohol will not mix with the blood drop. The puncture should be deep enough to allow a free flow of blood under gentle pressure. The first blood to exude should be wiped off.

"To make a thin film, a small drop of blood is collected on the face of a slide near one end. The end of a second or 'spreader' slide (preferably with a narrower end) is lowered onto the face of the first, in

an inclined position slightly in advance of the drop so as to make an angle of about 30°, and is then drawn back against the blood which spreads out at the line of contact. Then with a rapid, even motion the film is made by pushing the spreader toward the other end of the slide. A good thin film should show the erythrocytes well separated with no overlapping or massing. Parasites are found most easily along the outer edges and in the tails of the smear."[1] An identifying number may be written with pencil in the thicker portion of the thin film.

### B. Staining With Wright's Stain

1. Measure in a pipette the quantity of stain that will fully cover the smear. Pour this on the horizontal slide and let it stand for one minute. The quantity should be such that it will not evaporate to the extent of precipitation.
2. Add rapidly twice as much neutral distilled water (IV,C,2) as there was of stain. None of the diluted stain should run off the slide.
3. Mix the stain and water well by blowing gently along the length of the slide.
4. Allow the diluted stain to stand on the slide for 5 or 6 minutes. (The amount of time should be ascertained by testing each lot of stain on positive smears.)
5. Wash off the stain by flooding the slide with a rather strong flow of water (preferably neutral). This is best accomplished by bringing the stream of water to the level of the slide and letting it rush horizontally under the scum that forms on top of the stain. This will prevent precipitation. Never pour off the stain and then wash the smear.
6. Wipe the stain from the under side of the slide, and air dry. Blotting is not advised.
7. Examine with immersion oil.

*Wright's stain cannot be used successfully on thick films,* even though the slides are dehemoglobinized before staining. It does not give the right color contrast and stains cellular remains too deeply.

### C. Staining with Giemsa Stain

Giemsa stain can be used for thin films and gives a much more permanent and consistent stain than does Wright's stain. *The films must be fixed, before staining, with acetone-free methyl alcohol.* They should be washed, after staining, by dipping the slides only once or

twice in neutral distilled water, for much of the staining detail will be lost with prolonged washing. Such slides are excellent for class study. They should be protected from the light when not in use.

The same methods as for thick films may be used quite successfully (IV, C, 3, a and b).

### D. Staining with Wright-Giemsa Stain

### (IV, C, 3, b)

The thin films must be fixed with absolute methyl alcohol for this stain also.

## IV. THE THICK FILM

### A. Making the Film

#### 1. *Slides*

*For making thick films use only slides which are clear, unscratched noncorroded, and above all meticulously clean—i.e., free from grease, dust, acid, or alkali.* When slides are to be cleaned and re-used repeatedly a grade of glass should be used which will stand the cleaning process and the necessary handling without fogging or corroding, and which will not scratch too easily. Scratches in slides will hold the stain and give various false impressions; corroded slides have this disadvantage as well as that of cutting out part of the light necessary for exact microscopical work. New slides should never be used without cleaning, no matter how bright or shiny they look, for there may be left on them traces of oil put there in the glass polishing process and this will cause difficulty. Thin films will not spread evenly on an oily or dirty surface and thick films will not adhere to it. There may be on an unwashed new slide traces of soda, lime, or potash from the glass itself, and these particles can change the pH of the stain to such an extent as to make young malaria parasites and parts of older ones invisible. Great care must be exercised in handling clean slides, since oil from the fingers on the slide's surface will cause thick smears to slip off in staining.

For new slides the following process may be used: Wash the slides with warm water and mild soap, rinse well in warm running tap water, then in distilled water, dip in ethyl alcohol, (preferably 90 or 95 per

cent) and dry with a clean, lintless cloth. They will not be scratched if handled with reasonable care. After slides are cleaned they should be protected from dust. To accomplish this, groups of 25 slides or more may be wrapped in roll toilet tissue and bound with a rubber band. These packs are particularly convenient for field survey work, or they may be used in the laboratory. For the practising physician, who carries slides in his bag, it is suggested that he wrap them slide over slide so that the surface of only one slide at a time is exposed in unwrapping. This will keep the remaining slides clean for future use.

For cleaning used slides on which immersion oil has been used, the following method has been found quite satisfactory: Immerse the slides for 12 hours or more in dichromate cleaning solution. Rinse them well in running tap water. Let them stand in a solution of warm soapy water for about an hour. Scrub slide surfaces with a soft brush. Rinse the slides again in warm running tap water. Rinse in distilled water. Let stand in alcohol for a short period. Dry and shine with a soft lintless cloth.

### 2. *Cleaning the Area To Be Punctured*

*Be sure that the blood taken is free from grease, perspiration, or dirt which may be on the skin, and that the alcohol used for cleansing never be allowed to mix with the blood.* Cleanse the area to be punctured with gauze or cotton soaked in alcohol. Then rub dry with a piece of sterilized cotton or gauze (gauze is to be preferred since it does not leave lint on the skin). Alcohol on the skin or needle will " fix " the red blood cells and interfere with the dehemoglobinization of the red cells which is part of the staining process. Hence all alcohol must be wiped away or allowed to evaporate before blood is taken for the smear. Some workers prefer to prick through the alcohol on the skin and then wipe away the alcohol with the first drop of blood.

### 3. *The Puncture*

*Prick the skin deeply enough to allow the blood to well up in a large drop under gentle pressure but not deeply enough to cause excessive bleeding.* Everyone has a favorite instrument for this purpose. A needle with a pyramidal point has these advantages: it cuts as it pricks and the puncture consequently can be very shallow and practically painless, yet gives sufficient blood for a good thick film without squeezing.

### 4. *The Film*

***Near one end of the slide cover a space about the size of a dime with as much blood as will easily spread over this area without crackling and peeling when dry.*** This smear of about 3 to 5 average drops may be made in either of two ways: First method is to touch the under surface of the slide to the crest of the large rotund drop of blood and, without losing contact with the drop of blood or touching the finger, move the slide in narrow circles in the blood until a smear of the required size and thickness is made. The second method is to take several average size drops of blood quite near each other on the slide, and then, with the needle or with the corner of a clean slide, quickly puddle these into one fairly homogeneous drop about the size of a dime. One should be careful to take enough blood to make a smear which is several layers of erythrocytes thick and yet not so thick that it will contract and pull loose from the slide in drying. On the other hand, one should be sure not to make the smear too thin, else it will have no advantage over the thin film. Never use the second method without smearing the drops together and do not put them so far apart that the blood has to be spread over a large thin area in order to bring them together. *The ideal thick film is several layers of erythrocytes thick in the middle and has a thinner edge of one-cell thickness.* Ordinary printing can just be read through the wet center of a well made thick film when the slide is placed over the printed page. The smear is placed near one end of the slide (its edge about ¼ inch from the end of the slide) so that only a small amount of stain will be needed to cover the smear with the slide vertically immersed in the solution.

### 5. *Marking the Slide*

***On the end of the slide opposite the blood film put an identifying number or character with a wax pencil or other marking device.*** This should correspond with the name or number on a data slip, statistical record, or information blank, so that there will be no confusion in identifying the slide. Never write the number in the thick blood film, for this may cause the thickened blood to be loosened in places from the slide, or it may interfere with correct examination of the small area covered by the blood, sometimes actually obliterating the rare parasites.

### 6. *Drying the Film*

***Lay the slide flat to dry so that the blood may be evenly distributed, and have it well protected from dust and insects. Air dry without application of excessive heat.*** If the blood smear is tilted while drying, the greater part of the blood will collect in a much thickened line along one edge of the smear. This may peel off completely in staining and, if not, it will be too thick for ease in examination. Also, the remainder of the film will be so thin as to be valueless, in light infections, for diagnosis. A covered Petri dish may be used for drying slides in the laboratory. In field work the slides should be inserted *film-side* down in a slide box (capacity of 25)—which is held upright against the perpendicular of an inverted T block by means of a rubber band. (See Plate II) When this box is full it should be closed and the box kept in an upright position until the slides are dry. Stained dust particles, etc., may cause trouble for the microscopist and slow down examination time. Flies or cockroaches will eat away the blood or contaminate it with bacteria or other organisms. If possible, let the blood smear dry in the air for 8 to 12 hours, thus protected. Thick films stain most clearly when several hours old. Very fresh smears may not have had time to adhere well to the slide and hence part of the blood may be lost. They frequently show a meshlike, fine fibrinous arrangement in the background also. This does not interfere materially with diagnosis but does not allow so clear a picture as the same slide will present if it dries for a few hours before staining. However, if a report must be made immediately the slide may be stained as soon as visibly dry, provided it is gently handled in both the stain and rinsing water. To aid in quick drying the slide may be placed for a short while in an incubator at 37° C. Too long drying of this kind, it is believed, will harden the cells and prevent perfect staining. Drying by means of warm air from an electric hand hair dryer held not too close to the wet smears, is an ingenious method that has been suggested and used satisfactorily.[11] The stirring up of dust, etc., should be avoided if this method is employed. Direct excessive heat should never be applied for, like alcohol, it "fixes" the red blood cells.

### 7. *Shipping Films*

***When smears are taken outside the laboratory they should be sent immediately to the person who is to stain them—because of the adverse effect of summer heat or of age on the smears.*** Slides shipped by mail

should be sent either in the slide boxes (these in turn carefully packed in larger firmly made boxes), or should be wrapped *when dry,* slide over slide in toilet tissue in small packages. These are then placed with much protective packing in mailing containers to prevent breakage in shipping. The custom of dropping a slide into an envelope and mailing it, or sending slides in other ways in which they are easily broken in the mails, is deplorable. Broken slides are most difficult to stain and to examine, and no technician can do creditable work with such material.

Thick films may be hardened or partially fixed by age or by summer heat to the extent that they will not give up their hemoglobin. Such films are valueless. Old films never take the stain so easily or so brilliantly as fresh ones, but if they *are not fixed* by age, heat, or alcohol, they may be diagnosed provided they are carefully stained. Unstained slides may be successfully kept for a number of days during cool or cold weather and in our laboratory during the summer have been stored in a cold room over a week end. In the latter instance care should always be taken to prevent condensation of moisture on the slides, since this may loosen the smears and cause them to be lost during the staining process.

### B. Making Slides into Blocks or Groups for Staining

If a large number of slides is to be stained, the technician's time may be saved and uniformity in quality of staining assured by the simple method described by Barber and Komp.[12] Slides are made into blocks of as many as 25 by placing inch squares of cardboard (about the thickness of the glass slide) between the slides at the ends opposite the blood films, compressing the alternating slides and squares and *winding around that end of the block an inch wide strip of heavy paper* which is secured with a strong rubber band. The paper prevents the slides from cutting the rubber band and the tight rubber band keeps the free ends of the slides from touching and assures access of the stain to the surface of all the thick films. By this method hundreds of slides may be stained together, and consumption of stain diminished by using a container of the exact size necessary to hold the number of blocks to be stained. In survey work each pack may be identified by a cardboard square, containing necessary data, slipped under the rubber band on the outside of the pack.

It has been known for years by most persons doing large surveys and those staining and examining large numbers of clinical slides, that

transfer of parasites from one slide to another is possible in mass staining of thick films, but the amount of transfer in the average survey has not been considered statistically important. This transfer is likely to occur under several conditions. Very fresh thick smears wash from the slides quite easily, so should be handled with extreme care. If the slides are not *thoroughly* cleaned, parasites may be left over from a former smear on an improperly cleaned slide, or the fresh blood smear may not stick to the dirty slide and flakes from a positive smear may attach themselves to other smears. Smears that are made too thick may flake off very easily. Also if the hematocrit reading of the blood taken is low, it will not stick to the slide well. For this reason it is suggested that *clinical cases* with known high parasitemias be stained separately; and that the technician keep in mind that transfer of parasites is possible where two or more slides are stained together, though it may happen very seldom with a careful worker.

In examination one can often identify under the low power objective flakes that are superimposed on the blood film, also one can be assured that parasites are rather evenly distributed through a smear, so one does not find a true positive with a high number of parasites in one microscopic field or one small area and not a like distribution throughout the smear. Where individual parasites are carried over there may be danger of a wrong diagnosis. Brooke and Donaldson [13, 35] have done extended studies recently on this subject. They have found that where blood films face each other in staining, one is much more likely to become contaminated by flakes from the other, than if the slides are placed so that the back of one slide is adjacent to the face of the next. They have discovered,[36] also, that the addition of a 0.05 per cent concentration of Triton X-30 to the stain solution reduces the surface tension and practically eliminates the transfer of parasites from one smear to another.

## C. STAINING FILMS

### 1. *Stains*

The most dependable malaria stain, particularly for thick films, is obtained with a good quality of Giemsa stain solution diluted with distilled water of a pH from 7.0 to 7.2. Most laboratories will want to use commercial stains. Grübler's dyes have long given complete satisfaction in this work, but American dyes [14] are much more easily obtained now and are cheaper. When Giemsa stains made from American dyes are used, they should be those stains which are certified

by the Commission for the Standardization of Biological Stains. Lots of American Giemsa certified from 1940 to 1949 should conform to the needs of the malariologist, as the staining of malaria parasites in thin and thick film has been one of the tests used by the Commission prior to certification of these Giemsa stains. The Commission has recently begun certification of two types of Giemsa stain, i.e., Giemsa stain, Azure B type and Giemsa stain, Azure A type. The Azure B type is that suited to the use of persons staining for malarial parasites and should be requested when purchasing either powder or solution. If the Giemsa stain solution is made by the laboratory worker from powders, the best reagent methyl-alcohol (neutral, acetone-free) and glycerine C. P. (neutral) should be used. The glassware used should be chemically clean and dry.

*a. Directions for making stain from Giemsa powder*

600 mg. stain powder (Cert.) *
50 ml. methyl alcohol—acetone-free
50 ml. glycerine—neutral—from freshly opened bottle

* National Aniline certified dyes NGe 10—NGe 15 have proved satisfactory.

Grind powder in a mortar before weighing.

Weigh powder.

Grind the stain powder with part of the measured glycerine in a mortar, pour the top third off into a chemically clean flask, and add more glycerine and grind again. Repeat until most of the powder has been mixed with the glycerine and mixture poured into the flask.

Stopper the flask with a cotton plug, place a heavy paper cap over the opening and bind with a rubber band. Place flask in a water bath and let stand for 2 hours at 55° C. to 60° C. Shake gently at half-hour intervals.

After grinding powder and glycerine together in mortar, measure alcohol and use part of it to wash the last bit of stain from the mortar. Put the washings in a small airtight bottle.

After 2 hours remove glycerine and stain powder from water bath, allow to come to room temperature, add the measured alcohol and washings from the mortar, and shake well.

The stain can be filtered and used immediately, but it is preferable to let it stand about 2 weeks with intermittent shakings, then filter for use.

Shake bottle of stain before pipetting off required amount for staining. Never put a wet or soiled pipette in the stain.

From the stock bottle pour a quantity of stain into a small bottle for current use.

### 2. *Buffer Solutions*

Buffered distilled water for staining and washing insures that the blue and red elements in the stain shall be taken up in correct degree by the blood and the parasites, thus giving maximum color contrast and facilitating diagnosis. To obtain a pH of from 7.0 to 7.2 in the water used for the dilution of the stock stain as well as for washing the stained slides, buffer solutions should be added to the distilled water. Disodium phosphate, and either sodium or potassium acid phosphate in M/15 solutions are used and are prepared by dissolving the salts as follows:

$Na_2HPO_4$(anhydrous), M/15 = 9.5 gm. per liter; $NaH_2PO_4.H_2O$, M/15 = 9.2 gm. per liter; and $KH_2PO_4$, M/15 = 9.07 gm. per liter. The two stock solutions are kept in separate glass-stoppered Pyrex bottles from which are removed the following quantities to make the indicated amount of the buffered water:

| pH | M/15 $Na_2HPO_4$ | M/15 $NaH_2PO_4 \cdot H_2O$ or M/15 $KH_2PO_4$ | Distilled $H_2O$ |
|---|---|---|---|
| 7.0 | 61.1 ml. | 38.9 ml. | 900 ml. |
| 7.2 | 72.0 ml. | 28.0 ml. | 900 ml. |

The pH of the buffered water is then tested and adjustments are made if necessary. Brom thymol blue indicator solution and a set of brom thymol blue color standards are used for testing. Buffered water should be prepared each week. If this is inconvenient, the neutral water may be put into a large container provided with a pinch cook and tube, and the surface of this water may be covered with mineral oil to keep out the $CO_2$ from the atmosphere, which will change the pH. Of course, the water should be replenished before the oily surface reaches the bottom of the glass tube.

### 3. *Thick Film Staining Directions*

*a. Regular method*

Use one part of stain to 50 parts of neutral distilled water to make the amount of stain necessary to cover the slides as they stand on end in the staining dish—thick film downward.

Leave slides in diluted stain for 45 minutes.

Remove slides from the stain and stand them in neutral distilled water for 3 to 5 minutes. (The age and thickness of the smear and density of the stain are the controlling factors here. Very fresh smears require little washing, as do films that are not very thick. Older, thicker, or deeply stained films give a clearer picture if washed a longer period of time.)

Remove slides from the water and allow to air-dry, standing on end on absorbent paper. Never blot thick films. An electric fan will facilitate the drying but direct heat is not advised, since some of the excess stain, which might flow from the smear, may be dried upon it and cloud the background.

If thick and thin films are put on the same slide, they may be stained together. The thin film should be "fixed" by dipping that end of the slide in alcohol or by touching the thin film with a small cotton swab dipped in alcohol. Care must be exercised to keep the alcohol from touching the thick film, and a compromise must be made in washing. It is probably better to have a little deeper background than usual in the thick film and retain the details of the thin, so dipping two or three times for washing is advised.

Always clean the staining dish after use, as sediment from previous staining may precipitate part of subsequent stain. For maximum staining qualities use the stain only once.

*b. Rapid Staining Methods*

To make the thick film technique meet the needs of physicians in their offices and diagnosticians in clinics and hospitals, the following satisfactory methods are suggested. Smears should not be so thick as when stained for a longer period and should be stained as soon as thoroughly dry. Drying may be hastened in a 37° incubator.

(1) With Giemsa Stain—

Put 2 ml. of Giemsa stain solution into 50 ml. of neutral distilled $H_2O$ and stain the smear in mixture for 20 minutes. Dip the slides in neutral distilled $H_2O$ to wash them. Dry and examine.

(2) With Wright-Giemsa Stain—

Make solution of Wright-Giemsa stain [15] (as prepared below) in proportion of 1 part of stain and 9 parts neutral distilled $H_2O$. Pour this over slides in a staining dish.

Stain slides for 10 minutes.

Flush scum from top of the dish with neutral water, then remove slides and wash them for 1 minute in neutral distilled $H_2O$.

Dry and examine with oil immersion.

(a) *Preparation of Wright Giemsa stain*—

To make Wright-Giemsa stain, dissolve 2 gm. Giemsa powder, certified (National Aniline Division, Allied Chemical and Dye Corp., New York) in 100 ml. glycerine (C.P. from a freshly opened bottle). This may be done by heating in a water bath at 55° C. to 60° C. for 2 hours and mixing well at intervals with a glass stirring rod.* To this mixture add 100 ml. Wright's stain solution (aged solution of 2 gm. powder to 1,000 ml. methyl alcohol). Let stand overnight and then add an additional 800 ml. of aged Wright's stain solution. Filter and use.

## V. EXAMINING THE FILM

### A. EQUIPMENT

A good microscope, preferably a binocular for constant work, equipped with 5 X or 6 X oculars for searching and 10 X for closer study of questionable objects, together with an oil immersion objective of 1.8 to 2 mm., and a microscope lamp giving sufficient blue white light are essential. The low power ocular gives a sense of greater distance, consequently is more restful to the eye. Also, it gives a larger microscopic field and so allows examination of more blood in a given number of fields.

Instead of cedar oil for examination of slides, one of the following is suggested: Crown Immersion Oil obtained from Techni-Products Co., 5 Woodette Place, Buffalo, N. Y.; Shillaber's nondrying Immersion Oil from R. P. Cargille, New York; or heavy mineral oil mixed with alpha bromo-naphthalene (82 parts of the former and 18 parts of the latter). Each of these oils has the refractive index of glass, they do not dry on the film, and can be wiped from slide with facial tissue. They do not spoil the thick film for future examination as cedar oil will do, nor is it necessary to use xylol, which fades the stain, to remove the oil from the slide or from the microscope.

### B. IDENTIFICATION OF THE SPECIES IN THE THIN FILM

#### 1. *Plasmodium vivax (benign tertian malaria)*

Considering the parasites as they appear in stained thin films from peripheral blood, the youngest ring form consisting of a blue margin of cytoplasm and a rather heavy red dot of chromatin, which may be

* Avoid absorption of moisture by covering the mouth of the flask containing the mixture with a double thickness of paper secured with a rubber band and by removing the flask from the water each time the mixture is stirred.

located centrally or peripherally, is about one-third the diameter of a normal red blood cell. After a few hours the red blood cells, infected with *vivax* parasites, are always enlarged, pale, and may be very bizarre in shape. Also, in correctly stained films Schüffner's stippling * may be demonstrated in many of the parasitized cells at any stage after this period. As development proceeds the parasite may continue to show a ringlike appearance in stained films with much thickened cytoplasm and enlarged chromatin mass. However, it may very early exhibit pseudopodial processes indicative of ameboid movement, a characteristic which is very pronounced in this species of malaria and which gave rise to the parasite's specific name "*vivax*." After five or six hours the trophozoite begins to show yellowish-brown pigment granules. These are small, round, angular or rodlike, and increase in number with the growth of the parasite. In the young forms they frequently cannot be distinguished as separate granules or rods, but exhibit their presence by giving a yellowish tinge to portions of the cytoplasm. As the trophozoite develops, it may assume practically any shape within the enlarged cell, with projecting pseudopodia and one or several vacuoles. Meanwhile both cytoplasm and chromatin are increasing in amount. In a *vivax* infection this stage is far larger than the corresponding stage of the other species. At the end of about 40 hours the parasite fills, or practically fills the cell, which may be twice its original size. It has now completed its vegetative growth and prepares for reproduction. To this end it draws in its pseudopodia, ceases its movement, assumes a rather compact form, usually irregular in outline and with cytoplasm mottled in appearance as though unevenly massed. It still has a single nucleus which is compact and usually lies near the periphery of the parasite. This stage may appear much smaller than some of the ameboid forms which have preceded it, and due to its compactness it usually stains much more heavily.

Now the division of the chromatin begins. First, there are two divisions possibly brought about by binary fission, and then successive divisions until there are 12 to 24 segments, with 16 as a common number. At first these are irregular in shape but as division becomes complete the segments appear more regular in size and shape and rather small and compact, as compared to earlier appearances. In the mean-

* This consists of bright pink granules which appear evenly distributed throughout the part of the parasitized cell not occupied by the parasite. The dots are rather consistently fine and uniform in size. As the parasite grows the dots often become more pronounced, and take a somewhat deeper stain. This stippling is peculiar to *P. vivax* and *P. ovale*, hence is of diagnostic value. Careful staining is necessary to demonstrate the dots in the maximum number of infected cells and prolonged washing will obliterate them. Their exact nature is unknown.[16]

time the single mass of cytoplasm has gradually broken up, one portion adhering to each dot of chromatin, thus forming individual parasites. These are the merozoites. During this process the pigment has gradually collected into one or two loose masses, the complete but rather loose clumping of the pigment being a definite sign that segmentation in this species is complete. Frequently there is an uneven number of merozoites in the mature schizont. The entire growth from small trophozoite through mature schizont requires about 48 hours. Upon maturity the cell bursts, the merozoites attack new cells and begin another generation.

The majority of parasites attain maturity at about the same time. Not all do, however, some are earlier in maturing than others. Hence there are often several stages of the *vivax* parasite in the peripheral circulation at the same time.

Gametocytes of *P. vivax* are sometimes found with the appearance of the first parasites in the peripheral blood, but more frequently they appear after schizogony has continued through several generations.[17] It is thought that development of the gametocyte requires much longer than that of the asexual form. It has also been suggested that the gametocytes of *Plasmodia* may go through an intermediary step involving the production of *premacrogametocytes* and *premicrogametocytes,* before the final gametocytes develop. This might account for the additional time required.[18] The shape of the growing gametocyte changes very little, from the compact merozoite form, due to lack of the ameboid activity found in the trophozoite. The mature microgametocyte is often about the size of a normal red cell; the mature macrogametocyte is distinctly larger, sometimes almost double the size of a normal red cell. Both are in enlarged red cells just as mature schizonts are. The quantity of pigment granules in the mature gametocytes of both sexes is usually greater than in the schizont, and the grains and rods are usually darker in color in the female. They are always distributed rather evenly over the cytoplasm of the macrogametocyte and often in that of the microgametocyte. However, in the latter there is a tendency at times to slight aggregation.

The macrogametocyte possesses a densely blue-staining, generally homogenous cytoplasm. The nucleus is usually compact, and very rich in deep red chromatin. Around this chromatin there is sometimes a colorless area which is called the zone of carolymph and is thought to be the non-staining part of the nucleus. The nucleus is usually situated near the periphery of the parasite.

The microgametocyte contains less cytoplasm than the macrogametocyte; it stains more lightly than that of the female and may be gray-blue, greenish blue, pinkish blue, or at times practically colorless. There is a loose nuclear system with reticular distribution of the chromatin. Sometimes the nucleus is termed "stellate"; sometimes it extends in a broad spindle across the body. Most often it is centrally placed, is more or less rounded, and has a light staining quality. There is often in the microgametocyte an unstained vesicular area around the mass or grains of stained chromatin, making the entire nucleus quite large.

There is difficulty in differentiating between the full grown trophozoite (just before division of the chromatin) and the slightly immature macrogametocytes. These points can be of assistance: The cytoplasm of the trophozoite may present a mottled appearance or may even contain one or more vacuoles; the outline is usually irregular and may show deep indentations. The pigment, although scattered through the cytoplasm, is in small golden-brown grains. In the macrogametocyte the cytoplasm is rather homogeneous and contains no vacuoles. The outline as a rule is regularly circular or ovoid. Its pigment may be more abundant and in larger darker brown granules. The full grown macrogametocyte is usually larger than the grown trophozoite.

### 2. *Plasmodium malariae (quartan malaria)*

The young trophozoites of *P. malariae* are ring forms and are about the size of, or slightly smaller than, those of *P. vivax,* though they sometimes seem to have a broader circle of cytoplasm than young *vivax* rings. Double chromatin dots are rare. The vacuole of the ring stage disappears very soon after the parasite begins its growth. The growing stages may assume band forms which stretch across the red blood cell; but the trophozoites seen in stained smears may also be compact forms, angular or even round or ovoid in outline. The chromatin may be a rounded mass, though more frequently it is streaked or semi-circular in formation—even in the rounded parasites. The *malariae* parasites spend nearly two-thirds of the 72 hour cycle in the "filled-in" trophozoite stages, so these are the forms most frequently found in blood smears.[19] There is very little ameboid activity in the growing and older trophozoites, hence one seldom finds the irregular, tenuous, ameboid forms that are so characteristic of *P. vivax.* As the parasite becomes older it may grow into a wider band form or

have a more rounded or slightly irregular shape. Frequently, there is a peripheral arrangement of pigment, often along the edge opposite the nucleus. The pigment of *malariae* trophozoites appears early in the growth, is usually a darker brown than that of the trophozoites of *P. vivax* and the individual granules are usually larger. Mature forms of the parasite almost fill or completely fill the normal-sized red cell and may be rounded or band shape with rounded or elongate chromatin mass. The cells containing parasites of *P. malariae* are never enlarged —frequently even seem smaller than normal and sometimes darker in the early stages. The completion of the cycle requires 72 hours, hence the growth of the parasite is comparatively slow. When schizogony is complete there are from 6 to 12 merozoites (usually 8) sometimes arranged peripherally around the centrally clumped pigment (rosette formation), but more often in an irregular cluster.

*P. malariae* seems to have fewer gametocytes than the other two common species. They are frequently very difficult to find. When the young gametocytes are found in the peripheral blood, they are almost impossible to distinguish from growing trophozoites which, in their compact form, resemble them very closely. The gametocytes are smaller than those of *P. vivax* but their development follows the same course. Like those of *P. vivax* they are spherical or oval in shape and they have the same differences in sex and in staining qualities. The pigment is abundant, dark-brown, and coarser than in *P. vivax*. Growing gametocytes of *P. malariae* are said not to take the band formation.[20] Mature macrogametocytes are likely to be larger than the mature trophozoites and they may be ovoid in shape. The pigment granules in them are more numerous and prominent than in the trophozoites.

On occasions there have been demonstrated in the cells containing *P. malariae* certain pink-staining dots similar to Maurer's spots in *P. falciparum*. These have been named Ziemann's stippling. Investigators seem to agree that the stippling is usually pale and the dots slightly irregular in size, round and less distinct than in *vivax*. Also, they are as a rule, less developed than those found in *falciparum*. They seem to be best brought out by intensive staining or stain solution the pH of which is about 7.5. Since they have little differential diagnostic significance, it is thought to be sufficient in diagnosing smears to stain slides at the neutral point and ignore these possible granules.

*P. malariae* as a whole is the least often found of the species occurring in the United States, but in some vicinities the comparative proportion of this species is rather high.

### 3. *Plasmodium falciparum (estivo-autumnal malaria)*

The small trophozoites of *P. falciparum* are usually smaller than those of the other types, have quite a delicate threadlike line of cytoplasm and one or more rather small dots of chromatin. Double chromatin dots are much more frequently found in this species than in the others. The rings may vary in size but in their youngest stage are usually smaller and much finer than the equivalent stage of *P. vivax*, and when found in sufficient numbers this small size and delicacy are leading diagnostic characteristics. The young parasites may at times be quite irregular in form (round, rectangular, flame-shaped, or streaked). Flattened marginal forms and bridge forms are more common in *P. falciparum* than in any other species. Multiple infections of the single cells are also more common in this species, though certainly not confined to it.

As growth proceeds the parasites of *P. falciparum* usually retain a ring formation much longer than most *vivax* parasites. The older rings differ from the very young ones in the slightly increased size and in the increased amount of cytoplasm and chromatin; also in the fact that they contain traces of pigment, which give a yellowish tinge to the cytoplasm. These parasites, which correspond in age to the large ameboid forms of *P. vivax* may be confused, in a diagnosis of smears containing few parasites, with the younger ring forms of *vivax*, since they are so nearly the same size. Because *falciparum* parasites do remain as ring forms during much of their trophozoite growth, and because of the great number of parasites produced in a *falciparum* infection, there are likely to be more rings of *P. falciparum* in the peripheral circulation at one time than in either of the other species. Also, unlike *P. vivax* or *P. malariae*, it is the tendency of the parasites of *P. falciparum* to disappear from the peripheral blood in the ring stage and complete their growth and development in the capillaries of the internal organs. This accounts for the fact that, of the stages of the asexual parasite, usually only the ring forms are found in the peripheral blood. The exact stage at which the ring forms disappear from the circulation varies; sometimes they disappear very soon after they enter the red blood cells; at other times they remain for hours, attaining considerable size. With the foregoing facts, *one may state that when only a large number of ring forms are seen and no older trophozoites or schizonts can be found, the infection is in all probability falciparum.* Ring forms are found more readily after the chill. In *P. falciparum*,

PLATE I.—*P. vivax*

1. Normal sized red cell with marginal ring form trophozoite.
2. Young signet ring form trophozoite in a macrocyte.
3. Slightly older ring form trophozoite in red cell showing basophilic stippling.
4. Polychromatophilic red cell containing young tertian parasite with pseudopodia.
5. Ring form trophozoite showing pigment in cytoplasm, in an enlarged cell containing Schüffner's stippling.[1]

6, 7. Very tenuous medium trophozoite forms.

8. Three ameboid trophozoites with fused cytoplasm.

9, 11, 12, 13. Older ameboid trophozoites in process of development.

10. Two ameboid trophozoites in one cell.

14. Mature trophozoite.

15. Mature trophozoite with chromatin apparently in process of division.

16, 17, 18, 19. Schizonts showing progressive steps in division ("presegmenting schizonts").

20. Mature schizont.

21, 22. Developing gametocytes.

23. Mature microgametocyte.

24. Mature macrogametocyte.

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[1] Schüffner's stippling does not appear in all cells containing the growing and older forms of *P. vivax* as would be indicated by these pictures, but it can be found with any stage from the fairly young ring form onward.

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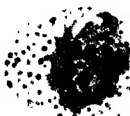

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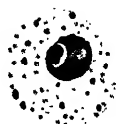

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INEZ DEMONET

PLATE II.—*P. malariae*

1. Young ring form trophozoite of quartan malaria.

2, 3, 4. Young trophozoite forms of the parasite showing gradual increase of chromatin and cytoplasm.

5. Developing ring form trophozoite showing pigment granule.

6. Early band form trophozoite—elongated chromatin, some pigment apparent.

7, 8, 9, 10, 11, 12. Some forms which the developing trophozoite of quartan may take.

13, 14. Mature trophozoites—one a band form.

15, 16, 17, 18, 19. Phases in the development of the schizont (''presegmenting schizonts'').

20. Mature schizont.

21. Immature microgametocyte.

22. Immature macrogametocyte.

23. Mature microgametocyte.

24. Mature macrogametocyte.

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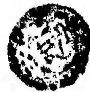
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## Plate III.—*P. falciparum*

1. Very young ring form trophozoite.

2. Double infection of single cell with young trophozoites, one a "marginal form," the other "signet ring" form.

3, 4. Young trophozoites showing double chromatin dots.

5, 6, 7. Developing trophozoite forms.

8. Three medium trophozoites in one cell.

9. Trophozoite showing pigment, in a cell containing Maurer's spots.

10, 11. Two trophozoites in each of two cells, showing variation of forms which parasites may assume.

12. Almost mature trophozoite showing haze of pigment throughout cytoplasm. Maurer's spots in the cell.

13. Aestivo-autumnal "slender forms."

14. Mature trophozoite, showing clumped pigment.

15. Parasite in the process of initial chromatin division.

16, 17, 18, 19. Various phases of the development of the schizont ("presegmenting schizonts").

20. Mature schizont.

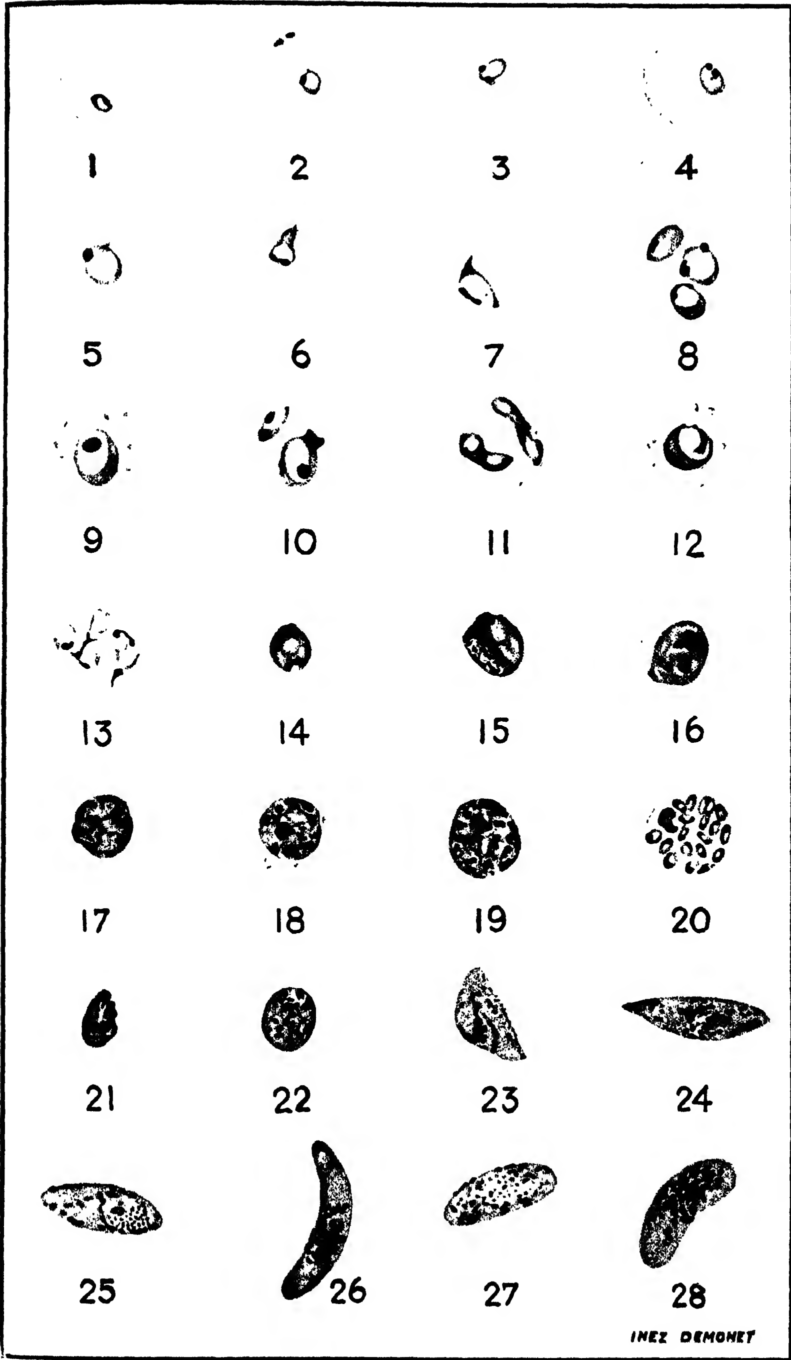
21, 22, 23, 24. Successive forms in the development of the gametocyte—usually not found in the peripheral circulation.

25. Immature macrogametocyte.

26. Mature macrogametocyte.

27. Immature microgametocyte.

28. Mature microgametocyte.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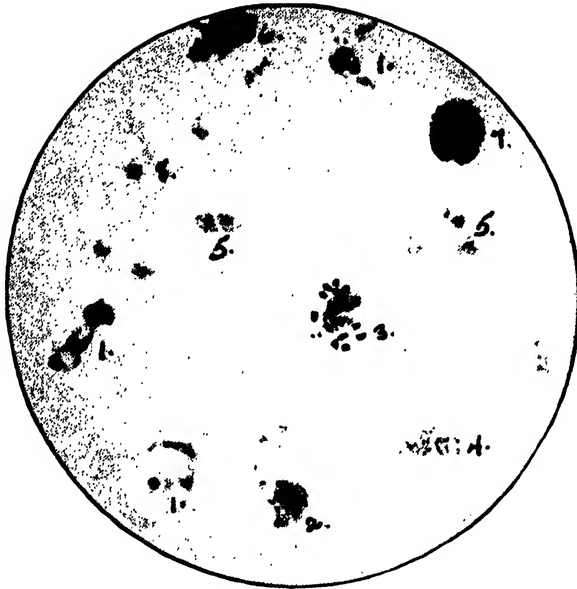
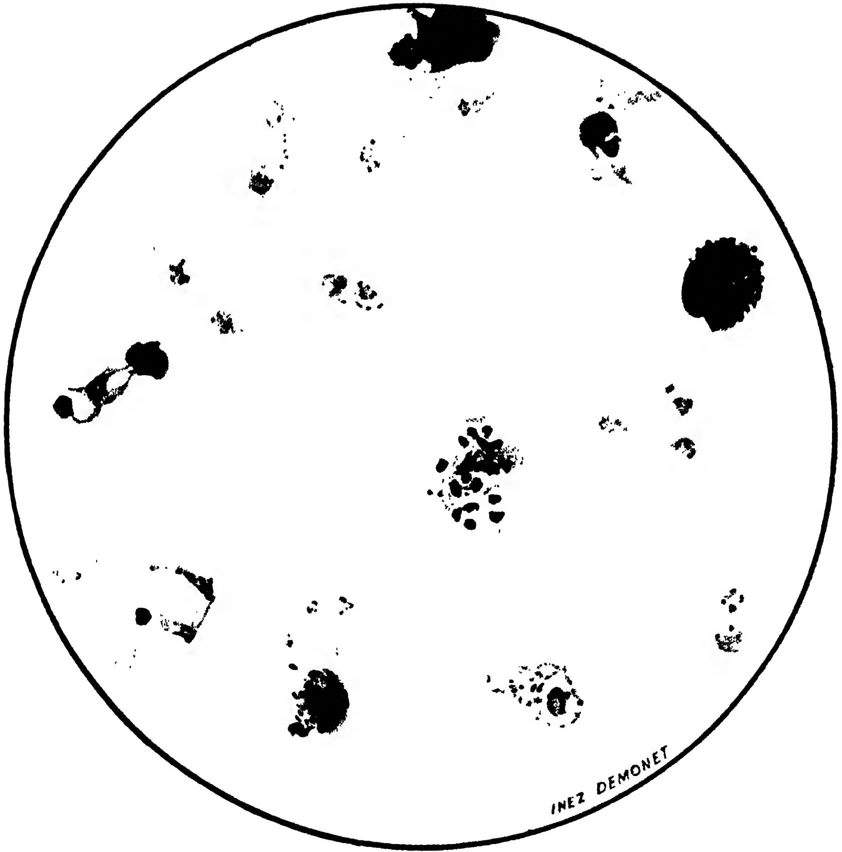


PLATE IV.—*P. vivax*—thick film

1. Ameboid trophozoites.
2. Schizont—2 divisions of chromatin.
3. Mature schizont.
4. Microgametocyte.
5. Blood platelets.
6. Nucleus of neutrophil.
7. Eosinophil.
8. Blood platelet associated with cellular remains of young erythrocytes.

PLATE IV.



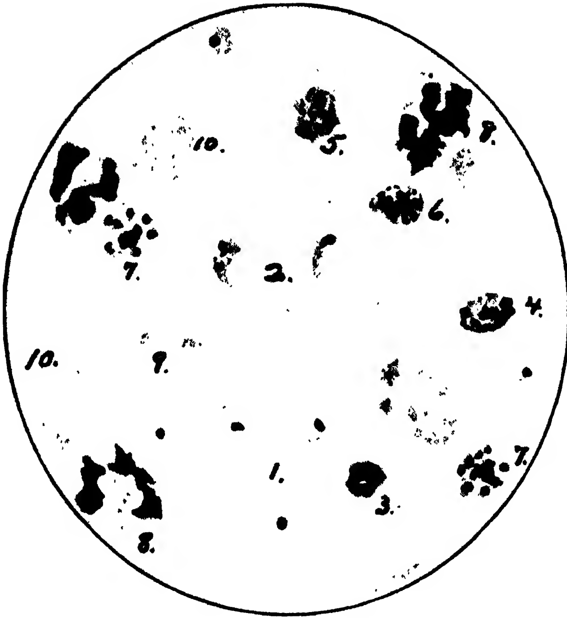


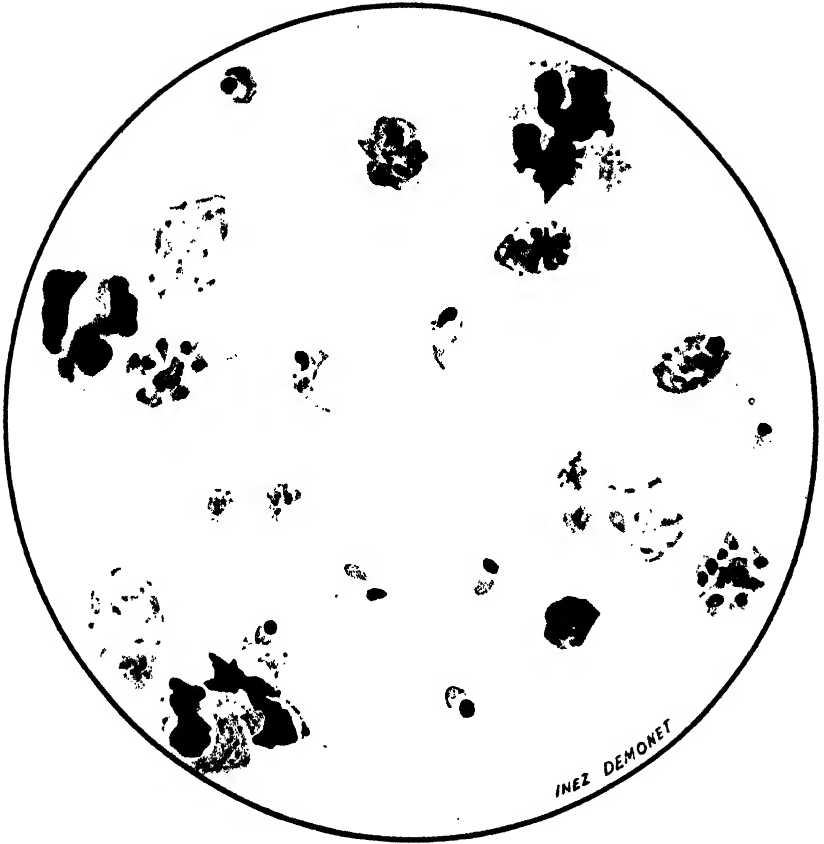

PLATE V.—*P. malariae*—thick film

1. Small trophozoites.
2. Growing trophozoites.
3. Mature trophozoites.

4, 5, 6. Schizonts (presegmenting) with varying numbers of divisions of the chromatin.

7. Mature schizonts.
8. Nucleus of leucocyte.
9. Blood platelets.
10. Cellular remains of young erythrocytes.

Plate V.



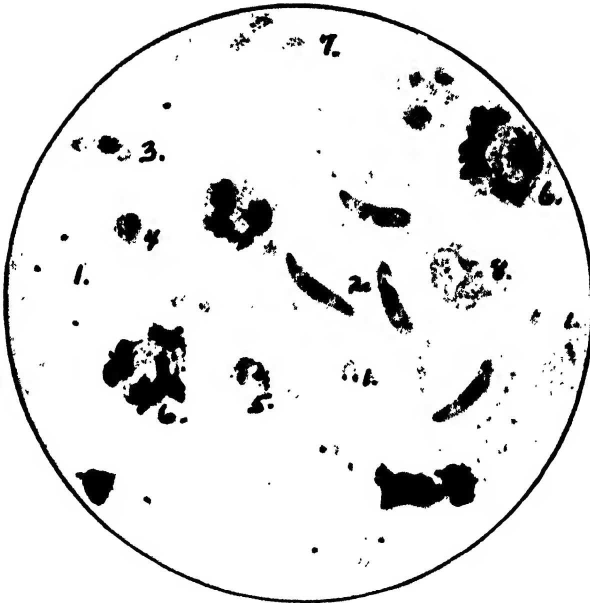
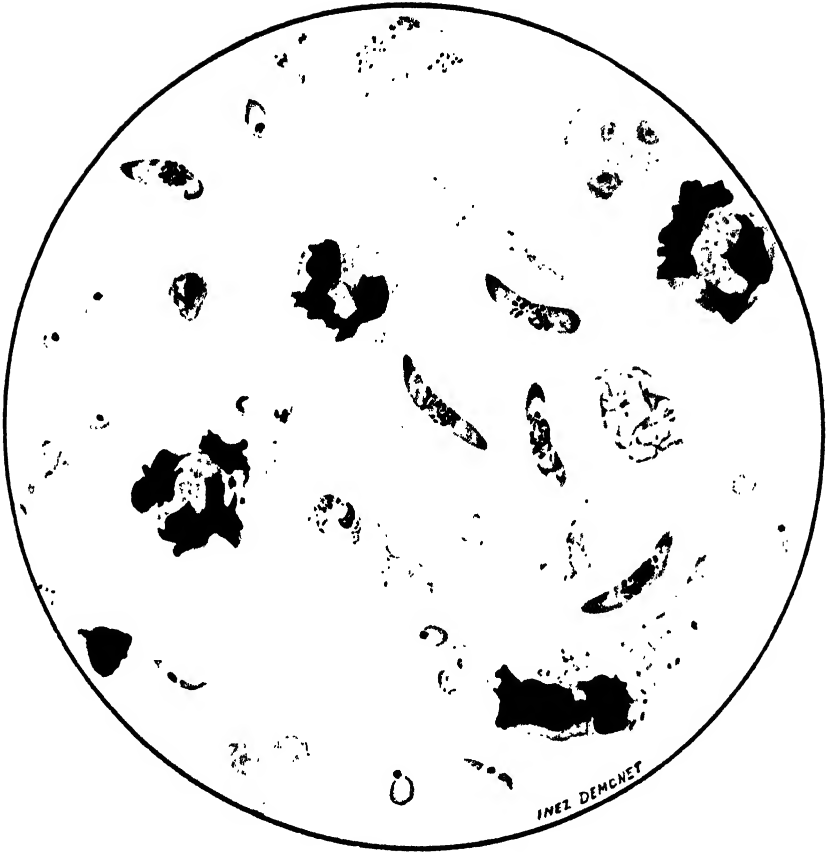



Plate VI.—*P. falciparum*—thick film

1. Small trophozoites.
2. Gametocytes—normal.
3. Slightly distorted gametocyte.
4. "Rounded-up" gametocyte.
5. Disintegrated gametocyte.
6. Nucleus of leucocyte.
7. Blood platelets.
8. Cellular remains of young erythrocyte.


INEZ DEMONET

if the case is running a tertian course, the parasite count will be much higher during the period of intermission between paroxysms than on the day of the paroxysm.

The more mature trophozoites and schizonts are, as a rule, not found in the peripheral blood. However, in some heavy or intense infections rare ones may occur usually along with large numbers of ring forms. The old trophozoites, when they are found, consist of a small bit of compact, light-staining cytoplasm, a granule of chromatin somewhat larger than in the ring and usually a dull blur or a small, dense, almost black block of pigment. The parasite at this stage is very small, sometimes hardly as large in circumference as the older ring forms. The presegmenting and segmented schizonts resemble those of the other species; but even in its complete segmentation the *falciparum* parasite fills only about two-thirds the diameter of the normal red cell. To differentiate the older forms of *falciparum* from those of *malariae* which they most resemble, one may keep in mind the following facts: the respective stages of *falciparum* are smaller, and they contain less pigment, which is usually black or almost black in appearance, and has a tendency toward early clumping (often before the division of the chromatin begins).

There are from 8 to 24 or more divisions (usually around 20) of the chromatin in *falciparum* and about 48 hours are required for maturation. The cell attacked by a *falciparum* parasite is not altered in size. Sometimes there may be observed in the cells containing the parasites red staining dots called "Maurer's spots." They are irregular in shape and size, though they are generally much coarser than the dots of Schüffner's stippling and not nearly so numerous. They are brought out in some slides with overstaining or with the staining solution on the alkaline side. Some films show them far better than others. The age of the parasite may have some effect on their staining. It is possible that they are peculiar to certain strains.

The gametocytes of *P. falciparum* are quite different from those of the other two species except in their youngest stage; at that time they are small compact bodies of cytoplasm with a small mass of chromatin, and like the other gametocytes they do not develop a vacuole. Their shape, from a rather early stage, however, is likely to be elongate or angular though rounded forms are found also. As the gametocyte grows the chromatin extends in a thick line, usually along one side of the body. The pigment in this stage is scattered through the cytoplasm. Occasionally, one finds long, thin spindle-shaped or

lanceolate forms in which the pigment is rather evenly distributed throughout the length of the parasite. These are young gametocytes, also. As the gametocyte gets older the chromatin and pigment generally pass to the center of the body and the parasite assumes an elliptical, slightly crescentic or sausage shape.

The mature macrogametocyte shows a dense blue cytoplasm and a compact, small red mass of chromatin, lying in or near the center, or near one of the poles. The pigment, in separate grains, closely adheres to the chromatin, surrounding it completely or on one side, sometimes even covering it. The macrogametocyte is often more slender and slightly longer, as well as more deeply stained than the microgametocyte. The microgametocyte, in comparison, is likely to be broader, shorter, and more sausage-shaped with lighter staining qualities throughout. The cytoplasm in the microgametocyte is usually pale, often grayish-blue or pink, the chromatin is loose and scattered, with the abundant brownish pigment rodlets and granules, irregularly through about half of the length of the crescent. As a rule, only mature crescents are found in the peripheral blood, but immature ones like the older asexual forms may be found in the circulating blood of persons seriously affected.

It is usually days before gametocytes of *P. falciparum* appear in fresh infections. On the other hand, in old or chronic cases, they are frequently found when no rings are to be seen. The gametocytes of *falciparum* seem to be produced in showers at intervals during the disease instead of being produced in proportionate number simultaneously with the asexual forms as in *P. vivax.*

The cells containing the crescent forms stretch as the longitudinal growth proceeds and frequently one can see in the stained thin film, on the concave side of the parasite, a faint bow-shaped projecting rim, representing the residuum of the infected red cell. Sometimes the remains of the red cell appear as a red zone around the crescent.

### 4. *Plasmodium ovale*

Most of the cases of malaria caused by *P. ovale* [21] have been reported from East Africa, though there are records of two cases in South America. So far none has been found in the United States. Those persons who have seen and worked with *P. ovale* advise microscopical comparison of well stained thin films of the classical species with some of this species in its various stages, in order to ascertain those characteristics which distinguish it. They state definitely that it cannot be identified in the thick film because of resemblance to both

*vivax* and *malariae* parasites. As in *vivax*, the parasite produces a tertian fever; i.e., the complete asexual cycle requires approximately 48 hours. As with *vivax*, the infected cell shows Schüffner's stippling and enlarges as the parasite grows older. As in both *vivax* and *malariae* all stages of the parasite appear in the peripheral blood.

Considering the young ring stage, the most definite means of identification seems to be a thick studding of Schüffner's stippling in almost 100 per cent of the infected cells. According to James, Nicol, and Shute,[21] these dots in *ovale* are numerous, even with the very young stages and are large and well defined, as compared with the rather sparse fine dots found in a few of the cells infected with young forms of *vivax*. The ring forms are, as a rule, darker in color and more solid than those of *falciparum*.

In the older trophozoite stages of *ovale* the parasite has a striking resemblance to that of *P. malariae*, since it has little ameboid activity. In actual measurement, it is larger and it has less pigment, which is lighter in color and not so conspicuous as that of *malariae*. A large proportion of the infected cells containing this stage and succeeding stages will be found to be rather longer than wide and to have an irregular fringed, not well defined margin drawn out into ragged points. The deeply stained Schüffner's stippling fills the cell even to the ends of these transparent points. While such cells might be found upon long search in *vivax*, they are frequent enough in *ovale* to be a characteristic of the species.

The presegmenting stage resembles closely the same stage of *P. malariae*, but it is a rounded parasite found in a definitely oval-shaped, enlarged cell, which may have the fringed edge spoken of above and usually has heavy Schüffner's stippling.

The segmenting schizont, as in *malariae*, usually has 8 segments often arranged in rosette form; the infected cell is enlarged, may be oval and fringed, and it nearly always has the heavy Schüffner's stippling.

The sexual forms resemble those of *vivax* and are difficult to distinguish from them. They are distinguished from *malariae* by the enlarged cell and presence of Schüffner's stippling.

### 5. *Mixed Infections*

It is believed that there are many more mixed infections than are usually determined in blood examinations, and that the possibility of a mixed infection is frequently overlooked by the microscopist. First of

TABLE 1

*Summary of Parasite Differentiation in Stained Thin Films*

| | *Plasmodium vivax* | *Plasmodium malariae* | *Plasmodium falciparum* | *Plasmodium ovale* |
|---|---|---|---|---|
| **Infected cell** | Larger than normal, pale, often bizarre in shape. Schüffner's dots very often present. Multiple infection of erythrocyte not uncommon. | About normal or slightly smaller. Sometimes darker in early stages. Ziemann's dots rarely seen. Multiple infection of erythrocyte rare. | Normal in size. Multiple infection of erythrocytes more frequent than in the other species. Maurer's dots sometimes seen (in overstained smears or when pH of $H_2O$ is on alkaline side). | Somewhat larger than normal, often with fringed or irregular edge and oval in shape. Schüffner's dots appear even with younger stages, stain more readily and more deeply than in *vivax*. |
| **Small trophozoite (early rings)** | Signet ring form with heavy chromatin dot and large cytoplasmic circle, possibly with fine pseudopodia. | Signet ring form with heavy chromatin dot and cytoplasmic circle which is often smaller, thicker, and heavier than that of *vivax*. | Small threadlike cytoplasmic circle, and one or two small chromatin dots. (Double chromatin dots more frequent than in other species.) Marginal and bridge forms are frequent. May disappear in this stage from peripheral circulation and return to internal organs for development. | Small, darker in color and more solid, as a rule, than those of *falciparum*. Schüffner's dots regularly present in almost 100% of infected cells. |
| **Growing trophozoite** | Same as above with gradual increase in amount of cytoplasm and chromatin. Often with more distinct pseudopodial processes. Small yellowish-brown pigment granules in cytoplasm, number increasing with age of parasite. | Chromatin rounded or elongated, cytoplasm in a compact form with little or no vacuole or in a narrow band form across the cell. Dark brown pigment granules—may have peripheral arrangement. | This stage remains in the ring form but chromatin and cytoplasm increase to the extent that in size the parasite resembles closely the small trophozoite of *vivax*. A few pigment granules give a yellowish tinge to the cytoplasm. This is usually the oldest asexual stage seen in peripheral circulation. | Resembles closely same stage of *P. malariae*, but is considerably larger. Pigment is lighter in color and less conspicuous. (applies to growing and large trophozoite) |
| **Large trophozoite** | One abundant mass of chromatin, loose, irregular or close compact cytoplasm, with increasing amounts of fine brown pigment. Parasite practically fills enlarged cell by end of 36 to 40 hours. | One mass of chromatin, often elongated, frequently less definite in outline than that of *vivax*. Cytoplasm dense, compact with few irregularities of outline; in rounded, oblong or sometimes band shape. Pigment granules larger, darker than in *vivax* with great tendency toward peripheral arrangement. Fills or almost fills normal cell. | Stage seldom seen in peripheral blood. Very small, solid, with one small mass of chromatin; lightly staining, compact cytoplasm; and with haze of pigment scattered through the cytoplasm or with very dark pigment collected in one small, dense block. | |

| | | | | |
|---|---|---|---|---|
| **Schizont (presegmenting)** | Chromatin divided into a number of masses; cytoplasm shows varying degrees of separation into strands and particles; pigment shows tendency to collect in parts of the parasite. | Same as *vivax* except that the parasite is smaller and shows fewer divisions of chromatin, as it approaches segmentation and more delayed clumping of pigment. | When found in peripheral blood this stage resembles the same stage of *P. malariae* but is smaller and the pigment is likely to be completely clumped in one small dark mass. | About 25% of infected cells are definitely of oval shape. Usual picture is that of a round parasite in center of an oval cell. Many cells with indefinite fringed outline. Pigment lighter in color and less coarse than in *P. malariae*. |
| **Mature schizont**......... | 12 to 24 divisions or merozoites, composed of a dot of chromatin and a portion of cytoplasm. The pigment is in one or two clumps. Parasite practically fills enlarged cell. | 6 to 12, usually 8 or 10 merozoites in a rosette or irregular cluster. Practically fills normal-sized cell. | 8 to 24 or more merozoites, which are very small compared to those of other species. Rarely found in peripheral blood. Fills about two-thirds of normal-sized cell. | Usually 8 merozoites arranged around a central block of pigment. |
| **Macrogametocyte** ....... | Dark blue, homogeneous cytoplasm with no vacuoles; small, compact, dark red, usually eccentric chromatin; abundant dark brown pigment scattered through cytoplasm. When grown usually fills or nearly fills enlarged cell. The outline is circular or ovoid and regular. | Cytoplasm and chromatin same as *vivax*. Pigment is abundant, dark brown, coarser than in *vivax*. When grown usually fills the normal sized cell. Outline circular or ovoid. | Cytoplasm possibly a deeper blue than in microgametocyte. Usually single dark red chromatin mass near center associated with concentrated aggregation of pigment, darker than in microgametocyte. Crescentic or sausage-shaped about 1½ times diameter of erythrocyte in length, possibly longer and more slender than microgametocyte. | Distinguished from *P. malariae* by size of infected cells and by Schüffner's dots. Less easy to differentiate from *vivax*. Seldom or never contained in an oval erythrocyte. (applies to macrogametocyte and microgametocyte) |
| **Microgametocyte** ........ | Light blue, gray, pink or almost colorless cytoplasm; large diffuse light red or pink chromatin—usually centrally placed, often with vesicular area around chromatin mass. Abundant yellowish-brown pigment throughout cytoplasm. When grown, about size of a normal cell. Usually circular in outline. | Same as *vivax* except in size. When grown, fills or almost fills normal sized cell. | Often the cytoplasm is paler than in macrogrametocyte — grayish blue or pink. Loose, diffuse, light staining granules or threads of chromatin scattered with numerous granules of pigment throughout central half or more of parasite. Parasite possibly broader, shorter, and with more rounded ends than those of macrogametocyte. | |
| **Length of asexual cycle**.. | 48 hours...................... | 72 hours...................... | 48 hours...................... | 48 hours |
| **Stages in peripheral blood** | All .......................... | All .......................... | Usually ring form trophozoites and gametocytes. Other stages rarely found except in severe cases. | All |
| **Remarks** ............... | More stages of growth likely to be seen in one film than in other species. Gametocytes appear early in cycle. | Parasites are usually more compact and hence appear more intensely stained than those of other common species. Gametocytes rarer than in other species, appear late. Least often found of 3 species in United States. | Parasites frequently more numerous than in other infections. Unlike other species—growth of asexual forms, following the ring stage, take place in internal organs. Gametocytes appear later — often after symptoms have disappeared. | Species not reported to date in United States. Differentiation not possible in thick films. |

all, it seems to be the tendency in mixed infections for one species to predominate over the other,[22] and the chance of finding numbers of the parasites of one species in a film and of missing completely the infrequent parasite of another species is quite possible. The fact is that many technicians look no further when definite parasites of a single species are seen. Of course, typical and characteristic forms of the respective species must be found to determine a mixed infection. In thin films these specifically distinctive forms in *P. falciparum* are the crescent shaped gametocytes. In *P. vivax* they are the large, distinctly ameboid trophozoites or any stage of the parasite in an enlarged cell containing Schüffner's stippling. In *P. malariae* they are the pigmented band forms or any heavily pigmented form beyond the ring stage in an unenlarged cell. The mixed infections in the United States are most frequently *falciparum* and *vivax;* sometimes, however, they are *vivax* and *malariae, falciparum* and *malariae,* or even on rare occasions, all three species.*

### 6. *Accompanying Blood Picture and Sources of Error*

In malaria the total number of white cells, as a rule is below normal, though the count may rise during the febrile period, or with some complicating factor such as pneumonia. In the stained thin film one frequently finds the picture of secondary anemia in greater or less degree. There may be increased polychromatophilia or basophilic stippling, central achromia of the red cells, variation in their size and shape, or even nucleated red cells. In the early stages of the disease there may be a reduction of the number of neutrophiles and an increase in lymphocytes and monocytes. During subsequent attacks there is a transient increase of neutrophiles with a display of many immature forms. After the attacks the neutrophiles decrease again and the monocytes increase. These latter play a part in combating the infection by ingesting the pigment and damaged cells. Clumps of malaria pigment in the white cells are almost as certain proof of malaria as the parasites themselves, but when this pigment is present, one usually finds plenty of malaria parasites, also.

In examining thin films for malaria the inexperienced observer may mistake a platelet which lies over a red cell for a young trophozoite, or a group of platelets between cells for a group of merozoites. The

* This information on parasites in the thin film is based partly on experience and partly on information obtained from Thompson and Woodcock,[30] Wenyon,[28] Stitt, Clough, and Clough,[24] Nocht and Mayer,[25] and James, Nicol, and Shute.[31]

granular texture of the platelets, as contrasted with the rather solid appearance of the chromatin dot of the ring or merozoite, should be a help here. Precipitated stain is often very misleading. Basophilic stippling in red cells and Howell-Jolly bodies, Cabot's rings, etc., are sometimes confusing. One may have to rule out bacteria from the skin, dust or plant spores from the air; and contaminants from distilled water which is not fresh or which has been placed in an unclean, unsterilized container.

## C. IDENTIFICATION OF SPECIES IN THE THICK FILM

### 1. *General Appearance of the Blood in Stained Thick Films*

The discussion of the differentiating points of the species of malaria is usually based upon their appearance in the thin film. In this kind of film the cells are spread over the slide in one layer and then by the application of alcohol, either as a separate process or in the stain itself, are fixed so that their outlines and the outlines of the parasites within them are preserved.

In the *thick film,* on the other hand, the blood cells are piled upon each other and removal of the hemoglobin from the cells is necessary to render the film sufficiently penetrable to transmitted light for microscopical examination. Dehemoglobinization takes place in the process of staining the unfixed film with an aqueous solution of Giemsa stain. The outlines of the red cells are thus obliterated and a microscopical picture presented which differs considerably from that of the thin film.

In the thicker portion of the thick film the background varies in color from a clear, light blue to a mottled gray-blue, depending upon stain factors, age of the smear, and individual blood variations; while at the edge, the thinner portion of the smear (many times only one cell in depth and about the width of one or two microscopical fields) often takes a pinkish color. Against the laked background the familiar purple nuclei of the white cells, in varying states of preservation, stand out clearly. Sometimes the cytoplasm of the white cells stains also, but it is ragged and uneven in appearance. The neutrophilic granules are often indistinct or absent but eosinophilic granules show rather distinctly in their characteristic color. Blood platelets are pinkish-violet in color, finely granular in texture and hazy in outline. They lie singly or in groups, and due to their distinctive granular appearance are not likely to be confused with parasites in the thick film. If the blood is taken from a free-flowing puncture there will be little likelihood

of excessive clumping of platelets. Very often, particularly in anemic bloods, the thick film shows in the background (the thicker the smear, the more evident it is) nuclear and reticular remains of immature red cells (cellular debris). These may lie singly, often filling a space the exact size and shape of the laked cell, or they may appear in blue clouds of fine skein-like masses or stippling. See "Sources of Confusion or Error" (V,C,8). Barber claims that the degree of anemia can be estimated by the degree or density of these blue clouds.[10] The technician with a well grounded knowledge of the stages and species of malaria parasites, as they appear in the thin film, will have no great difficulty in diagnosing malaria in the thick film, or in differentiating species. For the less experienced person, the thin edge of the thick film will be very valuable for study. Here the red cells frequently retain a ghost-like outline and one finds characteristics of infected cells and of parasites duplicating those in the thin film—for instance, the enlarged infected cells and distinct Schüffner's dots of *vivax,* band forms of *malariae,* marginal ring forms; doubly infected cells and perfectly formed gametocytes of *falciparum,* etc. The thin edge is particularly valuable for determining mixed infections. It is well in learning thick films to start in this thin edge and work in toward the thicker portion comparing the typical forms with the less characteristic ones. Schüffner's stippling, with good staining of the smear and a short washing period, may frequently be seen as individual granules in the infected cells at the edge of the film, whereas, in the thicker portion one sees sometimes a delicate pink area around the parasite which one interprets as the color caused by Schüffner's stippling. Maurer's and Ziemann's spots have not been observed in the thick film though pink areas around parasites have been seen in the thick film when these spots were found in the thin film.

Except in the very thin edge the parasites usually appear without the definite outline of their host cells. The chromatin and cytoplasm stain characteristically and the pigment retains its usual appearance, but in the thicker portion of the smear the parasites often seem smaller and more shrunken. This may be due in part to the destructive effects of lysis and also to the heaping and crowding of the red cells in the thick smear, which prevents the cells and parasites from flattening and spreading out as they do in the thin film. Also, in the slower drying thick film the parasites have an opportunity to draw in their pseudopodia, which gives them in the stained film a denser, smaller appearance.

Almost constant focusing is necessary to distinguish all the parasites in the thicker fields, as the depth of the blood gives varying ocular planes. Too much stress cannot be laid upon careful staining, for ease in examination and accuracy of diagnosis are completely dependent upon it.

A discussion of the identification of the parasite stages and species in the thick film follows, together with Table 2 giving the most noticeable characteristics of the parasites. No such table can be complete. There are certain things that can be learned only by study at the microscope, by comparison of similar stages of the various species in the thin and thick film of the same blood, and by practice in the diagnosis of smears. One *must* follow such a course to become proficient in the use of the thick film. Also one must take into consideration the composite picture of all the parasites found on the film, and not depend too much upon any one parasite found.

### 2. *Small Trophozoites (Rings)*

In young ring forms the red or purplish-red of the chromatin dot first strikes the eye and the cytoplasm is distinguished subsequently. Young trophozoites may be found in the thick film in the signet ring formation just as in thin films, but are frequently not complete in outline; i.e., the chromatin dot with only a portion of the cytoplasmic circle is visible. This portion may be connected to the chromatin dot on both sides in short, straight or curved dashes, giving the impression that the rings are turned sideways or are lying at an angle to the plane of the blood. The very descriptive term " swallow form " has been applied to these.[26] Often the cytoplasmic dash is visible adjoining only one side of the chromatin dot. This form resembles more or less an " exclamation mark " or " comma " and has been designated by these terms. Sometimes the cytoplasm follows a circular outline but is made up of seemingly disconnected small pieces or fragments and is called an " interrupted ring." At times the cytoplasm is in a solid semicircular piece and lies completely disconnected from the chromatin dot on the curve of the ring opposite the nucleus. This form, too, might come under the term " interrupted ring."

When only this ring stage of the parasite is present and the rings are infrequent or rare in the thick film specimen, it is often impossible to differentiate the species. If an older form of the parasite can be found, this may facilitate the diagnosis.

TABLE 2

*Summary Table for Diagnosis of Parasite Species in Stained Thick Film*

| *Stage of Parasite* | *Plasmodium falciparum* | *Plasmodium vivax* | *Plasmodium malariae* | *Comments* |
|---|---|---|---|---|
| Small trophozoite (early ring) | Small size rings, with small chromatin dot and delicate, scanty cytoplasm. Frequently rings have double chromatin dots. Tendency toward large number of rings. Many ring forms with no older stages—practically certain to be *falciparum* infection. Diagnosis on small number of rings may often be assisted by finding distinguishing gametocyte, though this stage is not necessarily present. | Larger, heavier, ring form than in *falciparum*, often with variety of cytoplasmic pattern and irregularities in shape. Usually older stages of the parasite can be found also. | Ring is likely to be heavy, with large dot of chromatin and small amount of cytoplasm, which is often "filled in," without a vacuole. Pigment forms early and may appear as haze in cytoplasm of this stage. Rings practically always associated with older forms. The ring phase is brief, so this stage is not found as often as older stages. | Ring forms often not complete circle — may be "swallow" forms, "exclamation mark," "comma" forms, or "interrupted rings." When rings only are present and number is small, it is practically impossible to differentiate species. |
| Growing trophozoite..... | Heavy large ring forms—resemble young rings of *vivax*. sometimes show pigment grains or haze rather clearly in cytoplasm. | Stage usually ameboid in appearance, with large variety of shapes. Cytoplasm frequently fragmented and arranged irregularly in cluster of varying sized pieces or streamers, about or close to a large chromatin mass. Small yellowish brown pigment granules scattered through parts of the cytoplasm. This is the most characteristic stage of *vivax*. Frequently other younger or older stages accompany this one. | Small, usually rounded compact forms, "like marbles in a ring." Profuse, heavy, dark, large-grained pigment. Forms frequently so solid that chromatin seems buried in the mass. This stage and the one that follows are the commonest forms of this parasite seen. | In heavily stained films and in films which have been kept for several days before staining, the "ghost" of the enlarged host cell and persistence of Schüffner's stippling or a pinkness remaining from the stippling, may assist in diagnosis of *vivax*. |
| Large trophozoite....... | Ring vacuole lost or almost lost. Parasite quite small and compact, cytoplasm often quite pale, irregularly circular or oval. One large chromatin dot. Pigment in blurred mass or small, very dark clump or clumps. Stage is usually found only when the infection is intense and usually accompanied by numbers of ring-form trophozoites. | Frequently quite solid and dark staining. More or less irregular in outline, possibly with one or more vacuoles. Fine brown pigment scattered throughout the cytoplasm. May be confused with macrogametocyte. | Compact, dark, larger than "growing" stages. Sometimes in thinner portion of the smear spreads to normal size. Profuse, fairly coarse, dark brown pigment—often masking the chromatin. May be confused with "rounded up" *falciparum* gametocyte or with gametocyte of *malariae*. | On rare occasions Maurer's spots have been observed in thick films of *falciparum*. The infrequently found stages of *falciparum* are, of course, more readily found in thick films. Band forms have tendency to become rounded in thick films of *malariae*—except perhaps in very thin edge of smear. |

| | | | | |
|---|---|---|---|---|
| Schizont (presegmenting) | Stage not often seen and is usually accompanied by large numbers of growing trophozoites when present. Parasite is very small. Contains 2 or more divisions of chromatin and very little cytoplasm (often pale) in which there is located one or more small, dense blocks of very dark pigment. | Irregular or compact clusters of chromatin divisions, often dark reddish-purple in color. Cytoplasm in irregular broken masses and wisps, containing light brown pigment granules which are clumping in spots. Usually accompanied by other stages. May be confused with same stage of *malariae*. | Much like *vivax* of the same stage except that parasites are often smaller with darker, larger pigment granules. Often so compact that internal structure is difficult to define. Usually accompanied by other stages. May be confused with presegmenting schizonts of *vivax*. | Schizonts are much like thin film forms of same stages—more compact, smaller in thicker portions of smear. This is most difficult stage (except infrequent ring forms) on which to diagnose species. |
| Mature schizont......... | Seldom seen except in severe cases. Always associated with many small trophozoites. Usually contains around 20 or more tiny merozoites clustered around a small, very dark, pigment mass. | Usually contains around 16 merozoites which are individually larger than those of *falciparum*. Usually relatively larger than other species. Nearly always associated with other stages. Not so often found as other stages. | Most distinctive stage of *malariae* in thick film. Often found in large numbers—usually with trophozoites or presegmenting forms or both. About 8 merozoites each with large chromatin dot and small amount of cytoplasm—may be compact or clearly separated. Frequently the chromatin and pigment only are seen, the chromatin dots being bare and well separated. The dark heavy pigment is more often concentrated though sometimes dispersed. | Usually smaller than same stage in thin film. |
| Young gametocyte....... | Sometimes long, slender and pointed with pigment scattered to the ends. Usually associated with many trophozoites. | When found is a small, compact, usually rounded parasite, with one chromatin mass which is often in the center of cytoplasm and frequently has unstained area around chromatin mass. Sex is almost impossible to determine. | Same as *vivax* except that parasite is even less frequently found and resembles compact trophozoite so closely that differentiation is absolutely impossible. | |
| Mature gametocyte...... | Differentiation of sex is difficult or impossible. As "crescent" or "sausage" shapes, may be quite diagnostic of species. In thicker portion of smear may take an oval or rounded, somewhat eroded appearance, which may be confused with *malariae* trophozoite or gametocyte. Ofttimes may be distinguished by difference in amount and appearance of pigment or by pink or red "flag" protruding from the edge of the parasite. May be accompanied by ring form trophozoites or appear alone and infrequently. Often appears in "showers." | Macrogametocyte is larger, as a rule, than in other species; pigment is light, delicate, well dispersed through non-vacuolated cytoplasm. Except in thin edge of film cannot be differentiated from some mature trophozoites of same species. Microgametocyte often distinguishable as large blob of chromatin (varying from pink to purplish-red) surrounded by halo of pale or colorless cytoplasm in which pigment granules are more or less evenly dispersed. Other stages of the parasite can usually be found. | As a rule, few in number, somewhat smaller than *vivax*, otherwise have the same distinguishing features except that pigment is coarser and darker. May resemble rounded *falciparum* gametocytes. | |

In *P. falciparum*, the young ring forms are very small and delicate, the chromatin dot tiny, and the cytoplasm threadlike. There is a tendency toward a large number of rings in *falciparum*, so if many small rings are found and no older form of the parasite is in evidence, one can be practically sure that the infection is *P. falciparum*. Double chromatin dot rings are readily picked up in the thick film and a high percentage of such rings is always suggestive of *falciparum*. The ring forms may or may not be accompanied by gametocytes.

The young rings of *P. vivax* are, as a rule, larger than the same stage of *P. falciparum* with a heavier chromatin dot and cytoplasmic circle. Sometimes, even in this stage, there is evidence on the stained smear of ameboid activity shown by tiny pseudopodial processes on the cytoplasmic circle. It is seldom that species diagnosis must needs be based on young trophozoites alone, however, since older forms of *vivax* can usually be found.[27]

The small trophozoite of *P. malariae* is usually heavier than that of *P. falciparum* and frequently not so large as that of *P. vivax*. Since the ring stage lasts only a short time, and the cytoplasmic circle has a tendency to "fill in" early, the young form of *malariae* in the thick film often shows as a large chromatin dot with a small concentrated mass or dash of cytoplasm. There is very little tendency on the part of the *malariae* ring to spread or show ameboid outlines. Pigment forms very early and may be seen as a haze over parts of the cytoplasm in some of the larger rings. A search, even when the parasites are scarce, will usually show older forms accompanying the ring stages in *malariae*.

### 3. *Growing and Large Trophozoites*

While the young and delicate ring form of *P. falciparum*, when found in sufficient numbers in the thick film, can often be differentiated from those of *P. vivax* and *P. malariae*, the growing trophozoites of *falciparum* present more difficulty in diagnosis of species. These heavy ring forms of *falciparum* are about the size of much younger forms of *vivax* and can be confused with the latter, yet they correspond in age to much larger ameboid parasites of *vivax*. The old trophozoite of *falciparum* (the form just before division of the chromatin) is not often found except in heavy infections and is consequently usually accompanied by large numbers of ring forms. When it is found, it is usually very small, compact, often nonvacuolated. The chromatin dot is larger than that in

the young ring, the cytoplasm seems lighter than that of the similar stage of *malariae*, and the pigment, even at this stage, is usually clumped in one or two very small dark or blurred masses.

In thick films showing the growing trophozoites of *vivax* one may still encounter ring formations much enlarged, with increased amounts of delicate, tenuous cytoplasm. There is a great variation in cytoplasmic pattern at this stage and there is a decided tendency, in the older ameboid forms of this species, for the cytoplasm to be fragmented and arranged irregularly in a cluster of varying-sized pieces with no visible connection. This cytoplasm is associated with a large round or irregularly shaped, red or magenta mass of chromatin. The pigment appears as a yellowish haze, or as small light-brown grains or delicate rods on the cytoplasm. This is the most characteristic stage of *vivax* in the thick film. The trophozoite stage of *P. vivax,* just before division of the chromatin, is frequently quite solid in the thick film with a dark staining appearance, often with a more or less regular outline. Its yellow-brown pigment, like that of the gametocyte, is rather evenly distributed through the cytoplasm. In the thick film these forms cannot be distinguished definitely from macrogametocytes.

Due to the fact that there is little ameboid activity in *P. malariae,* the growing trophozoites of this species are, as a rule, more compact looking in thick films than are those of *P. vivax.* This is the commonest form of *P. malariae* seen in blood examinations as the stage lasts for nearly two-thirds of the 72 hours cycle.[19] The profuse, heavy, dark pigment scattered through the solid cytoplasm gives a dense appearance to a majority of the parasites. The picture often presented in the thick film by the quarter or third-grown trophozoites of *malariae* is so distinctive that when they are present in sufficient numbers, diagnosis is practically certain. In this stage the inconspicuous body of chromatin is often imbedded in a small, tight, rounded, heavily pigmented mass of cytoplasm. At first glance, one does not distinguish the characteristic red, blue, and brown of the parasite, but to the eye trained to pick them up, close examination will reveal the colors. Old trophozoites of *malariae* are also often quite compact and dark. They are very much like the growing forms except that they are larger, and hence often show more morphology. They sometimes display quite nicely the elongated or streaky chromatin and peripheral arrangement of pigment, so characteristic of this stage in the thin film. Band forms of *malariae* usually round up in the thick film and cannot be certainly identified except perhaps in the thin edge.

### 4. *Schizonts (Presegmenting)*

The schizonts (presegmenting or immature) of all the species of malaria have much the same appearance as in the thin film, except that there is a tendency toward more compactness. There is division of chromatin material, and the fewer of these divisions or the further from complete segmentation the parasite is, the more irregular in appearance the chromatin masses usually are. These chromatin masses often appear quite dark, reddish-purple and are sometimes (in the thicker portion of the smear) distinguished with difficulty within the heavy cytoplasm. As the parasite approaches complete segmentation, the nuclear masses appear more regular in shape and the cytoplasm in the process of dividing may appear somewhat paler and in light wisps, particularly in *vivax*. The pigment is gradually collected into fewer and fewer groups as segmentation progresses in *P. vivax* but it has a tendency to concentrate late in *P. malariae*. The presegmenting schizonts are probably the ones most often passed over in the thick film by the inexperienced worker. The presegmenting forms of *P. vivax* and *P. malariae* are (aside from infrequent ring forms) the most difficult stage in which to differentiate species in the thick film. However, these stages will usually be found accompanied by more readily recognized stages on which diagnosis may be based. There are times when the presegmenting forms of *vivax* are definitely larger than those of *malariae*, but one cannot depend completely on size.

The presegmenting stage of *falciparum* is not frequently found in the peripheral blood. When found it is, as a rule, smaller even than that of *malariae* and very compact. There are 2 or more small purple-red divisions of chromatin usually in a slightly eroded small mass of cytoplasm, which is often pale in color. The best differentiating character is the small, rather prominent dark clumped mass of pigment. This form, when found, is nearly always associated with many young ring forms.

### 5. *Mature Schizonts (Segmented)*

Mature schizonts of the three species resemble very closely the same stages in the thin film, practically the only difference being the absence of the cell outline and a possible shrinkage of the parasite. In *vivax*, there are usually about 16 individual merozoites which often stand out from the group, each rounded mass of chromatin having its completely differentiated division or light blue zone of cytoplasm. Sometimes they

are more closely grouped. The pigment at this stage is usually clumped in the center or near the edge of the parasite. Mature schizonts of *P. vivax* are not as frequently found as are other stages of this parasite. *The mature schizont of P. malariae is the stage by which the inexperienced can diagnose this species most readily in the thick film.* It rather regularly has about 8 merozoites—often clearly separated, each with a large dot of chromatin and a group of heavy dark pigment granules concentrated or slightly dispersed. In many mature schizonts the cytoplasm accompanies each chromatin dot though frequently only the chromatin divisions and pigment show—with no sign of cytoplasm. Mature schizonts of *P. malariae* may appear in large numbers and are usually accompanied by young trophozoites or presegmenting schizonts. It is unusual to find the mature schizont of *P. falciparum* except in severe cases, but in this species the merozoites are smaller than in *P. malariae,* more numerous than in the other species, and there is a small, dark, closely knit mass of pigment. There are usually 14 to 20 or more tiny merozoites in each mature schizont.

### 6. *Young Gametocytes*

Young gametocytes are relatively infrequently found. In *vivax* and *malariae,* they are usually rather small, compact, rounded parasites, those of *malariae* being correspondingly smaller than those of *vivax.* It is frequently difficult and often impossible to distinguish them from compact trophozoites, or to distinguish sex. The chromatin often lies in the center of the compact cytoplasmic body and frequently has an unstained area between the chromatin mass and the surrounding cytoplasm. Young gametocytes of *falciparum* are sometimes long, slender and pointed, with pigment scattered to the ends of the parasite. When found, they are usually associated with many trophozoites. Occasionally even younger gametocyte stages will be found, but those in the thick film are most difficult to identify; the difference between them and the mature trophozoites being the fact that the pigment is scattered through the cytoplasm in the young gametocyte, whereas it is clumped in the trophozoite.

### 7. *Gametocytes*

In the thick film it is impossible to distinguish definitely the macrogametocytes of either *P. vivax* or *P. malariae* from the rounded or oval, mature trophozoites with a single mass of chromatin, rather regular,

dense cytoplasm and evenly dispersed pigment. Mature microgametocytes of these two species are more easily determined because of their very large, usually rounded or stellate nucleus, surrounded by a small amount of light staining or colorless cytoplasm containing numerous grains of prominent pigment. This nucleus often stains more deeply, comparatively speaking, than in the thin films. The parasite is often just a blob of chromatin with a halo of pigment granules. There is no other stage that resembles it closely. Occasionally, in thick films, exflagellation of fully-matured microgametocytes takes place on the slide before it dries. The gametocytes of *P. vivax* may be larger than those of *P. malariae* but this is not always so, as the thickness of the film frequently determines size of the parasite. Gametocytes of these two species are usually accompanied by other stages. The mature gametocytes of *P. falciparum* (crescents) are easily determined, as long as they retain their characteristic elongate or sausage-like shape, though it is often impossible in thick films to differentiate the sexes. In heavier portions of thick films, particularly when the blood dries slowly, mature gametocytes of *falciparum* assume a rounded shape, a change that would take place normally in the mosquito during the early stage of maturation. These forms may be confused with older trophozoites or gametocytes of *malariae,* but the stages with which they are associated in the blood will aid in diagnosis as will certain characteristics of the form. The pigment will be in distinct rodlets and will often be arranged compactly with a clear halo or fringe of blue cytoplasm. Frequently, also, there is found projecting from one portion of the mass, a flag or tongue of pinkish or reddish staining material. The nature of this material is not known. It might possibly be the cell wall which contained the parasite; it might be extruded chromatin; but at any rate it is found sometimes in thick films with normally shaped crescents as well as with the rounded or "balled-up" forms. Usually typical crescents will be found in the thinner edges of the films so that species identification is not dependent on the uncertain forms.

### 8. *Sources of Confusion or Error*

The inexperienced microscopist may be confused in the examination of thick films by bacteria or dirt from the skin; by dust particles on the slide; by vegetable spores, yeast cells or fungi from the air; and by bacteria, molds, protozoa, or other contaminants from the distilled water used in staining. Probably the chief source of dirt on the thick

film is from the improperly cleaned finger. If the glass slide is very clean, the skin is cleaned thoroughly before taking the blood, the slide is not allowed to touch the skin, and the blood is dried protected from insects and dust; if utensils used for water and for staining are kept clean, and if the water used in staining and washing is kept free from growth and contamination, these disturbing factors will be entirely eliminated or reduced to a negligible minimum. Artifacts which may deceive the inexperienced will be found frequently to lie above the blood plane, or they may be refractile and focus out of the field unevenly. Sometimes red dots appear in the preparation without any visible cytoplasm. It is possible that some of these are at times remnants of malaria parasites, but *one should never call a slide positive on these dots alone.* The dots may be staphylococci or other cocci or they may be products of degeneration of the red cells. If the dots are evenly distributed throughout the smear they are more likely to be associated with the cells, than if they are found in clumps or only in a part of the smear. If a red stained coccus from the skin or small nuclear remnant lies adjacent to a blue stained particle of cellular substance or fibrin, there may be a resemblance to a parasite. This rarely occurs more than once or twice on a single slide and numbers of free cocci will usually be found also, whereas, in well stained smears free chromatin dots are rarely found. As one becomes more familiar with the thick film he is less likely to mistake any foreign body for a parasite. According to Barber and Komp a good general rule is not to consider anything a parasite which may be interpreted as an artifact. Thick films should not be diagnosed as positive on what appears to be only one parasite. The parasite should be unmistakable, or search should be continued until others are found. If this is impossible later smears should be made.

## D. REPORTING RESULTS OF EXAMINATION

In reporting the results of examination of blood films for malaria one should not only specify, if possible, the species of parasites found, but should give some indication of the stages of the parasite and the number found. Thus one may report *P. falciparum, P. vivax,* or *P. malariae,* showing (many, few or rare):

1. Trophozoites (small, large)
2. Schizonts (presegmenting, mature)
3. Gametocytes (young, mature)

In the thick film we use the word "many" to designate the number of parasites when one finds them in every microscopic field; "few" when he finds them in every third to tenth field; and "rare" when he must search for them. A positive report can be made on one unmistakable parasite, but it is wise in cases of rare parasites to search carefully for additional forms to confirm the report. Sometimes, also, one picks up mixed infections by prolonging the search.

## E. ENUMERATION OF PARASITES

Boyd says[28] "The quartan parasitemias seldom exceed 10,000 per cubic millimeter and those of *vivax* seldom exceed 50,000 per cubic millimeter. On the other hand, the *falciparum* parasitemia has no potential limits and it is important to note that the prognosis is definitely bad if the count attains or exceeds 500,000 per cubic millimeter." Sometimes only a matter of a few hours will suffice to jump a count of 70,000 or 100,000 to 500,000 in *falciparum*. Consequently it is always wise to make a parasite enumeration in this species, and it may be desirable in the other species.

A simple method of enumeration of parasites which may not be accurate but which will give a rough estimation of the number of parasites without any special equipment is as follows: A thick smear of the blood is made at the same time that a white blood count is made. One hundred (or multiples of 100) white cells are counted on the thick film. In the same microscopic fields with these cells the malaria parasites are counted also. Then the parasites per cu. mm. are calculated by the following formula:

$$\frac{X \begin{pmatrix}\text{no. of parasites} \\ \text{per cu. mm.}\end{pmatrix}}{\text{White cell count per cu. mm.}} = \frac{\text{No. of parasites counted in the same fields with 100 white cells}}{\text{No. of white cells counted (100 in this case)}}$$

EXAMPLE:

$$\frac{X}{4000} = \frac{1200}{100}$$

$$100X = 4{,}800{,}000$$

$$X = 48{,}000$$

Probably a more accurate method of enumeration, but one not so easily used by the average technician, because of the equipment and acquired skill necessary, is described by Earle and Perez.[29]

## F. MAKING PERMANENT PREPARATIONS

1. *Materials Used:*

a. Giemsa stained blood films, clean and free from immersion oil.
b. "Diaphane"—Obtained from Will Corporation, Rochester, N. Y.
c. Clean No. 1 cover slips, large enough to cover entire film or better portion of each film.
d. Clear, water white lacquer—"Rogers"* or equivalent.
e. Small camel's hair brush.
f. "Diaphane Solvent."

2. *Using "Diaphane" With a Cover Slip:*

With medicine dropper or glass rod, place small drop of Diaphane in center of the blood smear. If blood film is elongated, use larger drop of Diaphane and pull it down the center of the film. Use *only enough* to spread to the edge of a cover slip, when it is placed carefully over the film. Press from the center of the cover slip outward with the eraser end of a pencil to spread the Diaphane to the edge of the slip and to remove bubbles.

If either the layer of Diaphane or the cover slip is too thick, focusing with the microscope on the smear will be difficult or impossible.

Let slides stand in horizontal position a short time; wipe away any excess Diaphane with Diaphane Solvent. Then dip a small camel's hair brush in clear lacquer and "ring" the cover slip, so that the brush stroke extends over the edge of the slip and the surrounding portion of the slide. Allow to dry, and use a second coat of lacquer, if necessary, to seal the slip to the slide.

3. *Using "Diaphane" without Cover Slip:*

Hold side edges of one end of the slide between thumb and forefinger of the left hand, and with a medicine dropper or smooth glass rod place about three drops of Diaphane at the left end of slide. With the side of the dropper or rod spread the Diaphane across the width of the slide, then with gentle, even pressure draw the medicine dropper or rod toward the opposite end of the slide, pushing the Diaphane ahead of it. This will spread a thin film over the length of the slide. There should be no excess. Stand the slide on end on a paper towel and allow to dry.

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* Detroit White Lead Works, Detroit, Mich., U. S. A.

The small amount of Diaphane that drains from the slide to the lower end may be cleaned away with a small brush or cloth dipped in Diaphane Solvent.

Solvent may be used to thin the Diaphane when it becomes too viscous. The Diaphane is neutral and should be kept that way by being sure that nothing acid or alkaline comes in contact with it.

NOTE: One of the immersion oils suggested (V, A, Equipment) should be used. Never use cedar oil on slides covered with cover glass or with Diaphane only, since the xylol necessary to remove it will affect the lacquer used in sealing the covered slide; and the oil can in no way be removed from the uncovered slides without spoiling the film of Diaphane.

## VI. COMMENT ON DIAGNOSTIC METHODS OTHER THAN BLOOD FILMS

The quotations below are from a *Critique of Diagnostic Methods* by Craig [30]:

" Fortunately, in the vast majority of symptomatic malarial infections the plasmodia may be demonstrated in either thin or thick blood films, so that it is rarely necessary to employ other diagnostic methods. It is in latent malarial infections that it is sometimes impossible to demonstrate the plasmodia in the peripheral blood and dependence must be placed upon some other method of diagnosis.

" The *puncture of the Spleen* or *liver* and *sternal puncture* have proven useful in the hands of some authorities but the writer has never had to resort to these measures for the diagnosis of malaria in an experience of over forty years and believes their use to be very rarely indicated. Of the three mentioned, sternal puncture is the safest and should be preferred.

" *Cultivation methods* are of very little use in the diagnosis of malaria and are very rarely employed for this purpose. In rare instances such methods might conceivably prove useful, as in infections in which the plasmodia cannot be demonstrated in either thin or thick blood films or in blood concentrates, but practically it has never been demonstrated that cultivation methods are of service in malarial diagnosis." Geiman and his associates have done extensive work on cultivation of parasites in the past few years.[31]

" *Serological reactions* have yet to prove their practical value in the diagnosis of malaria. Of the specific antigen-antibody tests, the *complement-fixation* test of Coggeshall and Eaton would appear to give the

best promise of being a valuable diagnostic measure, while *precipitin tests* have given results which are encouraging. At the present time, however, neither complement-fixation nor precipitin tests are employed in the routine diagnosis of malarial infections." The *complement-fixation* test, with *P. knowlesi* antigen, has been used extensively and with excellent results in research work in recent years.[32] However, no commercial antigen is available, so the test cannot, as yet, be used by the average diagnostic laboratory.

" Serological tests, nonspecific in nature, as the *Henry melano-flocculation* test, the *Chorine* test, and the *Proske-Watson protein-tyrosin test* are still of uncertain value. The melano-flocculation test of Henry has been very largely used in Europe and is believed by many to be a valuable diagnostic measure but cannot replace the demonstration of the plasmodia in the patient's blood as an accurate diagnostic method. The fact that all of these tests are positive in conditions other than malaria renders a diagnosis of malaria based upon the results of these tests alone always open to question."

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# Laboratory Diagnosis of Helminths and Protozoa

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# I. LABORATORY DIAGNOSIS OF HELMINTHS

## I. INTRODUCTION

THE diagnosis of helminths is usually readily accomplished by fecal examination. Each species of worm passes characteristic eggs or larvae which makes specific diagnosis possible. The different species of helminths vary greatly in the number of eggs they pass daily. A female ascarid, for example, passes approximately 200,000 each 24 hours and therefore is easily detected by even simple methods of fecal examination. Other species of worms pass relatively few eggs, and in the presence of light infections repeated examinations or concentration methods must be employed. Most helminth eggs can be accurately diagnosed with a 10X eyepiece and 16 mm. objective, although occasionally a 4 mm. objective may be necessary for the smaller eggs. (See Figure 1).

## II. SPECIES OF HELMINTHS

The following list includes the important helminths of man, the presence of all of which, except the filarids, may be detected by fecal examination (*S. haematobium* eggs are found in the urine). The filarial worms are usually diagnosed by the presence of microfilariae in the circulating blood.

### A. NEMATODA (ROUNDWORMS)

*Necator americanus*—new world hookworm } eggs similar
*Ancylostoma duodenale*—old world hookworm }
*Ascaris lumbricoides*—stomach or eel worm
*Trichuris trichiura*—whipworm
*Enterobius vermicularis*—pinworm, seatworm
*Strongyloides stercoralis*
*Trichinella spiralis* (fecal diagnosis of no practical importance)
*Wuchereria bancrofti* }
*Wuchereria malayi* }
*Acanthocheilonema perstans* } Microfilariae in the blood
*Mansonella ozzardi* }
*Loa loa* }
*Onchocerca volvulus*—Microfilariae in the skin, not found in the blood

### B. CESTODA (TAPEWORMS)

*Taenia saginata*—beef tapeworm } eggs similar
*Taenia solium*—pork tapeworm }
*Diphyllobothrium latum*—fish tapeworm
*Hymenolepis nana*—dwarf tapeworm
*Hymenolepis diminuta*—rat tapeworm

## C. TREMATODA (FLUKES) NOT NATIVE IN THE UNITED STATES

*Schistosoma haematobium* }
*Schistosoma mansoni* } blood flukes
*Schistosoma japonicum* }
*Clonorchis sinensis*—liver fluke
*Opisthorchis fileneus*
*Fasciolopsis buski*—intestinal fluke
*Heterophyes heterophyes*
*Metagonimus yokogawai*
*Paragonimus westermani*—lung fluke

No trematodes have been reported as naturally occurring in man in the United States; on the other hand, there are nine trematodes, several of which produce serious disease, which may be acquired in the tropics or Orient and be brought into the United States by their host. The three Schistosoma species reside in the bloodstream of man, Clonorchis lives in the liver, and Paragonimus in the lung; however, the presence of these parasites is detected by the presence of their eggs in the feces, although those of Paragonimus are most commonly found in the sputum. The eggs of *Schistosoma haematobium* are usually found in the urine.

Although the presence of intestinal helminths in general may usually be detected by the examination of the feces for eggs, the following exceptions should be noted:

1. *Enterobius vermicularis* only occasionally passes eggs while in the intestine. The female worm liberates the eggs around the anal area after migrating out of the intestine, hence perianal swabs are resorted to for diagnosis.

2. The gravid segments of *Taenia saginata* and *T. solium* are frequently passed in the stool before they have disintegrated and freed their eggs; hence proglottids rather than eggs will be present. Gross examination of the whole stool should be made for proglottids. The proglottids should be immersed in a mixture of 75 per cent carbolic acid, 25 per cent xylene until cleared in order to bring out the internal structures. *Taenia solium* gravid proglottids have from 8 to 12 main lateral uterine branches, while *T. saginata* has from 15 to 30 branches. Material suspected of being tapeworm proglottids may be pressed between two microscope slides and examined under low power for eggs.

3. The eggs of *Strongyloides stercoralis* usually hatch before being evacuated in the stool, hence the examination is for the larval form rather than the egg.

4. Early in the infection with *Trichinella spiralis* young adults may be passed in the stool, but the chance of finding one is extremely remote.

## III. FECAL SPECIMENS

Fecal specimens to be examined for helminth eggs and protozoan cysts may be collected in 1 oz. tin containers which have a cardboard top used for the recording of the patient's name and address or identification number. Heavy glass vials with tight fitting corks also make satisfactory specimen containers. These specimens may be shipped in a mailing tube conforming to the postal laws and should reach the laboratory within two days. Specimens should be stored in a refrigerator until examined. Adequate identification, including the name and address of the person furnishing the specimen should be included with the container.

Fecal specimens to be examined for protozoan trophozoites must be freshly passed and examined while still warm for satisfactory results.

## IV. REPORTING THE RESULTS

The results of examinations of stools for helminth eggs should be reported as negative or positive. When positive, the species of egg present should be given. Inasmuch as the various technics differ widely in their sensitivity, the method of examination should also be included in the report. In reporting the results of the Stoll dilution egg count, the number of eggs per ml. of feces should be given and the estimated number of worms the egg count indicates may be reported.

## V. DIAGNOSTIC METHODS FOR HELMINTHS

### A. Fecal smear

#### *Material*

1. Toothpick
2. Glass microscope slide
3. Cover slip
4. Physiological salt solution or tap water

#### *Preparation and examination*

A piece of feces the size of a rice grain should be thoroughly mixed with a toothpick in one or two drops of physiological salt solution or tap water on a clean slide and covered with a clean cover slip. The

feces should be mixed with enough water so that ordinary newsprint can be seen through the smear preparation.

A well prepared, carefully examined smear will detect an infection of as few as 25 hookworms or a single ascaris. Trichuris does not produce large numbers of eggs, and a light infection may not be detected by a single smear examination. Several smears should be examined before the stool should be reported negative by this method.

### B. BRINE FLOTATION

Helminth eggs have a specific gravity greater than water but will float up in concentrated salt solutions and may be collected from the surface of the solution. Such preparations have the advantages of concentrating the eggs from a quantity of stools and of eliminating the debris that makes the examination of smears difficult. Willis (1921) devised the original brine flotation method which is given here with several useful modifications.

#### *Material*

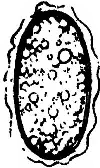
1. Toothpick or applicator stick
2. Saturated NaCl solution, specific gravity 1.197. Boil 320 gm. of unrefined NaCl in 1,000 ml. of water. Allow solution to cool and pour off supernatant fluid from the excess salt.
3. One oz. tin or glass container, approximately 1″ in diameter
4. Lipless test tube ½ to ¾″ in diameter
5. Glass microscope slide 1½″ x 3″
6. Cover slip ⅞″ square

#### *Preparation and examination*

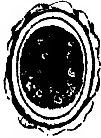
Thoroughly mix 1 gm. of feces with 1–2 ml. of saturated solution (specific gravity 1.197) of unrefined table salt (NaCl) in a 1 oz. tin or a glass container 1″ in diameter. Add 20–30 ml. of saturated salt solution and mix. Allow fecal suspension to stand 30 to 60 minutes. Transfer some of the surface film to a glass slide by means of the open mouth of a lipless test tube. Cover preparation with a clean cover slip. Examine under low power (16 mm. objective—5X or 10X eyepiece) focusing on the upper surface of the salt solution film.

The operculate eggs do not float in salt solution; hence this method is not useful in detecting fluke infections or the tapeworm Diphyllobothrium. Schistosome eggs shrink in concentrated salt solution making their recognition difficult. This technic is not satisfactory for helminth larvae.

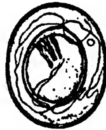
FIGURE 1



*Ascaris lumbricoides*
(Unfertilized)
ROUND WORM

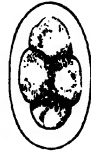


*Ascaris lumbricoides*
(Fertilized)
ROUND WORM

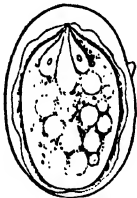


*Hymenolepis nana*
(*Taenia murina*)
DWARF TAPEWORM

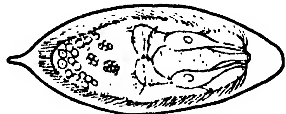
*Enterobius vermicularis*
(Oxyuriosis)
HUMAN PINWORM OR SEATWORM



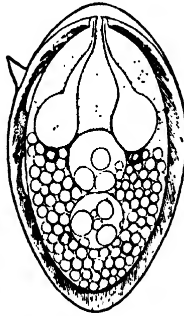
*Necator Americanus*
(Uncinariasis)
AMERICAN HOOKWORM



*Schistosoma japonicum*
ORIENTAL BLOOD FLUKE

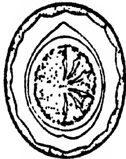


*Schistosoma haematobium*
VESICAL BLOOD FLUKE



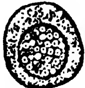
*Schistosoma mansoni*
MANSON S BLOOD FLUKE

*Trichuris trichiura*
WHIPWORM



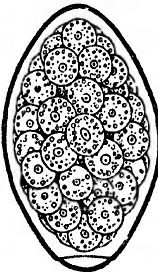
*Hymenolepis diminuta*
RAT TAPEWORM

*Trichinella spiralis*
TRICHINA



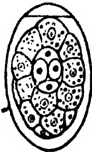
*Dipylidium caninum*
DOG TAPEWORM

*Taenia solium*
PORK TAPEWORM

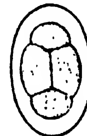


*Fasciolopsis buski*
LARGE INTESTINAL FLUKE

*Taenia saginata*
BEEF TAPEWORM



*Diphyllobothrium latum*
BROOD (FISH) TAPEWORM



*Ancylostoma duodenale*
"OLD WORLD" HOOKWORM

**EGGS OF PARASITES FOUND IN THE HUMAN** (Magnified approximately 400 times)

## C. ZINC SULFATE FLOTATION AND LEVITATION METHODS

See section on diagnostic methods for protozoa, IV, B.

## D. CONCENTRATION OF HELMINTH EGGS BY SEDIMENTATION

The inability of the various flotation technics to demonstrate regularly certain operculate eggs, unfertilized Ascaris eggs, and *S. japonicum* eggs has led to extensive trials of various sedimentation technics. Loughlin and Stoll (1946) have devised a centrifugation method that accomplishes the diagnosis of all types of helminth infections which show eggs in the feces. This technic has the advantage of considerable sensitivity in the finding of infertile Ascaris eggs and *S. japonicum* eggs.

### 1. *The acid-ether-xylol (AEX) method*

#### *Material*

1. Stoll dilution egg count flask or 100–200 ml. Erlenmeyer flask and rubber stopper
2. Glass beads
3. 20% hydrochloric acid
4. Ether
5. Xylol
6. 0.1 N sodium hydroxide
7. Capillary pipette
8. 1½" x 3" glass slide
9. 22 x 30 mm. or 25 mm. square cover slip

#### *Preparation and examination*

1. Measure 4 ml. (or gm.) of feces into a dilution counting flask which has been filled to the 56 ml. mark with water.
2. Add several glass beads (6 mm.), and after giving the preparation an initial shaking, set aside for several hours or overnight (preferably refrigerate); complete the comminution of the feces by vigorous shaking, so that all the eggs are free.
3. Shake to produce a thorough distribution of the eggs in the fecal suspension and transfer immediately 1.5 ml. of the suspension to a 15 ml. pointed centrifuge tube.
4. Add 3.5 ml. of 20 per cent hydrochloric acid,* put a rubber stopper in the tube, and shake for 1 minute; allow to stand for 2 minutes.

* 20 ml. of concentrated hydrochloric acid diluted to 100 ml. with distilled water. The net concentration of HCl in the centrifuge tube is reduced to 14 per cent.

5. Add 5 ml. of a freshly prepared mixture of equal parts ether and xylol and again shake for 1 minute.

6. Centrifuge at 1,800–2,000 r.p.m. for 2 minutes and allow the centrifuge to come to a stop gradually without interference.

7. Separate the semi-floating coagulum from the walls of the tube with a thin wooden applicator.

8. Decant quickly, permitting the sediment in the bottom of tube to remain undisturbed. Then, while holding the tube almost horizontally, clean any adhering coagulum from the inside of the tube with an applicator covered with gauze.

9. Add one drop of 0.1 N solution of sodium hydroxide to the sediment, mix thoroughly with a capillary pipette, transfer entire suspension to a glass slide and place a cover glass on it. If the density or amount of residue warrant it, make two drops of the material and cover each with a cover slip in order to secure a preparation which will allow the eggs to be seen easily.

10. Examine the whole preparation for eggs. Those found are from 0.1 gm. of the original fecal specimen.

### 2. *Sedimentation method of Faust, Ingalls, and See (1946)*

Relatively few eggs are passed by the schistosomes, and the irregularity with which they may find their way into the intestinal lumen makes it especially important to detect them when they are present, as this is a serious infection. It is obvious that the examination of a large amount of feces is highly advantageous. Methods that wash and sediment considerable quantities have proved especially sensitive. Faust, Ingalls, and See (1946) have devised a highly satisfactory sedimentation technic.

#### *Material*

1. Wire screening or Curity surgical gauze
2. 0.5% glycerine in tap water
3. Pipette
4. Microscope slides and cover slips
5. V-shaped sedimentation glass

#### *Preparation and examination*

1. Thoroughly homogenize the stool specimen, either manually or with an electric mixer, 5 gm. is usually a satisfactory amount.

2. Suspend the fecal material in 25 times as much 0.5 per cent glycerine in tap water, and then pour through a wire screen or 4 layers of Curity surgical gauze.

3. Allow the suspension to sediment and decant the supernatant liquid, repeating this process three times.

| | | |
|---|---|---|
| First sedimentation: | 1– 2 | hours |
| Second sedimentation: | 30–45 | minutes |
| Third sedimentation: | 30 | minutes |

4. The final sediment may be sampled with a pipette and placed on a slide for examination.

## E. DILUTION EGG COUNT (STOLL, 1923) FOR COUNTING THE NUMBER OF EGGS IN FECES

This technic is useful when one wishes a quantitative estimate of the helminth infection. It gives the approximate amount of infection and has been effectively used in field studies and hookworm control campaigns. It is also very useful in following the results of anthelmintics. Theoretically, this method should detect the presence of three or four female hookworms and easily detect one female ascarid.

### *Material*

1. Stoll displacement flask * graduated at 56 ml. and 60 ml. with a solid, number 4, rubber stopper
2. Stoll dilution egg count pipette * graduated at .075 ml.
3. Glass beads, 3 mm. diameter
4. Applicator stick
5. Sodium hydroxide solution N/10; 40 gm. NaOH to 1,000 ml. of water
6. Glass microscope slide 1½" x 3"
7. Cover slip 25 mm. square
8. Microscope with mechanical stage

### *Preparation and examination*

Fill the Stoll flask to the 56 ml. mark with N/10 sodium hydroxide solution. Using the applicator stick, place a sufficient amount of the stool specimen in the Stoll flask to raise the level of the fluid to the 60 ml. mark; i.e., add 4 ml. of feces. Care must be used to avoid smearing the neck of the flask above the upper mark. Add 15 to 20 glass beads, stopper tightly and shake vigorously. In shaking, hold

* Stoll flasks and pipettes may be secured from A. H. Thomas and Company, Philadelphia, Pa.

the thumb firmly over the rubber stopper and grasp the neck of the flask in the hand. It is best to prepare the flasks in the afternoon, shake them several times at intervals and allow them to stand overnight. This procedure usually results in complete dissolution of the fecal material. On the following morning before a sample of the material is removed for counting, the flask should be thoroughly shaken for approximately 1 minute and an examination made of the bottom of the flask to be sure no lumps of feces remain unbroken. Some densely formed stools require considerable shaking and standing to break up completely. When the fecal material has completely disintegrated, shake the flask for 1 minute and quickly remove 0.075 ml. of the specimen with the Stoll pipette and place it on the microscope slide, then carefully place a clean cover slip on the preparation.

To count the eggs in the preparation, a 7.5 or 10 power eyepiece and a 16 mm. objective are best. The eggs in the liquid around the edges of the slide should be counted first as this area may dry up before the rest of the slide is examined. The whole cover slip should then be covered systematically, beginning at the upper left hand corner, working back and forth across it in fields the width of the 16 mm. objective field.

To determine the number of eggs per ml. of stool, count the number of eggs in two 0.075 drops, add them together, and multiply by 100. In routine practice this method yields average counts accurate to within 10 per cent of the number of eggs in the fecal specimen. This method, however, is most useful in the study of infections of population groups.

### F. NATIONAL INSTITUTES OF HEALTH (NIH) CELLOPHANE-TIPPED SWAB METHOD OF EXAMINATION FOR ENTEROBIUS VERMICULARIS

Cram, Jones, Reardon, and Nolan (1937), and Brown (1932) have shown that routine stool examination for the eggs of *Enterobius vermicularis* is unsatisfactory as this parasite does not regularly pass eggs into the intestine, and hence they frequently are not present in the stool. Only approximately 10–17 per cent of the persons infected with pinworm give positive results by a single stool examination. The female Enterobius migrates around the perianal area liberating eggs; therefore, the presence of this parasite is best detected by perianal swabbing (Headlee, 1935; Brown, Sheldon, and Thurston, 1940). The studies of Sawitz, Odom, and Lincicome (1939), and others

indicate that a single NIH swab examination will detect approximately 72 per cent of the positives and that 6 examinations are necessary before the patient can be considered negative.

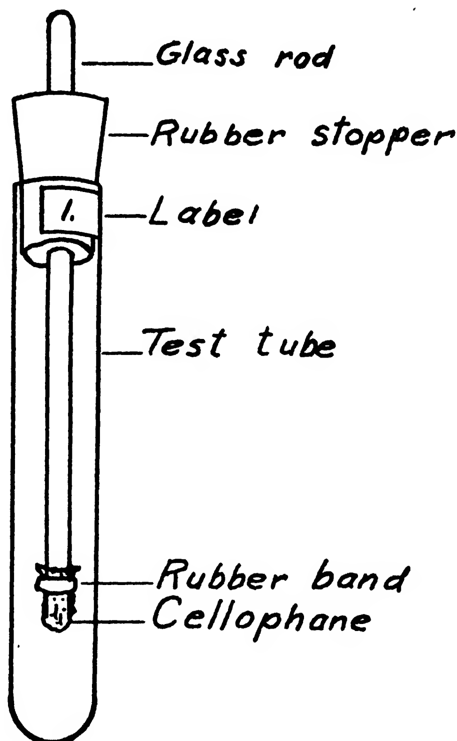



FIGURE 2

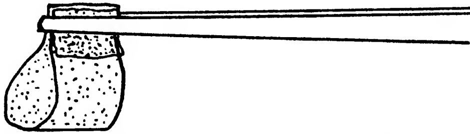
*Material*

1. NIH cellophane swab, Hall (1937), consists of a 20 mm. square of plain transparent non-moisture-proof cellophane wrapped about the tip of a small glass rod and held in place with a rubber band. The swab is inserted in a stopper and placed in a test tube which may be labelled for identification.
2. Glass slide
3. Cover slip
4. N/10 NaOH solution

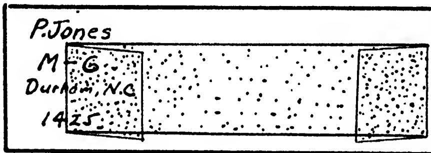
*Preparation and examination*

Collection of specimen: the swab is scraped through the perianal folds and may be transported in the test tube container.

To examine the specimen the rubber band is slipped up the glass rod and the cellophane placed feces side down on a glass slide in a drop of N/10 sodium hydroxide. A drop of the sodium hydroxide solution is placed on the cellophane and a cover slip added. Allow a minute or two for the cellophane to flatten out, and examine under low power of the microscope. After autoclaving, another cellophane tip may be added and the rubber band used again.



In forceps ready for use




Prepared for Examination.

SCOTCH CELLULOSE SWAB

FIGURE 3

## G. SCOTCH CELLULOSE TAPE SWAB FOR THE DIAGNOSIS OF ENTEROBIUS VERMICULARIS, GRAHAM (1940)

*Materials*

1. Piece of Scotch cellulose tape ½" wide and 8 cm. long
2. Forceps
3. Glass microscope slide

*Preparation and examination*

Fold back a centimeter of the piece of Scotch tape at each end, adhesive surfaces together, to form two grips for handling. Hold the

ribbon of cellulose in forceps in a loop, adhesive surface on the outer side of the loop. Pat the loop over the perianal folds in order to pick up ova deposited there. The tape is then placed lengthwise on a microscopic slide, sticky side down, and examined under the microscope without the use of a cover slip. The slides may be shipped in the usual slide containers.

## H. CONCENTRATION METHOD FOR SCHISTOSOMA HAEMATOBIUM EGGS IN THE URINE

### *Material*

1. Sedimentation glass or graduated cylinder
2. Pipette
3. Microscope slide
4. Cover slip

### *Preparation and examination*

Collect as large a specimen of urine as possible, pour into a sedimentation glass or graduate cylinder, and allow to stand for 1 hour. Pipette some of the bottom sediment onto the microscope slide, place a cover slip on it and examine under low power for typical terminal spined eggs. Centrifugation of the urine specimen is also a satisfactory rapid method of concentrating the eggs.

## I. THE EXAMINATION OF BLOOD FOR MICROFILARIAE

The adult filarid worms residing in man pass microfilariae that may be detected in the circulating blood. In general, blood drawn during the daytime is satisfactory; however, the microfilariae, *Wuchereria bancrofti,* one of the commonest and most widespread filarids, exhibits a marked nocturnal periodicity. Therefore, if this parasite is suspected, the blood should be secured from 10 p.m. to 2 a.m.

The microfilariae of *Onchocerca volvulus* are not usually found in the blood but must be sought in serum from an incision made near the adult worm or in a tissue snip teased out in warm saline.

### 1. *Blood smear examination for microfilariae*

### *Material*

Giemsa, methylene blue, or Bullards hematoxylin stain

### *Preparation and examination*

Fix the thin smears with methyl alcohol, but do not fix thick smears as this prevents laking of the red blood cells. Stain the smear with any of the above stains.

The microfilariae can be found under the 16 mm. objective of the microscope, but high power or oil immersion may be necessary to make a species diagnosis which is based on the presence or absence of a sheath and the position of the nuclei in the tail. (See Figure 4).

### 2. *A sensitive method for detecting microfilariae in the blood*

The usual blood smear which involves only a small amount of blood cannot be relied upon to detect light infections or the microfilariae of *Wuchereria bancrofti* in day blood, nor is the blood smear an accurate measure of the absence of microfilariae after therapy. In order to facilitate and increase the sensitivity of a daytime examination of blood for microfilariae, Knott (1939) devised the following technic:

a. Draw 1 ml. of blood from the vein and immediately expel this blood into a centrifuge tube containing 10 ml. of 2 per cent formalin solution.

b. The blood and formalin solution are thoroughly mixed by inverting the tube and shaking it. The solution kills the microfilariae which die in a stretched-out attitude and also lakes the red blood cells.

c. (1) Allow the tube to stand from 12 to 24 hours and the sediment will collect in the tip. *OR*

(2) Centrifuge the material for 5 to 10 minutes throwing the microfilariae and other solid blood constituents to the tip of the tube.

d. Decant the supernatant fluid.

e. With a long capillary pipette draw up the sediment from the bottom of the tube and spread it over a glass slide, covering an area uniformly approximately 2 x 5 cm.

f. Examination.

(1) If one wants an immediate diagnosis, the slide can be examined wet for microfilariae. *OR*

(2) Allow the slide to dry thoroughly and stain.

(a) Loeffler's methylene blue—stain for 2 minutes or more (saturated solution of methylene blue in alcohol 30 ml.; aqueous 1–10,000 solution of potassium hydroxide, 10 ml.), then rinse free of excess stain and dry; it is next counterstained for 1–2 minutes with 0.5 per cent aqueous solution of eosin, rinsed, and dried.

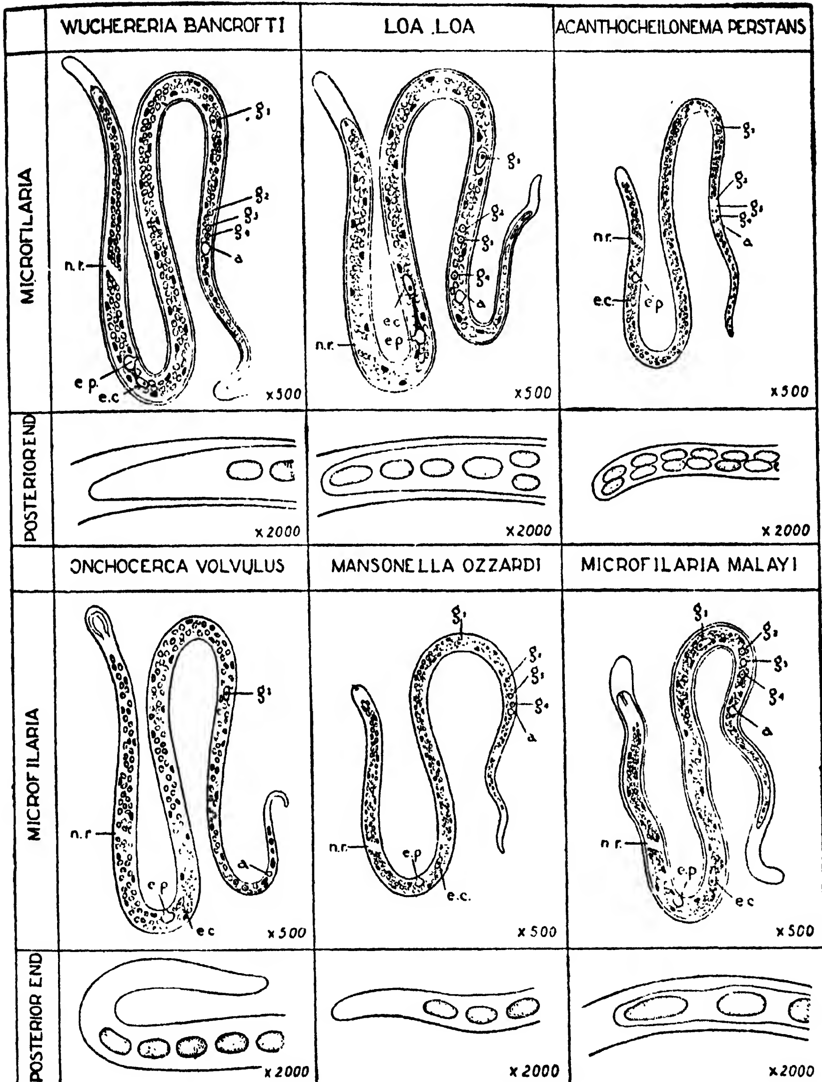



FIGURE 4
MICROFILARIA OF MAN
(Taken from Belding, *Clinical Parasitology*, 1942.)

(b) Giemsa—in our laboratory we have found Giemsa stain very useful. The technic of staining is essentially that used in staining thick smears for malaria parasites. We usually allow the slide to dry overnight and stain with Giemsa for 45 minutes (1 part concentrated Giemsa to 50 parts buffered pH 7.2 water). Destain 10–15 minutes in water, pH approximately 7.2. Allow to dry and examine.

# II. LABORATORY DIAGNOSIS OF INTESTINAL PROTOZOA

## I. INTRODUCTION

The laboratory diagnosis of intestinal protozoan infections, although complicated by the delicate nature of the organisms, especially the trophozoites, can be readily accomplished. The organisms may be few in number and hence careful, continued search must be made of smears or concentration methods utilized.

## II. SPECIES OF PROTOZOA

The intestinal protozoa are small and considerable magnification is necessary to study and identify them. A 10X eyepiece and an oil immersion lens is needed for this purpose. The protozoa may be located on the slide with either the 16 mm. or 4 mm. objective. The intestinal protozoa of man consist of the following species:

### A. AMEBAE

*Endameba histolytica*
*Endameba coli*
*Endolimax nana*
*Iodameba bütschlii*
*Dientameba fragilis*

### B. FLAGELLATA

*Trichomonas hominis*, *T. vaginalis* is found in the vagina
*Giardia lamblia*
*Chilomastix mesnili*
*Embadomonas intestinalis*
*Enteromonas hominis*

### C. SPOROZOA

*Isospora hominis*

### D. CILIATA

*Balantidium coli*

*Endameba histolytica* and *Balantidium coli* are definitely pathogenic, whereas there is some doubt as to the ability of the other intestinal protozoa to invade tissues and produce disease. All of the intestinal protozoa have a trophozoite or motile stage as well as a cystic stage except *Trichomonas hominis* and *Dientameba fragilis* which have only a trophozoite stage. The trophozoite stage of *E. histolytica* is present

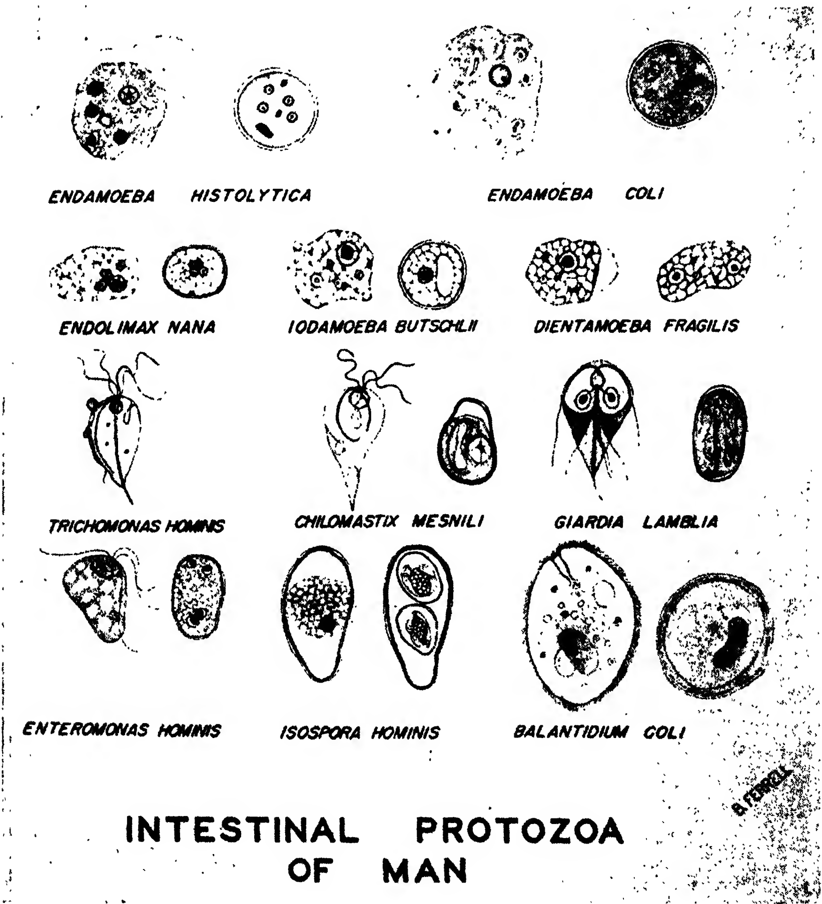



FIGURE 5

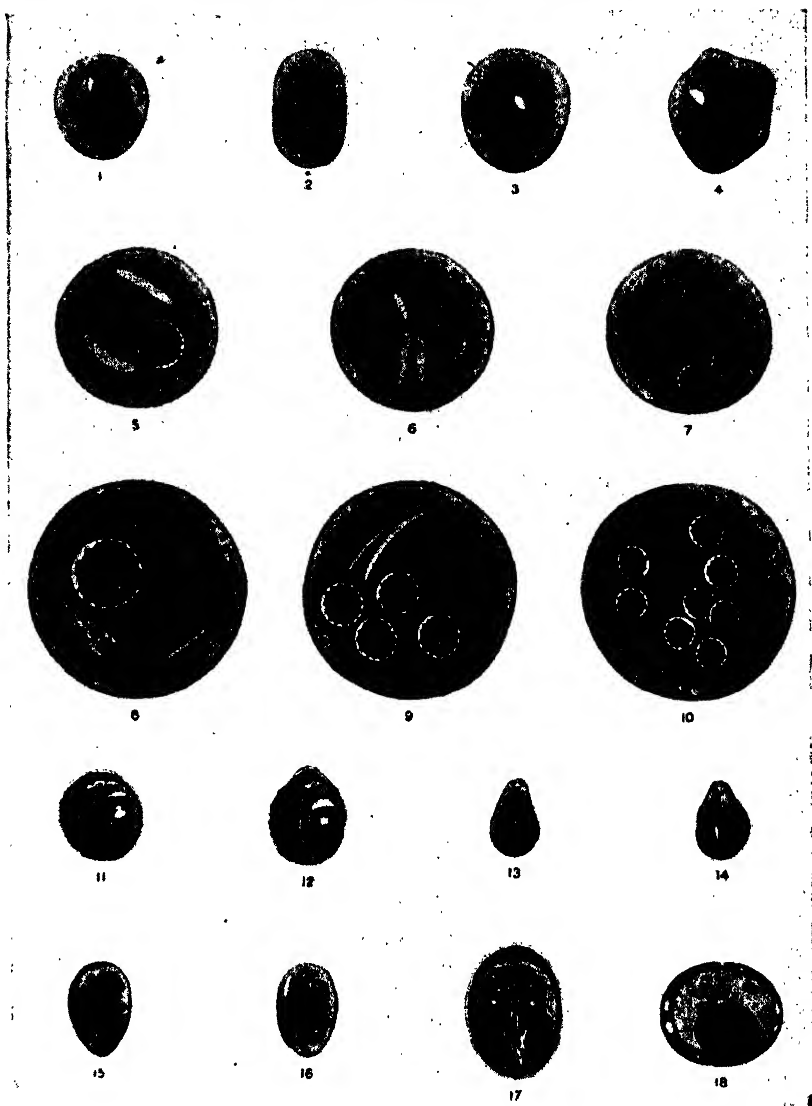



FIGURE 6

CYSTS OF INTESTINAL PROTOZOA TREATED WITH IODINE
(x 2000)

1 and 2, *Endolimax nana;* 3 and 4, *Iodamoeba bütschlii;* 5, 6, and 7, *Endamoeba histolytica;* 8, 9, and 10, *Endamoeba coli;* 11 and 12, *Chilomastix mesnili;* 13 and 14, *Embadomonas intestinalis;* 15 and 16, *Enteromonas hominis;* 17, *Giardia lamblia;* 18, *Blastocystis hominis,* a yeast resembling a protozoan cyst. (Taken from Belding, *Clinical Parasitology,* 1942.)

in the intestine when conditions are favorable for multiplication. The cystic stage is present when conditions in the intestine are presumably unfavorable and the trophozoite becomes motionless, rounds up, secretes a cyst wall, becoming a cyst. The morphology of the various stages of the several species may be sufficiently similar to make identification difficult. It is necessary, therefore, to be able to recognize all species to identify for certain the pathogenic forms (Figures 5 and 6).

The trophozoites of *Endameba histolytica* occur usually only in semi-fluid stools. Cysts will be found in the stools of more solid consistency. Trophozoites quickly lose their motility when cooled to room temperature; it is necessary, therefore, that examination for trophozoites be made upon a freshly passed warm stool, preferably a warm stage. Cysts of the various intestinal protozoa are resistant to external influences, and stools several days old are satisfactory for examination.

## III. REPORTING RESULTS

The species of protozoa and whether it was the trophozoite or cyst form should be reported.

Repeated stool examination of suspected *Endameba histolytica* cases should be made, as individual stools of an infected person may be negative or contain so few protozoa that they are overlooked. It is estimated that approximately one-third of the infections are detected with one stool examination. Six examinations will detect approximately 75 per cent of the infections. Stools passed following a cathartic are most useful in diagnosis. Magath (1934) and Andrews (1934) report that it is possible to detect *Endameba histolytica* in 80–89 per cent of infected individuals with the examination of a single stool following a magnesium sulfate purge. Stools following purgation should be examined immediately.

Material freshly secured from intestinal lesions by means of a proctoscope or a sigmoidoscope is especially useful and certain amebae will be found in a high percentage of such specimens. These specimens should be kept warm and moist during transportation to the laboratory.

Careful investigations indicate that the zinc sulfate flotation is the method of choice for the diagnosis of protozoan cysts. This method may be supplemented by direct examination of hematoxylin stained fecal smears for trophozoites. Examination of unstained fresh fecal smears for active trophozoites is also useful in the hands of an experienced worker.

# IV. DIAGNOSTIC METHODS FOR PROTOZOA

## A. FECAL SMEAR

### *Material*

1. Toothpick
2. Microscope slide
3. Cover slip
4. Physiological saline solution
5. D'Antoni's iodine solution

### *Preparation and examination*

1. *Examination for trophozoites*

A piece of freshly passed warm stool the size of a rice grain or material secured from the intestinal lesion by sigmoidoscopy should be thoroughly mixed with warm physiological saline solution with a toothpick on a warm slide and covered with a clean cover slip. The smear should be thin enough so that ordinary print can readily be seen through it.

Examine film under low or high dry power of the microscope. The oil immersion lens usually will be necessary for identification of the species.

2. *Examination for cysts*

Make smear as above except stool, saline, and slide need not be warm. Add one drop of D'Antoni's iodine solution to smear to stain the cysts. The cysts can usually be located under low power, but the high dry objective or the oil immersion lens is necessary for identification.

## B. CONCENTRATION TECHNICS FOR THE DETECTION OF CYSTS

Faust, et al. (1938) originally described the zinc sulfate centrifugal flotation method. This method is useful in detecting helminth eggs as well as protozoan cysts. Protozoan trophozoites are so greatly distorted by the zinc sulfate as to be unrecognizable, hence this technic is of absolutely no value in the detection of this stage. For routine laboratory work, this technic is the method of choice for the diagnosis of most-helminth and intestinal protozoa infections. Otto, Hewitt, and Strahan (1941), however, have greatly simplified the zinc sulfate technic without significant loss in accuracy.

1. *Zinc sulfate centrifugal flotation method of Faust, et al.*

*Material*

1. Zinc sulfate solution: 331 gm. zinc sulfate ($ZnSO_4 \cdot 7H_2O$) added to sufficient distilled water to make 1 liter of solution. Adjust to specific gravity of 1.18 by addition of water or the salt if necessary.
2. D'Antoni's standardized iodine stain. This stain is made by placing 100 gm. of potassium iodide (Merck's or Baker's c.p.) in a 1,000 ml. volumetric flask and adding distilled water up to the mark.

Two 25 ml. clean and absolutely dry volumetric flasks are balanced against each other on an analytical balance. Into one flask 25 ml. of the 10 per cent aqueous potassium iodide solution to be standardized is added, and the weight to the fourth decimal place recorded. For example:

| | |
|---|---|
| Theoretical weight of 25 ml. of 10 per cent potassium iodide solution | 26.9250 gm. |
| Actual weight of 25 ml. of 10 per cent potassium iodide solution | 26.7250 gm. |
| Difference in weight of solutions | 0.2000 gm. |

The difference in weight of solutions divided by the theoretical weight of 25 ml. of 10 per cent potassium iodide solution will give 0.007427, or 0.7427 per cent correction for actual percentage.

10 per cent minus 0.7427 per cent leaves 9.2573 per cent (actual percentage of potassium iodide solution).

100 gm. dissolved in 1,000 ml. gives a 9.2573 solution instead of the 10 per cent solution desired. Therefore, 100:9.2573 per cent = X:10 per cent.

X = 108.0229 gm. of potassium iodide of the particular sample used required to make a 10 per cent solution.

108.0229 minus 100 leaves 8.0229 grams to be added.

This amount of potassium iodide is added and 25 ml. of the solution weighed as before in dry volumetric flasks to determine the actual percentage solution. Results:

| | |
|---|---|
| Theoretical weight of 25 ml. of 10 per cent potassium iodide solution | 26.9250 gm. |
| Actual weight of 25 ml. of 10 per cent potassium iodide solution | 26.9248 gm. |
| Difference in weight of solutions | 0.0002 gm. |

The difference in weight of solutions divided by the theoretical weight of 25 ml. of 10 per cent potassium iodide will give 0.0000074, or 0.00074 per cent correction.

10.0000 per cent potassium iodide
0.0007 per cent correction

9.9993 actual percentage of potassium iodide

This stock solution of potassium iodide should be tightly stoppered and kept in the dark.

To prepare the iodine stain for use, add 10 ml. of the stock solution to 90 ml. of distilled water. Add 1.5 gm. of powdered iodine to this solution and allow to stand for 4 days. Filter the solution and keep in dark, tightly stoppered bottles for periods of not over 4 weeks. The solution should always be filtered before use and must not be allowed to remain unstoppered, as volatilization of the iodine will occur.

3. Wassermann tube
4. Glass slide 1" x 3" and cover slip
5. Applicator stick
6. Cheesecloth or surgical gauze
7. Small glass funnel
8. Bacteriological loop

*Preparation and examination*

a. Prepare a fecal suspension by comminuting one part of stool (the size of a marble) in ten parts of lukewarm tap water.
b. Strain approximately 10 ml. of the suspension through one layer of wet cheese-cloth, held in a small glass funnel, into a Wassermann tube.
c. Centrifuge the suspension in the Wassermann tube for 1 minute at 2,640 r.p.m. or top speed of the International clinical centrifuge. The supernatant fluid is poured off and 2 to 3 ml. of tap water added, and the sediment completely broken up by shaking the tube or with an applicator, and additional water is added to fill up the tube. Repeat this two or three times until the supernatant fluid is clear.
d. When the last supernatant is poured off, add 3–4 ml. of the zinc sulfate solution of 1.180 specific gravity. Break up the packed sediment and add enough zinc sulfate solution to fill the tube to within ½" of the rim.
e. Centrifuge for 60 seconds at top speed.
f. Using a bacteriological loop, remove several loopfuls of the diagnostic

material floating on the surface onto a clean slide. Add one drop of D'Antoni's iodine stain, agitating the preparation to insure a thorough mixing.

g. Mount preparation with a cover glass and examine under the microscope.

### 2. Zinc sulfate levitation method, Otto, et al. (1941)

*Material*

1. Zinc sulfate solution, specific gravity 1.180
2. D'Antoni's standardized iodine stain
3. Levitation tube: glass shell vial 5 cm. deep, 1.8 cm. in diameter
4. Glass slide, 1½" x 3"
5. Cover slip ⅞" square
6. Toothpick or applicator
7. Medicine dropper

*Preparation and examination*

a. Thoroughly comminute a portion of the fecal specimen the size of a kidney bean with the zinc sulfate solution in the shell vial.

b. Add zinc sulfate solution with a medicine dropper until the meniscus is rounded over the edge of the tubes. Place a cover slip on the surface of the liquid and allow to stand 1 hour.

c. Remove cover slip and place specimen side down on glass microscope slide on which one drop of D'Antoni's iodine solution had previously been placed.

d. Examine under microscope.

## C. Staining method for examination of intestinal protozoa

Well stained fecal smears are most useful in the accurate diagnosis of intestinal protozoa. Many different stains have been proposed, and all of them require considerable practice before good preparation will be obtained. The most important factor in preparing a good stained preparation is proper fixation of the specimen which must be done before the specimen is allowed to dry on the microscope slide. All definite structures necessary for differential diagnosis are quickly destroyed by drying. For the preparation of stained specimens of trophozoites, or motile forms, it is essential that the material be freshly passed, as old or refrigerated specimens are unsatisfactory for this purpose. Cysts of the various protozoa are suitable for staining even though the stool is several days old if it has not dried out.

### *Material*

1. Microscope slide and cover slip
2. Toothpick
3. Schaudinn's fixing solution: Prepare 200 ml. of a saturated solution of mercuric chloride in distilled water. Add to the above solution 100 ml. of 95 per cent or absolute alcohol. This solution will keep indefinitely. Add 5 per cent glacial acetic acid just before using.
4. Four per cent iron alum solution: Two gm. iron alum (sulfate of iron and ammonium) in 50 ml. of distilled water. Use only violet crystals in making this solution.
5. Hematoxylin solution, 0.5 per cent in distilled water. The staining qualities are enhanced by ripening the stain for several weeks in a flask plugged with cotton placed in a warm place.
6. Alcohol, 70 per cent to which sufficient iodine has been added to give it a port wine color.
7. Alcohol, 50, 70, 80, 90, and 100 per cent
8. Xylol
9. Balsam or clarite

## D. PREPARATION OF SMEARS FOR STAINING

Smears of fecal material for staining may be prepared either upon cover glasses or microscope slides. An emulsion of feces and saline should be prepared and the material smeared with a toothpick and care taken so that the resulting preparation is not too thick or too thin. This can be learned only through experience.

The smears must be placed immediately into Schaudinn's fixing solution which has been heated to 60° C. (140° F.). The smears should be left in the fixing solution for 5 minutes, which is sufficient to fix the protozoa present. On removal from the fixing solution, the smears should be immersed in 50 per cent alcohol to rinse off the fixative. They should then be placed for 10–15 minutes in the 70 per cent alcohol to which sufficient iodine was added to give it a port wine color. This solution removes the mercuric chloride from the specimens which would seriously interfere with staining. *At no time during the process of fixation or staining should the smears be allowed to dry* or they will be worthless.

## E. STAINING PROTOZOA WITH IRON HEMATOXYLIN

1. Take smears from 70 per cent alcohol and immerse in 50 per cent alcohol for 2 minutes.

2. Wash smears in slowly running water for 2 minutes or two changes in distilled water.

3. Immerse smears in 4 per cent aqueous iron-alum solution 2 minutes at 40° C. (104° F.) or 12–24 hours at 20° C.

4. Wash smears in gently running water for 2 minutes or two changes in distilled water.

5. Stain in 0.5 aqueous hematoxylin (Grubler's or National Anilin) for 2 to 5 minutes.

6. Wash in gently running water for 2 minutes or two changes in distilled water.

7. Differentiate in cold aqueous 1 per cent iron-alum solution. Follow this process under the microscope. The destaining process is the most critical step in the entire procedure and will vary somewhat with the species of protozoa and the fecal specimen. Trophozoites require less destaining than cysts; however, only experience will determine the optimum for the various species and stages.

8. Wash in gently running water for from 10 to 15 minutes until smear appears blue.

9. Immerse smears for 2 minutes each in 70, 80, 90 and 100 per cent alcohol.

10. Clear the smears with xylol.

11. Mount smears in balsam or clarite, using a clean cover slip.

12. At no time during these various steps in the staining process should the preparations be allowed to dry.

13. Locate protozoa under low or high power and study under oil immersion.

### F. CULTIVATION OF ENDAMEBA HISTOLYTICA

St. Johns (1926), Tonney, Hoeft, and Spector (1933), Svensson and Linders (1934) have all reported that cultural methods of diagnosis greatly increase the percentage of positive finding of *Endameba histolytica.* It is suggested, therefore, that if repeated, direct smear examinations are negative for trophozoites, and if the zinc sulfate flotation is negative for cysts that cultures of the stool may be made, especially from patients with a history suggestive of amebiasis. These cultures should be examined at 24 hour intervals for several days.

The fecal specimen to be cultured should be placed in the medium as soon as possible. Exposure to cold kills or attenuates the trophozoites so they will not reproduce in culture.

A number of culture media have been devised in which *E. histolytica* will multiply. St. Johns' (1932) medium (C. M. No. 59) is one of the simpler media, yet one of the best for the growth of the organisms and diagnostic purposes. The other intestinal amebae, intestinal flagellates, and *Blastocystis hominis* grow poorly or not at all in medium.

### *Material*

1. Standard bacteriological test tubes
2. Cotton
3. Glass slide
4. Cover slip
5. Bacto Beef Heart Dehydrated. (Digestive Ferments Company, Detroit, Mich.)
6. Ralston's whole wheat flour
7. Filter paper
8. Funnel
9. 1 ml. pipette with large bore
10. Modified Locke's solution
    Sodium chloride—8.0 gm.
    Calcium chloride—0.2 gm.
    Potassium chloride—0.2 gm.
    Magnesium chloride—0.1 gm.
    Sodium phosphate ($NaH_2PO_4$)—0.1 gm.
    Sodium bicarbonate—0.4 gm.
    Distilled water—1,000.0 ml.

### *Preparation of medium*

Boil for 1 hour in a double boiler 1 gm. of Bacto Beef Heart Dehydrated in 1,000 ml. of the modified Locke solution. The extract is filtered through filter paper and tubed in 5 to 10 ml. amount and autoclaved at 15 lbs. pressure for 30 minutes. Just before the tubes are inoculated, add 0.05 ml. of Ralston's whole wheat flour, using a clean sterile pipette, to the contents of each tube, and thoroughly mix with the medium by shaking. The flour should be sterilized before using by wrapping it in tinfoil, autoclaving it at 15 lbs. pressure for 30 minutes. It should then be dried overnight in an oven at 55° C., and finally heated in a hot air oven at 80° C. for 30 minutes. The reaction of the completed medium does not need adjusting.

### *Technic of cultivation*

A piece of feces the size of a pea is thoroughly mixed with the fluid medium. If the feces are fluid or semiformed, 0.5 ml. of this material should be introduced into the medium with a pipette. The tubes should be incubated at 37° C. and examined microscopically at the end of 24 and 48 hours. To maintain *E. histolytica* in cultures it is necessary to make transfers to fresh media every four or five days. In making transfers, it is necessary to observe aseptic precautions as it has been found that the introduction of new bacteria into the culture medium may result in the death of the amebae.

### *Examination of culture*

Using a pipette, secure a large drop of material from the bottom of the test tube, place on a warm glass slide, and cover with clean cover slip. The ameba may be located on the slide with the low power of the microscope and studied under the high dry or oil immersion lens. At least six preparations from the culture should be fully and carefully examined before it should be considered negative. Three of these preparations should come from the 24 hour culture, and three additional ones made on the culture after 48 hours if the 24 hour examinations are negative. Two to four amebae per microscopic field from the 24 hour culture represent a good growth, although there will, of course, be many fields without amebae.

The morphology of *Endameba histolytica* in culture is essentially the same as in the stools. Cultured specimens may be a little larger than amebae found in stools and the food particles within them will be bacteria and flour particles rather than red blood cells. The amebae from the culture exhibit sluggish movement, and they tend to drag detritus along with them. The cysts of *E. histolytica* are usually not present in cultures kept at 37° C., but if their temperature is allowed to drop to room temperature, the trophozoites will encyst. Cysts containing more than four nuclei are not infrequently observed in cultures.

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# Trichinosis

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I. Introduction—Description of the Disease
II. Laboratory Diagnosis
  A. Eosinophilia
  B. Detection of the Etiological Agent
    1. In stools
    2. In blood
    3. In spinal fluid
    4. In muscle biopsy tissue
    5. In meat and autopsy material
  C. Biological Reactions
    1. Precipitin test
    2. Intradermal test
III. Example of the Application of Diagnostic Methods
IV. References

## I. INTRODUCTION

TRICHINOSIS is essentially a systemic disease due to infection with *Trichinella spiralis*. Infection occurs when meat containing encysted larval forms of the organism is ingested in the raw or undercooked state. Human infections are almost always derived from pork products. The larvae mature rapidly in the small intestine and the females, after fertilization, imbed themselves in the mucosa and give off larvae which enter the systemic circulation and are thus disseminated throughout the body. They are able to survive only in striated muscle, but they set up inflammatory reactions not only in the muscle but also at times in the heart and central nervous system.

In a classical, fairly severe case, gastroenteritis occurs during the first week after infection, preceding the dissemination of the larvae. It is due to the irritation set up by the adult parasites. It may continue into the stage of generalization. As the larvae begin to enter the circulation, and lodge in the tissues, there is usually edema of the

periorbital tissues and face and sometimes of other parts of the body, pain in the skeletal muscles, and fever. The systemic phase lasts one to several weeks.

Most of the cases now seen in the United States are not severe, and the initial gastroenteritis is absent in the majority. Some cases have only slight fever and muscle pain, or fever and edema of the eyes without muscle pain.

## II. LABORATORY DIAGNOSIS

During the phase of gastroenteritis, laboratory diagnosis is rarely possible. The demonstration of eosinophilia is the most practical preliminary laboratory test for establishing the diagnosis. It must usually be supplemented by other more definite tests in suspected cases. However, if a group of individuals are ill with symptoms consistent with trichinosis and eosinophilia is present in all, the diagnosis may be considered established beyond reasonable doubt. This is of importance to the epidemiologist since it is the confirmatory test which is most consistently positive and positive early.

### A. EOSINOPHILIA

Eosinophilia is often absent during the first week after infection, when the gastroenteric symptoms occur. It usually appears within a very few days of the onset of generalization, that is, of the periorbital edema. It rarely fails to appear within the first week of generalization. Intercurrent infection, like pneumonia, may cause disappearance of the eosinophiles, and patients in extremis may also lose their eosinophilia. Otherwise it is a remarkably constant phenomenon, and the diagnosis should be seriously doubted in any case which fails to manifest it by the end of 2 weeks after the date of infection. Eosinophilia rises to a peak in the 3rd and 4th week and then declines, but it may still be present in some degree 8 months later. Eosinophile percentages of 40 per cent are not unusual and they may be as high as 85 per cent.

The total leucocyte count is not elevated in the milder cases. In severe cases an increase in the white blood cells is the rule, usually between 10,000 and 20,000 per cu. mm. It is desirable to determine the total white blood count as well as the differential so that the absolute number of eosinophiles per cu. mm. can be found. A total of 500 eosinophiles per cu. mm. or more is generally accepted as evidence of eosinophilia. When it is not feasible to obtain more than

a blood smear, one is justified in assuming that the total leucocytes are at least 5,000. Therefore, the presence of 10 per cent or more eosinophiles may be considered definitive but results between 5 and 10 per cent do not necessarily prove eosinophilia.

## B. Detection of the Etiological Agent

As in all infectious diseases, diagnosis is most satisfactorily established when the etiologic agent is demonstrated. Trichinella larvae have been demonstrated in muscle tissue, blood, spinal fluid, milk, pus, pleural and ascitic fluid. Trichinella adults have rarely been demonstrated in the stools.

For diagnosis in trichinosis, most clinicians have become conditioned to muscle biopsy. However, in order to get the best results, one must wait for 2 to 3 weeks from the onset of the illness in order that the bulk of the larvae may have reached the muscles.

### *Larvae in Stools*

From the standpoint of early diagnosis, the first place in which organisms might be demonstrated is in the stools. This has sometimes been done by sedimenting the stool after suspension in water and examining the sediment after repeated washing. This is a tedious and difficult procedure and very likely to give negative results.

### *Larvae in Blood*

The vast majority of cases are seen during the phase of generalization, and demonstration of the larvae in the circulating blood may be possible. This technique has been somewhat neglected, but it is simple and should be tried, at least in fairly severe cases in which there is a reasonable chance of success in the first 2 weeks following the appearance of edema.

#### *Method*

1. Add 5 ml. of blood to 50 ml. of 3 per cent acetic acid (or tenth normal hydrochloric acid) in a centrifuge tube.
2. Allow to stand 10 minutes.
3. Centrifuge at 2,000 revolutions per minute for 1 minute.
4. Spread the sediment on a slide.
5. Fix with methyl alcohol.
6. Stain with Giemsa stain, diluted 1:40 for ½ hour.
7. Study under low power of microscope for larvae.

### *Larvae in the Spinal Fluid*

Stiff neck may be present in trichinosis owing either to inflammation of the cervical muscles or to actual meningeal irritation. In such cases spinal fluid will often be obtained for diagnosis. It is sometimes possible to demonstrate larvae in the fluid by centrifuging it at 2,000 r.p.m. for 3 minutes and observing the sediment. Usually the spinal fluid is otherwise normal.

### *Larvae in Muscle Biopsy Tissue*

Muscle biopsy has several disadvantages. As already stated, it must be deferred until after 3 or 4 weeks from the onset of the illness for optimum results. It is an operative procedure and as such is likely to be resisted by the patient, especially since he is often well by the time it can be proposed and thus has nothing to gain. It is difficult to carry out unless the patient is hospitalized. It is far from 100 per cent efficient as a diagnostic procedure, at least as generally performed. In 61 cases derived from several outbreaks, it was positive in only 39.[1]

If muscle biopsy is performed, provision should be made for examination of the tissue by compression and digestion methods as well as histological section. If only the latter is used, larvae may be missed owing to the small volume of tissue examined. Without compression and digestion almost 50 per cent of cases may give negative results.

### *Method*

A piece of muscle one inch long is obtained, usually from the gastrocnemius muscle. A small portion is fixed for histologic section and the remainder sent in physiological saline to the laboratory. This portion is compressed between two glass plates by means of clamps and examined under the low power of the ordinary or dissecting microscope for the presence of the coiled larvae. Gould [2] has excellent illustrations of the apparatus needed. If this examination is negative or the apparatus is not available, digest the tissue as follows:

1. Cut the muscle into small pieces and place in a Petri dish with 20 ml. of digestion fluid of the same composition as described below for the examination of meat.
2. Place in incubator at 37° C. and allow time for complete dissolution, usually 2 or 3 hours.
3. Filter through one layer of gauze, 20 or 22 mesh, into one or two centrifuge tubes.
4. Centrifuge for 5 minutes at 2,500 r.p.m. and pipette off all but 2 ml. of sediment.
5. Examine this with the low power of the microscope for larvae.

### *Larvae in Meat and Autopsy Material*

The demonstration of larvae in meat specimens is of interest not only to the sanitarian but also to the epidemiologist and to the clinician in those cases in which specimens of the meat consumed can be obtained. Demonstration of larvae in autopsy material is of interest in medicolegal and survey work.

#### *Method*

1. Remove as much fat and connective tissue as possible from the muscle tissue. Pass the muscle through a grinder which will divide it finely and weigh out about 50 gm.
2. Prepare a 2 liter funnel on a stand. Attach a length of rubber tubing with a pinch cock to the outlet. Attach a short length of glass tubing to the rubber. Place a circular porcelain plate with large perforations in the funnel. Then line the funnel with two layers of gauze, 20 or 22 mesh.
3. Prepare digestion fluid as follows and pour it into the funnel:

| | |
|---|---|
| Pepsin (spongy granular) | 5 gm. |
| Hydrochloric acid, sp. gr. 1.19 | 7 ml. |
| Tap water | 1,000 ml. |

   Dissolve the pepsin in the water and then add the HCl.
4. Spread the chopped muscle on the gauze. Place the whole apparatus in the incubator at 37° for 18 to 24 hours. The larvae gravitate to the bottom of the funnel as they are digested free.
5. Draw off 15 ml. of the sediment into a centrifuge tube and a second 15 ml. into another. Most of the larvae will be in the first.
6. Centrifuge for 5 minutes at 2,000 r.p.m. and draw off all but 2 ml. of fluid from each tube with a pipette.
7. Examine all remaining material drop by drop under the low power of the ordinary dissecting microscope.

## C. BIOLOGICAL REACTIONS

For technical or practical reasons, it is often not possible to demonstrate the causative organism in cases of suspected trichinosis. In such cases, serological and intradermal tests are valuable in substantiating the diagnosis. Both precipitin and complement-fixation reactions can be obtained. The latter has not come into very general use. The writer has no experience with it. Those interested should consult the publication of Witebsky and his associates.[3]

Positive precipitin and intradermal reactions may often be obtained before the appropriate time for muscle biopsy has been reached. Furthermore, the patient may refuse to permit biopsy. For those reasons as well as the fact that muscle biopsy sometimes fails in positive cases, it is the writer's opinion that precipitin and intradermal tests should be carried out in all suspected cases.

### *Precipitin Test*

The precipitin test for trichinosis has the advantage of simplicity from the standpoint of the clinician, since it involves only the drawing of 5 ml. of blood, which is a familiar procedure. Its great disadvantage is the frequency of false positive reactions. These have been observed in a wide variety of clinical conditions including Hodgkin's disease, leukemia, and periarteritis nodosa. In the experience of the writer, the precipitin reaction tends to become positive as a general rule before the intradermal, most usually in the second week of illness.

In view of the high incidence of subclinical trichinosis in the general population demonstrated by autopsy studies, there is always the possibility that a positive reaction may be related to an old trichinosis and not the illness under study. If a negative test is obtained early in the illness and a positive result subsequently, it has much greater diagnostic value. A precipitin test is not considered positive unless the reaction occurs with a dilution of 1:320 at least of the antigen.

### *Method*

Five ml. of blood are drawn from the patient in the fasting state. If the patient is not fasting, the serum may be turbid and cannot be used.

Antigen dilutions of 1:80, 1:160, 1:320, 1:640 and 1:1,280 are prepared.

In each of six narrow precipitation tubes, 0.05 ml. of the patient's serum is placed. The same amount of each of the antigen dilutions is layered over the serum in the first five tubes with a fine capillary pipette. To the sixth tube, 0.05 ml. of physiological saline is added.

The tubes are observed for the formation of a bluish white ring of precipitate. In positive cases this may occur as early as 5 minutes; the final readings are made at 2 hours. The highest dilution in which there is a ring is recorded. The saline control should be negative.

### *Preparation of Antigen*

The following is a modification of the method of Bozicevich.[4]

1. Rats are infected by feeding chopped meat from other infected animals sprinkled with cheese. Alternatively, isolated larvae may be fed to the rats by pipette. An attempt is made to feed approximately 600 larvae to each rat.
2. The rats are killed by any method desired at the end of 6 weeks. If they die from the infection between 3 and 6 weeks they may be used.
3. The diaphragms are inspected to prove the presence of heavy trichinella infection.

4. The rats are skinned and eviscerated, care being taken to remove stomach, liver, kidneys, heart, and lungs. The remainder of the carcass, including the head, legs, and tail is put through a meat grinder.
5. Seventy gm. of the ground tissues is digested in the same way as described for the detection of larvae in meat with the following changes:
   a. A 4 liter funnel and 3 liters of the digestion fluid are used.
   b. A centrifuge tube is attached to the stem of the funnel by means of a short piece of rubber tubing. The pinchcock is left open so that the larvae collect in the centrifuge tube. The pinchcock is closed at the time the tube is removed when digestion is completed.
6. The supernatant fluid is pipetted off. The tube is filled with 0.5 per cent soda bicarbonate solution to neutralize the acid. The larvae are allowed to settle for 1 hour and then washed in a similar manner five times with physiological saline.
7. The settled larvae are pipetted into a small agate mortar. With a fine hand lens all debris, cotton threads or fluff, rat protein (brown particles) are suctioned off using a very fine bore pipette. The excess of saline is then removed. At no stage are the larvae centrifuged.
8. The mortar is put on the tray of a desiccator underneath which are four halves of Petri dishes filled at the last minute with phosphoric anhydride (pentoxide). The desiccator is sealed with stopcock grease and attached to a hyvac pump. In about 20 minutes the layer of larvae has dried and curled at the edges to a thin disc. Disconnect the hyvac pump after shutting off the valve of the desiccator. Put the desiccator in an icebox for further drying.
9. The powder is stored in sealed tubes. For use in the precipitin test it is made up into a stock solution of 1:20. This is centrifuged to clear it and placed in sealed tubes. These tubes are placed in the water bath for 2 hours at 56° C. on each of two successive days. The antigen is then ready for the preparation of the standard dilutions.

### *Intradermal Test*

The intradermal test has the advantage of being more specific than the precipitin test, but it requires more time and attention on the part of the physician or epidemiologist and becomes positive usually later than the precipitin test. It is usually positive in the third week of illness, sometimes in the second, and only rarely in the first. A positive reaction may persist several years. Consequently, as with the precipitin test, a single positive result may not be related to the illness under study but to a pre-existing infection. Therefore, it is more significant if a negative result has been obtained early in the illness.

Positive reactions may occur in the presence of other helminth infections, especially filariasis and strongyloidiasis. A few cases of trichinosis fail to manifest a positive skin reaction at any time.

*Method*

Antigen for intradermal tests is available commercially in a 1:10,000 dilution.

One forearm is cleaned with alcohol. With intradermal needles, injections of 0.1 ml. each of antigen and control are made about 2 inches apart. (A record should be kept of the arm used so that a different skin area may be used if the test is repeated.)

Readings are made after 10 and 20 minutes. If the test is positive, there will be a distinct wheal, larger than the injection wheal, and surrounding erythema, at the site of the antigen injection. Pseudopods are frequently not present.

If the result is negative, another reading should be made at 18 to 24 hours. In some cases, usually relatively early in the illness, there will be an area of solid erythema (in contrast to the mottled erythema about the wheal), slightly infiltrated and sharply demarcated. This is a delayed positive reaction and has the same significance as an immediate positive.

## III. EXAMPLE OF THE APPLICATION OF DIAGNOSTIC METHODS

The practical application of the diagnostic tests for trichinosis in an average case may be illustrated as follows:

A patient is seen after 5 days of illness beginning with headache and periorbital edema and associated with high fever. The edema has subsided by the time he is seen. A blood count is ordered. This shows 10,000 white blood cells per cu. mm. with 15 per cent eosinophiles. With the patient in the fasting state, 10 ml. of blood are drawn. Five ml. are tested for the presence of larvae and the remainder used to obtain serum for the precipitin test. An intradermal test is carried out. The precipitin and intradermal reactions will probably be negative. If larvae are found in the blood the diagnosis is made. If that test is also negative on one or more trials, the intradermal and precipitin tests are repeated after 1 week or 10 days. If they are still negative or doubtful, muscle biopsy is performed.

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# Pathogenic Fungi

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## I. INTRODUCTION

THE identification of pathogenic fungi is based on the morphology of the organisms as they are seen both in clinical materials and in culture. Many of the fungi, particularly those causing systemic infections, are dimorphic in that the morphology of the tissue or parasitic phase of the fungus is quite different from the morphology of the cultural or saprophytic phase of the fungus. Where these differences are great, descriptions of both forms will be given to facilitate an identification.

Biological methods of identification, e.g., fermentations, etc., are of value only in the separation of species in the genus *Candida (Monilia)*. Such methods do not lend themselves to other fungi. Differential staining, e.g., Gram's method, is of little value as all fungi are Gram-positive. An occasional organism, however, may be separated from closely related forms by being acid-fast, e.g., species of *Nocardia* (aerobic actinomycetes).

The nomenclature used is that found in the *Manual of Clinical Mycology.*[1] To a great extent, this nomenclature has been widely adopted with but few disagreements that await changes acceptable to the majority of those working in the field of medical mycology. The terminology used in mycology, however, is no more difficult than that found in the fields of parasitology and bacteriology, and familiarization with the names employed quickly dispels all mystery and confusion usually applied to the human pathogenic fungi. The following books should be consulted for detailed information:

Conant, N. F., Martin, D. S., Smith, D. T., Baker, R. D., and Callaway, J. L. *Manual of Clinical Mycology*. Philadelphia: Saunders, 1944.

Henrici, A. T. (Skinner, C. E., Emmons, C. W., and Tsuchiya, H. M.) *Molds, Yeasts and Actinomyces*. New York: Wiley, 2nd ed., 1947.

Lewis, G. M., and Hopper, M. E. *An Introduction to Medical Mycology*. Chicago: The Year Book Publishers, Inc., 3rd ed., 1948.

Swartz, J. H. *Elements of Medical Mycology*. 2nd ed. New York: Grune and Stratton, 1949.

## II. MATERIALS NEEDED FOR MYCOLOGY

The material needs of a mycological laboratory for the study and identification of fungi are very simple. In many cases, the usual bacteriological equipment found in most laboratories is sufficient. An inoculating needle holder with interchangeable wire loop and straight wire, the latter slightly bent at the end, serves for all culture transfers and the obtaining of material from cultures for microscopic examination. Two dissecting needles are needed for manipulating fragments of filamentous cultures for the purpose of untangling and spreading out the filaments in a mounting medium for better microscopic examination.

Lactophenol-cotton blue is a universal mounting medium for most fungi. A dropping bottle of this material should be available at all times as well as a dropping bottle of 10 per cent potassium hydroxide which is used for clearing clinical materials such as skin, hair, nails, and sputum. India ink should be available also for the demonstration of budding cells with capsules in yeast-like cultures, spinal fluid, ventricular fluid, sputum, or pus.

The usual bacteriological stains and blood stains are occasionally used. The Gram's stain, acid-fast stain and Wright's, Wilson's or Geimsa stains should be available for staining pus, sputum, spinal fluid, or blood films.

## III. FUNGI CAUSING INFECTION OF HAIR

### 1. COLLECTION OF SPECIMENS

A. In suspected *Tinea capitis* or *Tinea barbae* the hairs of the scalp or face are best examined in a dark room under a Wood's light. A peculiar greenish fluorescence is produced by most of the infected hairs. This allows a better selection of material for examination and culture. Fluorescent hairs should be epilated with forceps, placed between two flamed microscopic slides and the slides wrapped in paper for transport to the laboratory. The patient's name, history number, location from which hair was selected, and date of collection may be written on the package. A number of specimens may be collected in this way from different patients seen in the clinic.

B. In suspected *Piedra,* hairs of the scalp and beard should be inspected under ordinary light and in *Trichomycosis,* hairs of the axillary and pubic regions also should be inspected under ordinary light. Hairs showing nodules adherent to the shaft should be cut off with scissors,

placed between flamed microscopic slides, wrapped in paper, and carried back to the laboratory.

### 2. Examination of Infected Hairs

The Wood's light may be used for selecting infected fluorescent hairs for examination. Place fragments of hair in a drop of 10 per cent potassium hydroxide on a microscopic slide. Place cover glass over the preparation and gently heat the slide over a low flame of a Bunsen burner. Repeated gentle heating will drive out air bubbles and clear the specimen for microscopic examination. Examine all such preparations with the low and high dry objectives.

### 3. Key to Fungi by Direct Examination of Hair

A. No nodules formed on hair; fungus confined to bulb of hair and a short distance up the hair shaft.

I. Fungus outside of hair shaft.

1. Sheath surrounding base of hair formed of mosaic of spores 2 $\mu$ in diameter—Plate I, a.
*Microsporum* (species)

2. Sheath surrounding base of hair formed of parallel rows of spores 3–4 $\mu$ in diameter—Plate I, b.
*Trichophyton* (ectothrix, small-spored)

3. Sheath surrounding base of hair formed of parallel rows of spores 4–6 $\mu$ in diameter—Plate I, c.
*Trichophyton* (ectothrix, large-spored)

II. Fungus inside of hair shaft.

1. Fungus inside of hair shaft seen as parallel rows of filaments breaking up into spores—Plate I, d.
*Trichophyton* (endothrix)

2. Tunnels and spaces which fill with air seen inside of hair shaft due to disintegration of fungus—Plate I, e.
*Trichophyton* (favus)

B. Nodules formed on hair shaft; fungus at a distance from base of hair:

I. Hair of head shows nodules along shaft some distance from the base *(Tropical distribution)*.

1. Black, discrete, hard, adherent mycelial masses on hair shaft composed of wide, short-celled hyphae—Plate I, f.
*Piedraia Hortai* (black Piedra)

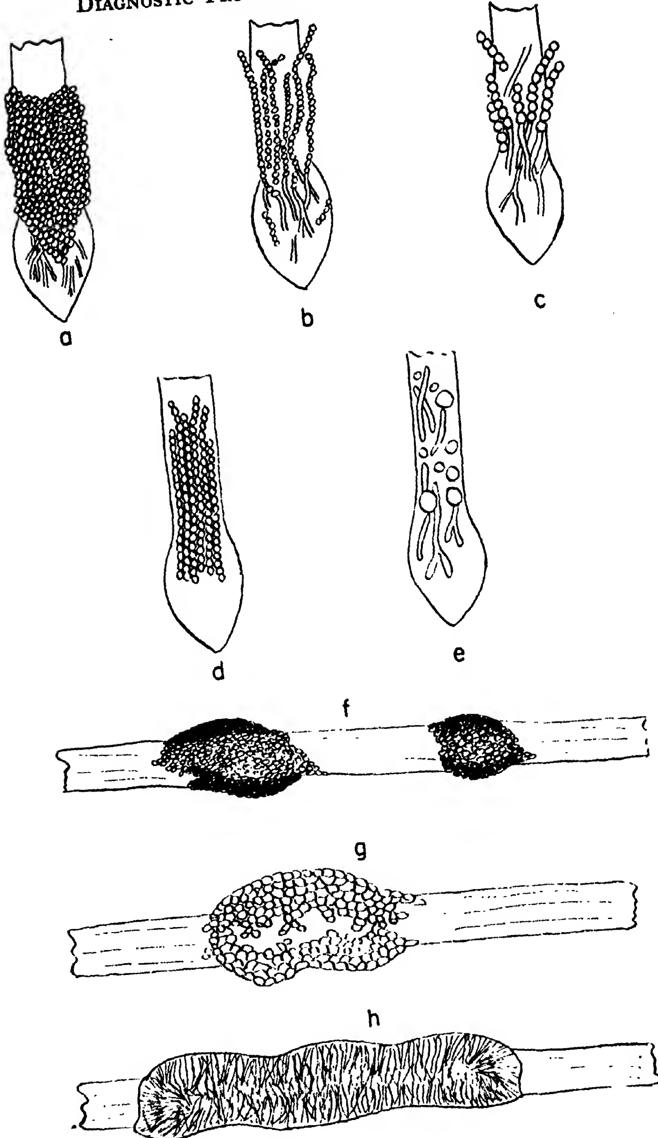


PLATE I

Fig. a—*Microsporum* hair
Fig. b—*Trichophyton* hair, ectothrix microides
Fig. c—*Trichophyton* hair, ectothrix megaspore
Fig. d—*Trichophyton* hair, endothrix
Fig. e—*Trichophyton* hair, favus type
Fig. f—*Piedraia Hortai*, black piedra type
Fig. g—*Trichosporon Beigelii*, white piedra type
Fig. h—*Nocardia tenuis*, trichomycosis hair

2. White, less discrete, softer mycelial masses on hair shaft composed of wide, short-celled hyphae—Plate I, g.
*Trichosporon Beigelii* (white Piedra)

II. Hair of axillary or pubic region shows nodules along shaft some distance from the base *(Universal distribution)*.

1. Yellow, red, or black nodules on hair shaft composed of masses of delicate hyphae 1 $\mu$ in diameter—Plate I, h.
*Nocardia tenuis* (trichomycosis)

### 4. CULTURE OF INFECTED HAIRS

Hairs from *Piedra* and *Trichomycosis* are sufficiently distinctive by microscopic examination to make an immediate identification without resorting to cultural technics. Since only one fungus, *Piedraia Hortai,* causes black piedra and only one fungus, *Trichosporon Beigelii,* causes white piedra, identification of these two fungi may be made on the appearance of the nodules on the infected hair. Likewise, the single fungus *Nocardia tenuis* causes trichomycosis and a careful microscopic examination of the nodules on the hair shaft is sufficient for identification. While the fungi from piedra may be cultured, the hairs from trichomycosis are never cultured.

Although the appearance of the infected hairs in tinea capitis often allows generic identification of the invading fungus (see key), the species involved can be identified only by accurate identification of the fungus obtained in culture. Infected hairs should be planted on Sabouraud's dextrose (C.M. No. 43) agar slants or crystal violet oxgall agar (C.M. No. 45) in Petri dishes. Inoculate slants with three or four fragments of hair so separated that they give isolated colonies. Inoculate crystal violet oxgall agar (C.M. No. 45) with several well separated hairs. All cultures should be maintained at room temperature for at least 2 weeks. Transfers from the edge of developing colonies on both slants and Petri dishes should be made to sterile Sabouraud's dextrose agar slants for pure cultures and identification.

### 5. EXAMINATION OF CULTURES

Fragments of cultures should be obtained from the slants with a holder having a wire with slightly bent end; bacteriological loops are unsatisfactory for obtaining material from filamentous cultures. Place the material in a drop of lactophenol-cotton blue on a microscopic slide and tease carefully with dissecting needles to make a thin prep-

aration. Place a cover glass over the preparation and heat the slide gently over a low flame of a Bunsen burner to drive out bubbles and get better penetration of the stain. Examine the preparation microscopically with the low and high dry objectives.

## 6. KEY TO IDENTIFICATION OF FUNGI BY EXAMINATION OF CULTURES

A. Culture filamentous, cottony to powdery, with *orange* pigmentation in the agar. This group contains the *Microsporum* species.

I. Colony slow-growing with dense, matted mycelium close to the agar surface and dull orange pigmentation in the agar. Old cultures sometimes have radial grooves. Spores when present [yeast-extract added to medium increases spore production [2] ] are large, spindle-shaped, multiseptate, imperfectly formed macroconidia; pectinate hyphae and racquette cells numerous—Plate II, a.

*Microsporum Audouini*

II. Colony fast-growing with wooly aerial mycelium and bright orange pigmentation in the agar. Numerous spindle-shaped, thick-walled, multiseptate (6–14 cells) macroconidia (8–15 x 40–150 μ) with roughened ends are produced: racquette hyphae numerous—Plate II, b.

*Microsporum (lanosum) canis*

III. Colony fast-growing with close, brown, powdery surface and dull orange to light brown pigmentation in the agar. Numerous ellipsoidal, rough, thin-walled, multiseptate (4–5 cells) macroconidia (8–12 x 30–50 μ) are produced; racquette hyphae numerous—Plate II, c.

*Microsporum (fulvum) gypseum*

B. Culture filamentous, cottony to powdery or granular with no pigmentation or a dilute yellow or purplish pigmentation in the agar. This group contains the "gypseum," "rubrum," and "crateriform" Trichophytons.

I. Colony fast-growing, powdery, light brown to buff in color with dilute yellow pigmentation in the agar. Numerous subspherical microconidia are produced in grape-like clusters (en grappe) or along the sides of the hyphae (en thyrses); a few to several clavate, smooth, thin-walled, multiseptate macroconidia (4–6 x 10–50 μ) are also found. Coils, antler-like hyphae, nodular bodies and racquette cells are numerous (from small-spored ectothrix hair)—Plate II, d.

*Trichophyton (gypseum) mentagrophytes*

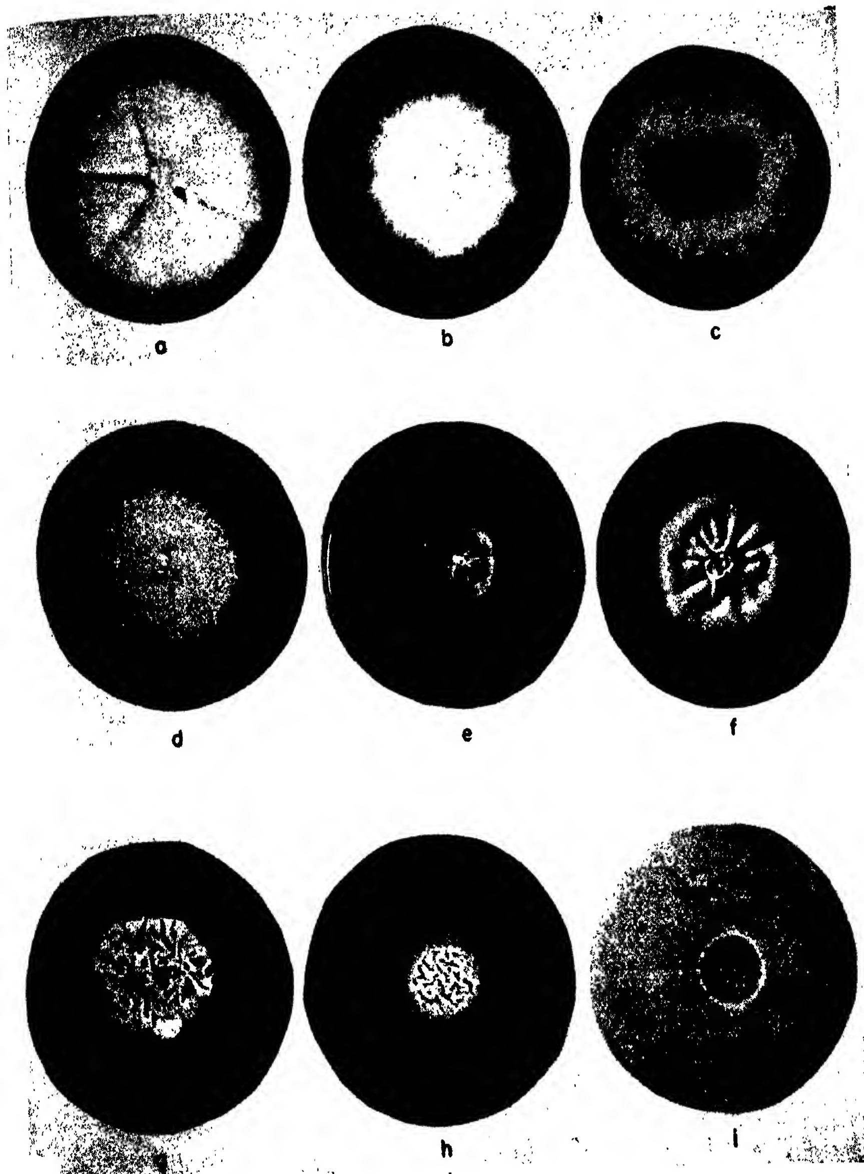


PLATE II

Fig. a—*Microsporum Audouini*, colony on Sabouraud's dextrose agar, 21 days
Fig. b—*Microsporum canis*, colony on Sabouraud's dextrose agar, 10 days
Fig. c—*Microsporum gypseum*, colony on Sabouraud's dextrose agar, 7 days
Fig. d—*Trichophyton mentagrophytes*, colony on Sabouraud's dextrose agar, 10 days
Fig. e—*Trichophyton rubrum*, colony on Sabouraud's dextrose agar, 12 days
Fig. f—*Trichophyton tonsurans*, colony on Sabouraud's dextrose agar, 35 days
Fig. g—*Trichophyton Schoenleini*, colony on Sabouraud's dextrose agar, 27 days
Fig. h—*Trichophyton ferrugineum*, colony on Sabouraud's dextrose agar, 14 days
Fig. i—*Trichophyton violaceum*, colony on Sabouraud's dextrose agar, 19 days

(See page 481 for references)

II. Colony may be fast-growing with cottony white aerial mycelium and deep red to purplish pigmentation in the agar or slower growing, somewhat velvety with deep red to purplish pigmentation in the aerial growth as well as in the agar. Numerous clavate microconidia are produced from the sides of the hyphae; a few to several clavate [on Difco Blood Agar Base Medium [3] ] thin-walled, multiseptate macrocondidia (4–6 x 10–30 μ) are also found (from small spored ectothrix hair on body)—Plate II, e.

*Trichophyton (purpureum) rubrum*

III. Colony slow-growing, heaped and folded; at times cerebriform, crateriform or acuminate with the surface covered with a short, velvety or powdery aerial mycelium and a dilute yellow pigmentation in the agar. Clavate microconidia are produced from the sides of the hyphae (from endothrix hair)—Plate II, f.

*Trichophyton tonsurans*

C. Culture slow-growing, glabrous, smooth and waxy, heaped or folded with a tendency to grow deep into the medium and split the agar. The surface may become velvety on prolonged growth or transfer. The microscopic morphology is poor in that spore forms are lacking. Some species, on enriched media, produce microconidia and macroconidia. (This group contains the "faviform" *Trichophytons.*)

1. Colony slow-growing, heaped, folded, glabrous and waxy with cream colored to light brown pigmentation. The surface may become velvety on repeated transfer. Typical "favic chandeliers" and chlamydospores are numerous. On grain media or enriched media a few clavate microconidia are produced [4] (from favus hair) —Plate II, g.

*Trichophyton (Achorion) Schoenleini*

II. Colony slow-growing, heaped, with a glabrous but sometimes velvety surface, white to light ochraceous in color. Such cultures are typically produced from large-spored, ectothrix *Trichophyton* hairs. They are animal species (bovine), difficult to obtain on initial isolation and are nonspore-producing on Sabouraud's dextrose agar. On enriched media [5, 6] the colonies grow more rapidly and produce aerial mycelium in which microconidia and macroconidia are developed (from large-spored ectothrix hair).

*Trichophyton faviforme*
syn. *T. album*
*T. discoides*
*T. ochraceum*

III. Colony slow-growing, heaped, folded, glabrous and waxy with deep reddish-yellow to orange pigmentation (from microsporum hair)—Plate II, h.

*Trichophyton (Microsporum) ferrugineum*

IV. Colony slow-growing, folded, glabrous and waxy with deep violet pigmentation (from endothrix hair)—Plate II, i.

*Trichophyton (Achorion) violaceum*

## IV. FUNGI CAUSING SUPERFICIAL INFECTION OF THE SKIN AND NAILS

### 1. Collection of Specimens

Lesions on the glabrous skin should be cleansed with an alcohol (70 per cent) sponge to remove surface bacterial and fungus contaminants and the area allowed to dry before collecting specimens. Materials obtained for examination and culture should be placed between two flamed microscopic slides which may be wrapped in paper for transport to the laboratory. After proper preparation (see below) the materials are examined microscopically with low and high dry objectives with the exception of material from *Tinea versicolor* and *Erythrasma* when oil immersion is used.

a. *Tinea petis*—The macerated skin between the toes should be removed with paper toweling or other materials which may be discarded. Skin should be collected for examination from the advancing border of the lesion between the toes with forceps or by scraping with a scalpel or the edge of a microscopic slide.

Brownish, discolored plaques occurring over the ball of the foot and heel should be scraped.

Material from vesicular or vesicopustular lesions should be obtained by removing the tops of the vesicles with scissors.

b. *Tinea cruris*—Material should be obtained from the erythematous advancing border of the lesion by scraping from the edge of the lesion toward the healthy skin. Also, the tops of vesicles should be obtained.

c. *Tinea corporis*—Typical ringed lesions, with healing centers and erythematous, vesicular borders (ringworm) are usually seen on the skin. Material should be scraped from the periphery of the lesions.

d. *Tinea nigra* (Tropical distribution)—Material should be scraped from the black areas occurring on the palmar aspect of the hand.

e. *Tinea unguium*—Material should be obtained from the discolored,

thickened areas of the infected nails by scraping the surface, digging out friable material from pits or grooves, and by removing accumulated material from beneath the nail. Also, if there is paronychial involvement, obtain exudate if present.

f. *Tinea versicolor*—Material should be obtained from the fawn-colored, furfuraceous patches by scraping with the edge of a microscopic slide. Hold another slide perpendicular to and against the skin directly below the lesion being scraped to insure catching the extremely thin and greasy scales.

g. *Erythrasma*—Material should be obtained from the reddish or reddish-brown lesions by scraping the area with a scalpel or the edge of a microscopic slide. Hold another slide against the skin as in *Tinea versicolor.*

### 2. Examination of Infected Materials

Fragments of skin or nail scrapings should be placed in a drop of 10 per cent potassium hydroxide on a slide. A cover glass should be placed over the preparation and the slide gently heated over the low flame of a Bunsen burner. Thick pieces of skin or nail should be pared with a scalpel and these fragments examined in potassium hydroxide (10 per cent).

Scales from *Erythrasma* and *Tinea versicolor* should be treated with ether to remove the fat before examination in potassium hydroxide. Such scales are so thin, they may be stained directly with methylene blue or lactophenol-cotton blue and examined microscopically after removal of the fat.

### 3. Key to the Fungi by Direct Examination of the Skin or Nails

A. Branching, wide, septate mycelium in the material.
  1. Colorless mycelium in the skin at different levels—
     Plate III, a — *Trichophyton species*, *Microsporum species*, *Epidermophyton floccosum*
  2. Colorless mycelium in the nail at different levels—
     Plate III, a — *Trichophyton species*, *Epidermophyton floccosum*
  3. Dark colored mycelium in the skin at different levels—
     (tinea nigra) *Cladosporium Wernecki*

B. Branching, delicate ($1\mu$) mycelium in the skin
   (Erythrasma) *Nocardia minutissima*

PLATE III

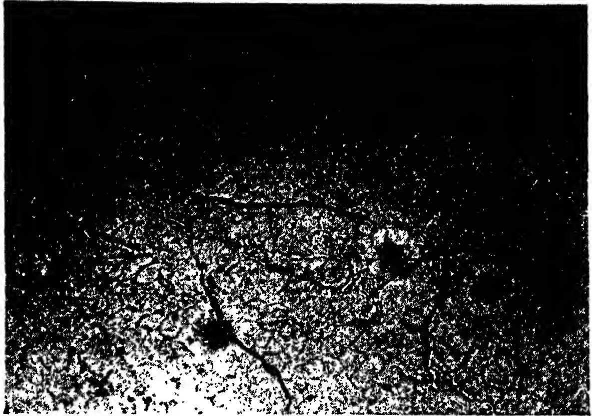

Fig. a — Potassium hydroxide preparation of skin showing fungus hyphae. x125

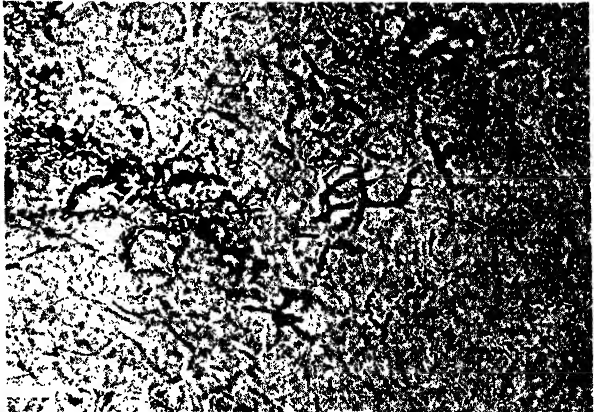

Fig. b — Potassium hydroxide preparation of skin showing mosaic fungi, an artifact. x245

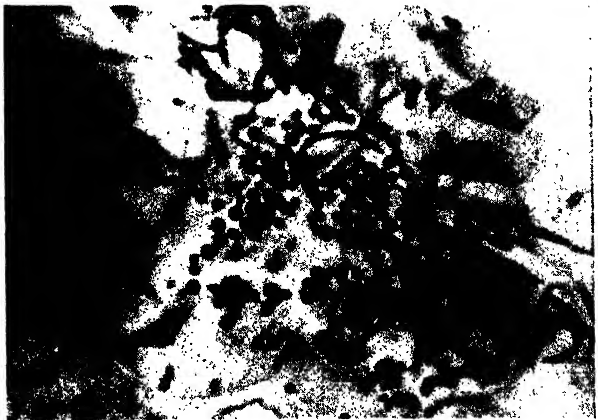

Fig. c — Lactophenol-cotton blue stained skin showing *Malassezia furfur* (tinea versicolor). x725

PLATE IV

Fig. a—*Epidermophyton floccosum*, colony on Sabouraud's dextrose agar, 12 days

Fig. b—*Trichophyton concentricum*, colony on Sabouraud's dextrose agar, 18 days

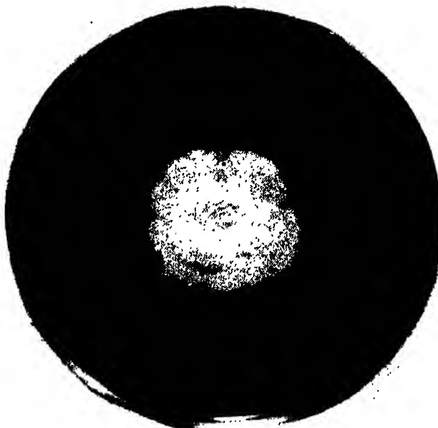

Fig. c—*Candida albicans*, colony on Sabouraud's dextrose agar, 21 days

(See page 482 for references)

C. Short, angular, septate hyphae intermixed with thick-walled, *round* budding cells (3–8μ) in the skin—Plate III, c
(tinea versicolor)*Malassezia furfur*

D. Small, oval, budding, yeast-like cells (2–4μ) in skin or nails
*Candida albicans*

E. Amorphous or crystalline material in mosaic arrangement outlining epidermal cells—Plate III, b Mosaic fungus (an artifact)

### 4. Culture of Infected Skin and Nails

No attempt is made to culture skin from *Tinea versicolor* or *Erythrasma*. The appearance of *Malassezia furfur* and *Nocardia minutissima* in potassium hydroxide or stained preparations is sufficiently distinctive to allow direct microscopic identification of these two fungi. The appearance of the dermatophytes *(Microsporum, Trichophyton* or *Epidermophyton)* in skin or nails, however, is not distinctive. All appear as identical, septate, branching hyphae, and the material must be cultured for the identification of the causative organism.

In addition to the dermatophytes there are several miscellaneous fungi which may infect the skin or nails. These are included in the key below.

Large pieces of skin should be cut into fragments with a scalpel and these planted on Sabouraud's dextrose agar slants (C.M. No. 43) and crystal violet oxgall agar (C.M. No. 45) in Petri dishes; each piece of inoculum should be partly submerged in the agar. Thick nail parings should be shaved with a scalpel and the thin shavings planted on the above media. All cultures should be maintained for 2 weeks at room temperatures, and transfers made to Sabouraud's dextrose agar slants from the edge of the developing colonies for pure cultures and identification.

### 5. Examination of Cultures

Preparations of the cultures should be made in lactophenol-cotton blue for microscopic examination.

### 6. Key to Identification of Fungi by Examination of Cultures

Since many of the dermatophytes which infect the hair also invade the skin and nails, a description of such species of *Microsporum* and *Trichophyton* may be found in the key to the identification of fungi which infect the hair. Some of the dermatophytes, however, do not

infect the hair (*Trichophyton concentricum* and *Epidermophyton floccosum*) and species of *Microsporum* do not infect the nails.

A. Culture filamentous, cottony to powdery: colony fast-growing, powdery, greenish-yellow and quickly overgrown with a cottony, aerial mycelium; numerous clavate, smooth, thin-walled, multi-septate (2–4 cells) macroconidia (7–12 x 20–40$\mu$) are produced —Plate IV, a. *Epidermophyton floccosum*

B. Culture glabrous, smooth and folded; the surface may become velvety on prolonged growth; colony slow-growing, glabrous, folded, at first white, but later deep brownish in the center. No spore forms are produced—Plate IV, b.
(from tinea imbricata, tropical distribution)
*Trichophyton concentricum*

C. Culture dark colored, at first yeast-like, but later filamentous; extremely pleomorphic; colony fast-growing, black, glistening, soft and yeast-like; later, aerial mycelium produced which is olivaceous to black. Black, oval, yeast-like cells in young cultures; dark colored, thin hyphae in old cultures produce *Cladosporium*-like spores. (From *tinea nigra)*
*Cladosporium Wernecki*

D. Culture cream colored, soft, with distinct yeast-like odor; colony fast-growing (48–72 hours) with oval, budding cells on the surface of the medium and pseudomycelium penetrating the agar; chlamydospores produced on corn meal agar (C.M. No. 44)—Plate IV, c.
*Candida albicans*

## V. FUNGI CAUSING INFECTION OF MUCOUS MEMBRANES, SKIN, AND SUBCUTANEOUS TISSUES

### 1. COLLECTION OF SPECIMENS

No general statement can be made concerning the collection of materials from the variety of lesions to be found on the mucous membranes, skin, or in subcutaneous tissues. The technics found to be most useful will be described below in a discussion of the diseases which are encountered. A portion of all materials collected should be examined microscopically in *fresh* or *stained* preparations and the remainder should be cultured on suitable media.

a. *Actinomycosis*—Pus from draining sinuses should be obtained by allowing this material to run into a sterile test tube held at the border of the lesion. Small quantities of pus should be obtained with a sterile loop. The roof of an abscess should be removed with a scalpel and

the small quantity of underlying pus collected with a sterile loop. The walls of a sinus may be scraped or biopsied.

b. *Maduromycosis*—As above for *Actinomycosis*.

c. *Rhinosporidiosis*—Lesions on the conjunctivae or polypoid masses on the mucous membranes of the nose should be swabbed, lightly scraped, or the tissue may be squeezed gently with forceps to release the sporangia and spores from the surface of the lesion.

d. *Coccidioidomycosis*—Pus should be aspirated from subcutaneous abscesses with sterile needle and syringe. Microabscesses at the border of verrucous lesions should be opened and the small quantity of pus obtained. Biopsy of the border of the lesion also should be made.

e. *Chromoblastomycosis*—Pus from ulcerated lesions should be obtained with sterile loop or with a swab. Crusts from verrucous lesions should be obtained with scalpel or forceps. Biopsy of ulcerated or verrucous lesions should also be made.

f. *Histoplasmosis* (cutaneous and mucosal lesions)—Material should be obtained by scraping or swabbing the ulcerated lesions on the mucous membranes of the nasopharyngeal areas or on the mouth and tongue. Biopsy of such lesions or granulomatous skin lesions also should be made.

g. *Moniliasis* (Thrush-Vaginitis)—Material should be obtained from the grayish patches on the mucous membranes of the mouth or vagina by scraping or with swabs.

h. *Sporotrichosis*—Material should be obtained with swabs from the primary, ulcerated, chancre-like lesion on the skin. Pus should be obtained with sterile needle and syringe from unopened subcutaneous nodules along the lymphatics. A biopsy of such a nodule also may be obtained.

i. *Cryptococcosis*—Material from ulcerated skin lesions should be obtained with a sterile bacteriologic loop or swab. Granulomatous skin lesions should be biopsied.

j. *Blastomycosis*—Pus should be aspirated from subcutaneous abscesses with sterile needle and syringe. Microabscesses *at the border* of verrucous lesions should be opened and the small quantity of pus obtained. Biopsy of the border of the lesion also should be made.

## 2. EXAMINATION OF INFECTED MATERIALS

Exudates and pus from ulcerated lesions or from subcutaneous abscesses or nodules should be examined as so-called *fresh preparations*. Such preparations are made by placing a cover glass over a small

quantity of the material on a microscopic slide. If the material is too opaque, it may be diluted with sterile saline or cleared with a drop of 10 per cent potassium hydroxide. Crusts should be cleared by being placed in a drop of 10 per cent potassium hydroxide on a slide, cover glass added, and the preparation gently heated over a low flame of a Bunsen burner. Examine all preparations with the low and high dry objectives.

The amount of light used during microscopic examination of clinical materials should be carefully regulated. Too bright a light often obscures fungus bodies in fresh preparations of pus or other exudates.

When actinomycosis or moniliasis is suspected, smears also should be made and stained by Gram's method. When histoplasmosis is suspected, smears should be stained with Wright's, Wilson's or Giemsa's stain. If cryptococcosis is suspected, pus or exudates should be emulsified in a drop of India ink and examined as a wet preparation to demonstrate the diagnostic capsule of *Cryptococcus neoformans*.

Some of the material obtained by biopsy should be prepared for sectioning and the remainder prepared for direct examination and culture. For direct examination, a small fragment of tissue should be ground in saline and examined as for pus or exudates.

### 3. KEY TO FUNGI BY DIRECT EXAMINATION

A. Granules present.

I. Granules, with or without clubs, composed of delicate hyphae, 1 μ or less in diameter, which, when crushed, break up into bacillary, diphtheroid and short branching elements.

1. Granules light colored, composed of Gram-positive, non-acid-fast, bacillary and branching elements—Plate V, a, b. *Actinomyces bovis*
2. Granules light colored, composed of Gram-positive, acid-fast, bacillary elements. *Nocardia asteroides*

II. Granules, without clubs, composed of wide, septate hyphae, 2–3 μ in diameter, containing numerous chlamydospores; the hyphae do not break up into bacillary elements. *Monosporium apiospermum*

B. Granules not present.

I. Fungus bodies do not reproduce by budding.

1. Large, round, hyaline, thick-walled sporangia, 40–300 μ in diameter, containing small, round, hyaline spores 7–9 μ in diameter (cannot be cultured). *Rhinosporidium Seeberi*

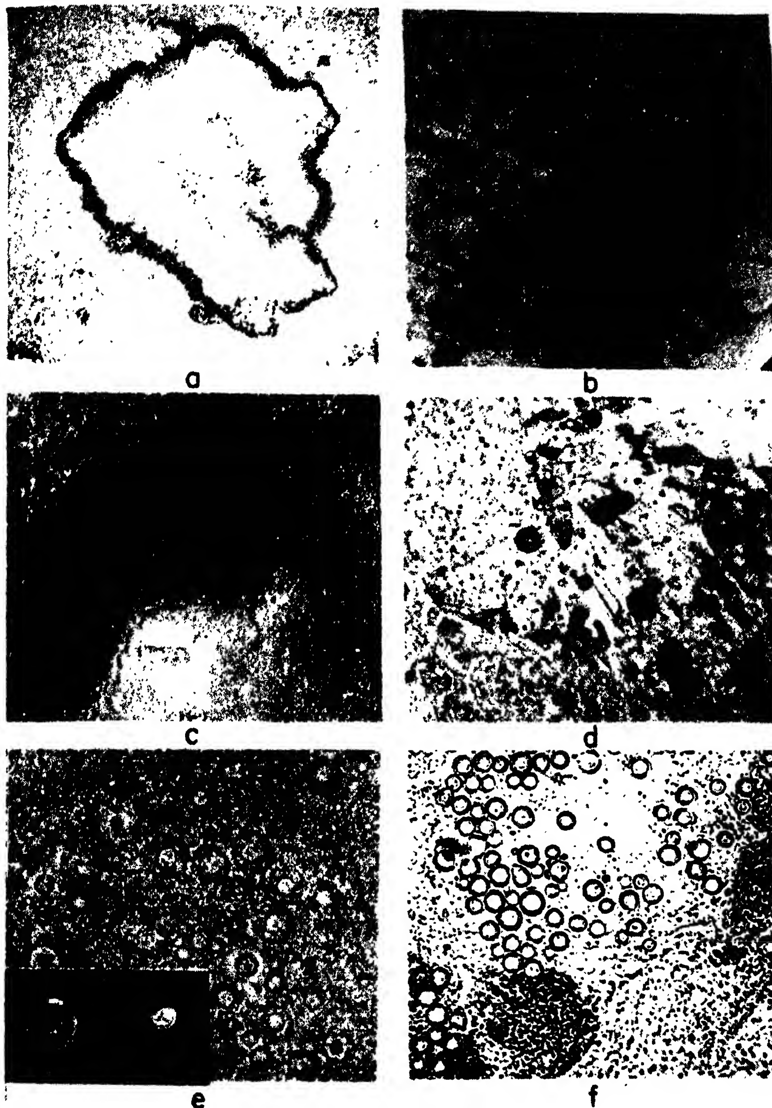


PLATE V

Fig. a—*Actinomyces bovis*, granule in pus. x450
Fig. b—*Actinomyces bovis*, granule crushed and stained by Gram's method. x1300
Fig. c—*Coccidioides immitis*, endospore-filled spherule in pus. x700
Fig. d—*Hormodendrum Pedrosoi*, dark brown fungus bodies in pus. x825
Fig. e—*Cryptococcus neoformans*, budding, encapsulated cells in pus; fresh preparation (x684) and India ink preparation (x684).
Fig. f—*Blastomyces dermatitidis*, thick walled budding cells in pus. x650

(See page 482 for references)

2. Large, round, hyaline, thick-walled spherules, 15–80 μ in diameter, containing numerous endospores—Plate V, c.
*Coccidioides immitis*
3. Large, ovoid to rounded, hyaline, thick-walled cells, 8–15 μ in diameter, and rectangular cells (arthrospores) 4–6 x 8–12 μ in size. *Geotrichum candidum*
4. Small, round, brown, thick-walled bodies, 6–12 μ in diameter, which reproduce by splitting—Plate V, d.
*Hormodendrum Pedrosoi*
*Hormodendrum compactum*
*Phialophora verrucosa*

II. Fungus bodies reproduce by budding.
1. Small, oval, thin-walled, budding cells, 2–4 μ in size, usually intracellular in large macrophages and best demonstrated by blood stains. *Histoplasma capsulatum*
2. Small, oval, thin-walled, budding cells, 2.5 x 4–6 μ in size, occasionally accompanied by pseudomycelium.
*Candida (Monilia)* species
3. Small, thin-walled, fusiform bodies, 1.5 x 4 μ in size, both intracellular and extracellular best demonstrated by Gram's stain (infrequently seen in human materials).
*Sporotrichum Schenckii*
4. Large, thick-walled, round, budding cells, 5–15 μ in diameter, surrounded by a wide capsule best demonstrated in India ink preparations—Plate V, e. *Cryptococcus neoformans*
5. Large, thick-walled, round, *single* budding cells, 5–20 μ in diameter, lacking capsule—Plate V, f.
*Blastomyces dermatitidis*
6. Large, thick-walled, round, *multiple* budding cells, 10–60 μ in diameter. *Blastomyces brasiliensis*

### 4. CULTURE OF INFECTED MUCOUS MEMBRANES, SKIN, AND SUBCUTANEOUS TISSUES

No attempt is made to culture *Rhinosporidium Seeberi*, the cause of rhinosporidiosis. The characteristic appearance of this fungus in microscopic preparations and, especially, in section, is diagnostic.

Granules from draining sinuses, abscesses, or from biopsies should be cultured anaerobically and aerobically. Thioglycholate broth (C.M. No. 58), ground meat medium (C.M. No. 14) and deep shake cultures of beef infusion dextrose agar (C.M. No. 5a) should be inoculated with crushed

granules and incubated at 37° C. Also, Bacto brain-heart infusion agar plates should be streaked and incubated at 37° C. under anaerobic conditions with the addition of 5 per cent $CO_2$.[7] Such cultures should produce growth of the microaerophilic *Actinomyces bovis*. Granules also should be cultured on beef infusion dextrose agar slants, blood agar plates, and Sabouraud's dextrose agar slants at 37° C. Such cultures should produce growth of the aerobic species of *Nocardia*.

Pus, exudates, and ground tissue from lesions suspected of containing *Candida albicans, Cryptococcus neoformans, Blastomyces dermatitidis, Blastomyces brasiliensis, Histoplasma capsulatum* or *Sporotrichum Schenckii* should be cultured on blood agar or Francis's cystine blood agar (C.M. No. 35) at 37° C. and Sabouraud's dextrose agar at room temperature. When the material to be cultured is contaminated, 20 units of penicillin and 40 units of streptomycin per ml. of medium should be added. If *Coccidioides immitis* is suspected, it may be isolated on a selective medium containing 1 per cent ammonium chloride, 1 per cent sodium acetate, 0.8 per cent tribasic potassium phosphate, 0.04 per cent cupric sulfate, and 2 per cent agar.

Materials from chromoblastomycosis should be cultured on Sabouraud's dextrose agar at room temperature.

With the exception of *Candida species, Cryptococcus neoformans,* and *Sporotrichum Schenckii* the pathogenic fungi develop slowly in culture. All cultures should be held for at least two weeks before discarding.

## 5. Examination of Cultures

Microscopic preparations of yeast-like cultures are best examined in a drop of water; filamentous cultures are best examined in lactophenol-cotton blue preparations. Most fungi may be handled using ordinary sterile technics and the usual precautions. *Coccidioides immitis,* however, becomes very powdery with the development of arthrospores in old cultures and should be handled with extreme caution.[8] Transfers should be made only under a hood after wetting down the mycelium with sterile saline to which has been added a loopful of Tween 80.

## 6. Key to Identification of Fungi by Examination of Cultures

A. Culture bacterial-like, some closely resembling pigmented, saprophytic acid-fast bacteria.
   I. Culture *anaerobic,* raised, rough, grayish to yellowish on streaked plates of brain-heart infusion agar at 37° C. under 5 per cent

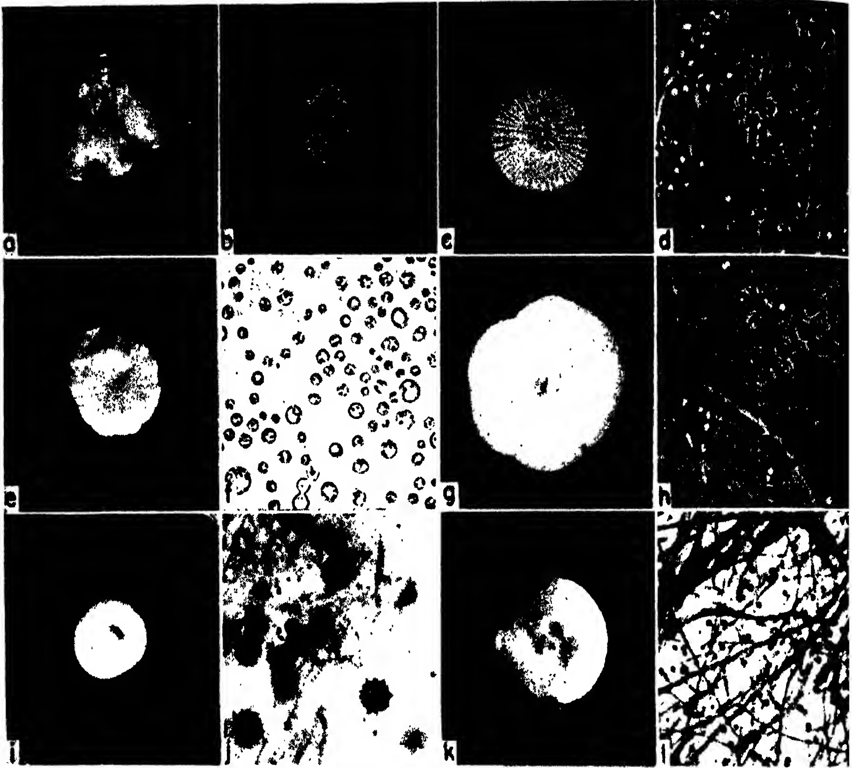

PLATE VI

Fig. a—*Actinomyces bovis*, colony on beef infusion dextrose agar, pH 7.8, 5 days. x10
Fig. b—*Nocardia asteroides*, colony on Sabouraud's dextrose agar, 14 days
Fig. c—*Candida albicans*, colony on Sabouraud's dextrose agar, 20 days
Fig. d—*Candida albicans*, from Sabouraud's dextrose agar. x658
Fig. e—*Cryptococcus neoformans*, colony on Sabouraud's dextrose agar, 12 days
Fig. f—*Cryptococcus neoformans*, from Sabouraud's dextrose agar. x700
Fig. g—*Geotrichum candidum*, colony on Sabouraud's dextrose agar, 10 days
Fig. h—*Geotrichum candidum*, from Sabouraud's dextrose agar. x658
Fig. i—*Histoplasma capsulatum*, colony on Sabouraud's dextrose agar, 12 days
Fig. j—*Histoplasma capsulatum*, from Potato dextrose agar. x864
Fig. k—*Blastomyces dermatitidis*, colony on Sabouraud's dextrose agar, 12 days
Fig. l—*Blastomyces dermatitidis*, from Sabouraud's dextrose agar. x700

$CO_2$; colonies bacterial-like, 1–3 mm. in diameter in 4–6 days, with delicate hyphae, 1 $\mu$ or less in diameter, which form tangled, branching Gram-positive masses or fragment into bacillary or diphtheroid Gram-positive elements—Plate VI, a.

*Actinomyces bovis*

II. Culture *aerobic,* wrinkled, granular, glabrous, and yellow to orange in color on Sabouraud's dextrose agar; colony slow growing

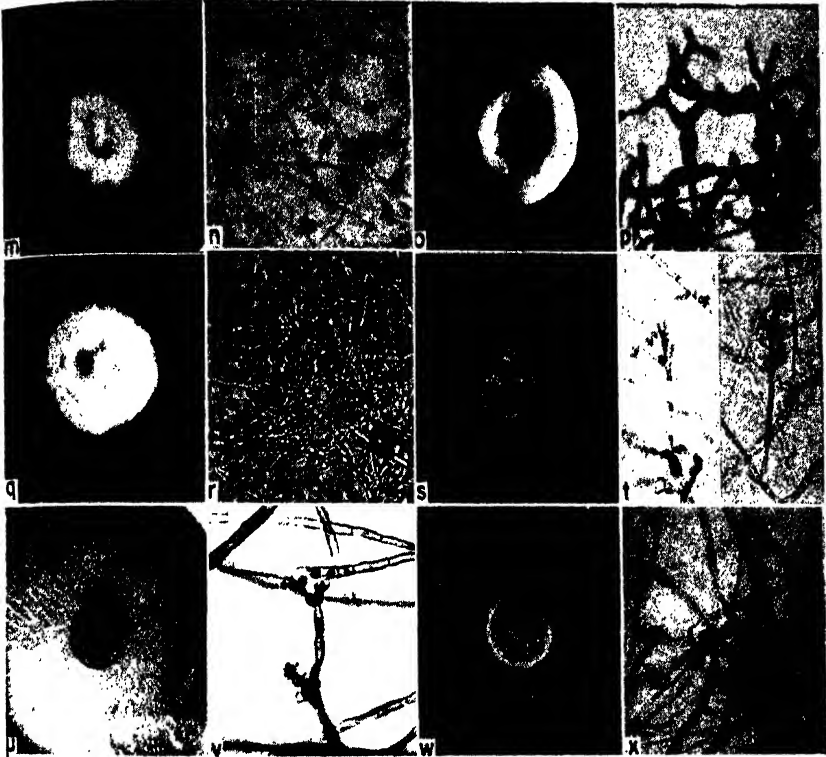


PLATE VI (Cont.)

Fig. m—*Sporotrichum Schenckii*, colony on Sabouraud's agar, 12 days
Fig. n—*Sporotrichum Schenckii*, from Sabouraud's dextrose agar. x658
Fig. o—*Coccidioides immitis*, colony on Sabouraud's dextrose agar, 7 days
Fig. p—*Coccidioides immitis*, from Sabouraud's dextrose agar. x736
Fig. q—*Monosporium apiospermum*, colony on Sabouraud's dextrose agar, 15 days
Fig. r—*Monosporium apiospermum*, from Sabouraud's dextrose agar. x305
Fig. s—*Hormodendrum Pedrosoi*, colony on Sabouraud's dextrose agar, 20 days
Fig. t—*Hormodendrum Pedrosoi*, from Sabouraud's dextrose agar. x700
Fig. u—*Hormodendrum compactum*, colony on Sabouraud's dextrose agar, 20 days
Fig. v—*Hormodendrum compactum*, from Sabouraud's dextrose agar. x816
Fig. w—*Phialophora verrucosa*, colony on Sabouraud's dextrose agar, 20 days
Fig. x—*Phialophora verrucosa*, from Sabouraud's dextrose agar. x410

(See page 482 for references)

(14–20 days) with delicate hyphae, 1 $\mu$ or less in diameter, which fragments readily into bacillary and short branching Gram-positive or acid-fast forms—Plate VI, b. *Nocardia asteroides* *

B. Culture yeast-like at both incubator and room temperature.

I. Culture cream-colored, soft, with distinct yeast-like odor; colony fast growing (48–72 hours) with small, oval, thin-walled, budding

* For identification of species of *Nocardia*, see Table 1.

cells, 2.5 x 4–6 μ, on the surface of the medium and pseudomycelium penetrating the agar; chlamydospores produced on corn meal agar—Plate VI; c, d. *Candida albicans* †[9, 10]

II. Culture cream-colored to light tan, mucoid, glistening and without odor; colony slow growing (10–12 days) with large, round, thick-walled, budding cells 5–15 μ in diameter having distinct capsules; no mycelium produced—Plate VI; e, f. *Cryptococcus neoformans*

III. Culture white to cream-colored with dry, mealy surface; colony fast growing (4–5 days) with septate mycelium and numerous rectangular and rounded cells, 4–12 μ in diameter, formed by fragmentation of the hyphae—Plate VI; g, h. *Geotrichum candidum*

C. Culture yeast-like at incubator temperature but filamentous at room temperature.

I. Culture yeast-like, smooth, white to cream-colored, and moist at 37° C. on sealed blood agar slants of Francis's cystine blood agar slants; colony fast growing (4–5 days) with small, oval, thin-walled, budding cells, 2 x 4 μ in size. Culture filamentous, cottony and white at first then buff to brown on Sabouraud's dextrose agar at room temperature; colony slow growing (10–14 days) with septate hyphae, numerous pyriform to round, thick-walled, tuberculate spores 8–20 μ in diameter. Culture converted to yeast-type by growth at 37° C.—Plate VI; i, j. *Histoplasma capsulatum*

II. Culture yeast-like, wrinkled, waxy and white on blood agar slants at 37° C.; colony fast growing (5–7 days) with large, thick-walled, round, *single* budding cells 5–20 μ in diameter. Culture filamentous, cottony and white at first then light brown on Sabouraud's dextrose agar at room temperature; colony slow growing (10–14 days) with septate hyphae, numerous small, smooth, round to pyriform lateral conidia 5–8 μ in size. Culture converted to yeast-type by growth at 37° C.—Plate VI; k, l. *Blastomyces dermatitidis*

III. Culture yeast-like, wrinkled, waxy, and white on blood agar slants at 37° C.; colony fast growing (5–7 days) with large, thick-walled, round, *multiple* budding cells 6–30 μ in diameter. Culture filamentous, small, heaped, with short nap of aerial mycelium on Sabouraud's dextrose agar at room temperature; colony slow growing (14–21 days) with septate hyphae, many chlamydospores and

† For identification of species of *Candida*, see Table 2.

few, if any, conidia. Culture converted to yeast-type by growth at 37° C. *Blastomyces brasiliensis*

IV. Culture yeast-like, soft, moist and cream-colored on Francis's cystine blood agar at 37° C; colony fast growing (5–7 days) with small, fusiform, oval, budding cells. Culture filamentous, smooth, leathery, lacking cottony aerial mycelium, cream-colored to dark brown or black on Sabouraud's dextrose agar at room temperature; colony fast growing (5–7 days) with delicate, septate hyphae 1–1.5 μ in diameter and small, pyriform conidia, 2–4 μ, borne in clusters on the ends of lateral branches and occasionally from the hyphae. Culture converted to yeast-type by growth at 37° C—Plate VI; m, n. *Sporotrichum Schenckii*

D. Culture filamentous at both incubator and room temperature.

I. Culture filamentous, cottony, white at first, then tan to brown on Sabouraud's dextrose agar; colony develops 7–14 days with septate hyphae segmenting into numerous oblong, rectangular spores (arthrospores), 2.5–3 x 3–4 μ in size—Plate VI; o, p. *Coccidioides immitis*

II. Culture filamentous, cottony, white at first, then grayish on Sabouraud's dextrose agar; colony develops 7–14 days with septate hyphae with ovoid to clavate conidia 5–8 x 8–10 μ, borne singly on the ends of conidiophores.—Plate VI; q, r. *Monosporium apiospermum*

III. Culture filamentous, flat, covered with short, felt-like aerial mycelium, dark green to black in color on Sabouraud's dextrose agar; colony slow growing (14–20 days) with black to olivaceous septate hyphae and *three* spore types; ovoid spores in loose, branching chain formation from the terminal cells of conidiophores (*Hormodendrum* type), spores from the sides of swollen conidiophores (*Acrotheca* type), and spores from a cup-shaped tip of small conidiophores (*Phialophora* type)—Plate VI; s, t. *Hormodendrum Pedrosoi*

IV. Culture filamentous, heaped, brittle, with coarse aerial mycelium, olive-black in color on Sabouraud's dextrose agar; colony slow growing (14–20 days) with black to olivaceous septate hyphae and *two* spore types: subspherical spores in compact, branching chains from the ends of conidiophores (*Hormodendrum* type) and, rarely, spores from a cup-shaped tip of small conidiophores (*Phialophora* type)—Plate VI; u, v. *Hormodendrum compactum*

V. Culture filamentous, flat, covered with short, felt-like aerial

mycelium, dark brown to black in color on Sabouraud's dextrose agar; colony slow growing (14–20 days) with black to olivaceous, septate hyphae and a *single* spore type; small, oval, thin-walled spores, 1.5–4 $\mu$, from a cup-shaped tip of small conidiophores—Plate VI; w, x. *Phialophora verrucosa*

TABLE 1

*Comparison of Five Species of Nocardia* *

| *Species* | *Granule* | *Pigment on Czapek's Agar* | *Fragmentation of Mycelium* | *Acid fast* | *Liquefy Gelatin* | *Coag. Milk* |
|---|---|---|---|---|---|---|
| *N. asteroides* (Eppinger) Blanchard, 1896. | Yellowish white | Yellow to orange ochraceous | + | + | − | − |
| *N. brasiliensis* (Lindenberg) Cast and Chalmers, 1913. | Yellowish white | Yellow to orange ochraceous | + | + | + | + |
| *N. madurae* (Vincent) Blanchard, 1896. | Yellowish white | Cream to pinkish | − | − | + | + |
| *N. Pelletieri* (Laveran) Pinoy, 1912 | Red | Coral Red | − | − | + | − |
| *N. paraguayenis* (Almeida) Conant, 1947. | Black | Dark cream | − | − | + | − |

* From *Zinsser's Textbook of Bacteriology*, New York: Appleton-Century-Crofts, 9th ed., 1948, p. 838.

## VI. FUNGI CAUSING PULMONARY INFECTIONS

### 1. COLLECTION OF SPECIMENS

A morning specimen of sputum should be obtained after the patient has washed or rinsed the mouth thoroughly with an antiseptic. The sputum may be collected in a sterile Petri dish, or sterile 1 oz. wide mouth, screw cap jar for transportation to the laboratory.

Bronchoscopic material should be sent to the laboratory immediately, after wrapping the trap with sterile gauze. Tissue obtained by biopsy during bronchoscopy should be divided for microscopic examination and culture and for sections. Pleural fluid should be obtained with sterile needle and syringe and transferred to a sterile test tube.

Pus from thoracic sinuses should be collected by allowing the pus to run into a sterile test tube held against the skin at the sinus opening. The skin around the opening should be cleansed with iodine and washed with alcohol (70 per cent) to avoid surface contaminants.

TABLE 2

*Differential Diagnosis of Species of Candida* *

| | *Pathogenic* | | | *Nonpathogenic* | | | |
|---|---|---|---|---|---|---|---|
| | *C. albicans* | *C. tropicalis* | *C. pseudo-tropicalis* | *C. krusei* | *C. parakrusei* | *C. stell-atoidea* | *C. Guill-iermondi* |
| Sabouraud's agar | Creamy growth | Not charac-teristic | Not charac-teristic | Flat, dry | Creamy | Creamy | Creamy growth |
| Sabouraud's broth | No surface growth | Narrow surface film with bubbles | No surface growth | Wide surface film | No surface growth | No surface growth | No surface growth |
| Blood-agar | Medium-sized, dull gray colonies | Large, gray colonies sur-rounded by mycelial fringe | Colonies small, not characteristic | Colonies small, irregularly shaped, flat, or heaped | Colonies small, brilliant white | Colonies star-shaped | Medium-sized, dull gray colonies |
| Corn meal | Branched, tree-like mycelium with chlamy-dospores | Mycelium well developed, branched, bearing numerous blastospores, no chlamydospores | Mycelium poorly developed, no chlamydospores | "Crossed Sticks" myceluim, no chlamydospores | Mycelium well developed, no chlamydospores | Mycelium with large, ball-like clusters of blastospores | Mycelium well developed, no chlamydospores |
| Glucose | AG | AG | AG | AG | AG † | AG | — ‡ |
| Maltose | AG | AG | — | — | — | AG | — |
| Sucrose | A | AG | AG | — | — | — | — |
| Lactose | — | — | AG | — | — | — | — |

* From Martin, D.S., Jones, C. P., Yao, K. F., and Lee, L. E., Jr., *J. Bact.* 34:99, 1937.

† Occasionally acid only.

‡ Langeron and Guerra report acid and gas produced in glucose and sucrose when cultured at 25° C. and held 20 days.

Stomach contents should be obtained by gastric lavage in the morning before eating and the material collected in a suitable sterile container.

## 2. Examination of Materials Collected

Sputum, bronchoscopic specimens and pus from draining thoracic sinuses should be examined as so-called *fresh preparations* by placing a cover glass over a small quantity of the material on a microscopic slide. Microscopic examination with low and high dry objectives and reduced light from the microscope condenser should reveal fungus bodies if present. When sputum or pus is too dense, it may be diluted with sterile saline or cleared in a drop of 10 per cent potassium hydroxide for microscopic examination.

Sputum or pus from sinuses also should be smeared and stained with Gram's method for easier identification of the budding cells and pseudomycelium of *Candida* species and the delicate, branching hyphae of *Actinomyces bovis* or *Nocardia* species. All such materials showing Gram-positive, branching filaments of bacterial dimension also should be stained with Ziehl-Neelsen's method to demonstrate the acid-fast, branching filaments of *Nocardia asteroides*.[11]

If cryptococcosis is suspected, a loopful of material should be mixed with a drop of India ink for a proper demonstration of the diagnostic capsule of *Cryptococcus neoformans.*

Gastric washings and pleural fluids should be centrifuged at high speed for 30 minutes and the sediment examined in fresh preparations for fungus cells and in smears for Gram-positive or acid-fast branching filaments.

Tissues obtained during bronchoscopy should be ground in sterile saline and fresh preparations examined for fungus cells.

## 3. Key to Fungi by Direct Examination

With the exception of *Monosporium apiospermum, Rhinosporidium Seeberi, Hormodendrum Pedrosoi, Hormodendrum compactum* and *Phialophora verrucosa,* all of the fungi listed in the section on Fungi Causing Infection of Mucous Membranes, Skin and Subcutaneous Tissues V, 3 may produce pulmonary infections. Therefore, the key to the identification of fungi found in that section also will serve to identify the fungi found in pulmonary infections.

#### 4. CULTURE OF FUNGI FROM PULMONARY INFECTIONS

With the exception of the fungi mentioned above, the cultural technics described for the isolation of fungi from mucous membranes, skin, and subcutaneous tissues Section V, 4 also are applicable to fungi causing pulmonary infections. However, sediment from centrifuged gastric lavage should be cultured immediately. It also should be borne in mind that all of these materials, except pleural fluid, may be heavily contaminated with bacteria. Therefore, they should be cultured on media containing penicillin and streptomycin, Section V, 4.

#### 5. EXAMINATION OF CULTURES

Microscopic preparations are made as described for fungi from mucous membranes, skin, and subcutaneous tissues, Section V, 2.

#### 6. KEY TO IDENTIFICATION OF FUNGI BY EXAMINATION OF CULTURES

With the exception of *Monosporium apiospermum, Hormodendrum Pedrosoi, Homodendrum compactum,* and *Phialophora verrucosa,* the key found in Section V, 6 is applicable to fungi isolated from pulmonary infections.

## VII. FUNGI CAUSING MENINGITIS

#### 1. COLLECTION OF SPECIMEN

Spinal fluid obtained by lumbar puncture should be transferred to a suitable sterile container for transportation to the laboratory.

#### 2. EXAMINATION OF SPINAL FLUID

Spinal fluid should be centrifuged at high speed for 30 minutes and the sediment examined as a fresh preparation. Such preparations are suitable for demonstrating *Blastomyces dermatitidis, Coccidioides immitis, Candida albicans, and Cryptococcus neoformans.* Occasionally, however, *Cryptococcus neoformans* is confused with the large macrophages, especially mononuclear cells, and the individual fungus cells are counted with this type of cell in cellular counts of spinal fluid. This may be avoided if India ink preparations are made of centrifuged sediment to demonstrate the diagnostic capsule of *Cryptococcus neoformans.*

Stained smears of the centrifuged sediment should reveal the Gram-positive filaments of *Actinomyces bovis* and the acid-fast filaments of *Nocardia asteroides*. Organized granules of these two fungi are rarely, if ever, found in the spinal fluid.

### 3. Key to Fungi by Direct Examination

The following fungi may be encountered in spinal fluid: *Actinomyces bovis, Nocardia asteroides, Coccidioides immitis, Blastomyces dermatitidis, Blastomyces brasiliensis, Cryptococcus neoformans* and *Candida albicans*. The most frequent fungus causing meningitis is *C. neoformans* (*Torula histolytica—Cryptococcus hominis,* etc.). Meningitis caused by this fungus simulates exactly that caused by *Mycobacterium tuberculosis* and can be differentiated only by laboratory studies during which one or the other of these organisms can be demonstrated either in the spinal fluid or by culture.

The fungi listed above may be identified by the key found in Section V, 3.

### 4. Culture of Fungi from Spinal Fluid

The fungi mentioned in the preceding section may be isolated from spinal fluid using the technics described in Section V, 4.

### 5. Examination of Cultures

Microscopic preparations are made as described for fungi from mucous membranes, skin and subcutaneous tissues in Section V, 5.

### 6. Key to Identification of Fungi by Examination of Cultures

The key in Section V, 6 is applicable to cultures obtained from spinal fluid.

## VIII. FUNGI RECOVERED FROM BLOOD OR BONE MARROW

Although many fungi cause systemic diseases and undoubtedly metastasize by hematogenous spread, very few attempts are made to obtain cultures from the blood. In rare cases species of *Candida* have been isolated.[12]

In cases of histoplasmosis, however, *Histoplasma capsulatum* may be demonstrated in blood or bone marrow. Smears and cultures of these

materials should be made. In the smear, the fungus appears (in mononuclear or occasionally in polymorphonuclear cells) as intracellular small, oval, encapsulated cells, 2–4 μ in size, with a deeply stained crescent area in the broadest end of the oval cell. This staining differentiates *H. capsulatum* from *Leishmania donovani* in which the nucleus and kinetoplast are stained clearly.

Blood or bone marrow should be cultured in beef infusion dextrose broth incubated at 37° C. Small, discrete, granular to fluffy colonies develop slowly and the cultures should be held for at least 3 weeks before discarding as negative. Transfers of the developing fungus should be made to Sabouraud's dextrose agar slants for better development of the typical and characteristic tuberculate chlamydospores.

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# Blood Cultures

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## I. INTRODUCTION

THE routine blood culture technic is a commonly employed laboratory test that has given invaluable aid to the clinician in the diagnosis and prognosis of disease. Furthermore, this test has increased our knowledge concerning the pathogenesis and development of many disease processes. In many instances, the interpretation of the bacteriological findings of the blood is not difficult, but frequently the findings are complex and difficult to associate with the clinical observations. Since the development of the sulfonamide drugs and their derivatives, the final interpretation of the blood culture is even less understood. In spite of the numerous blood culture studies that have been made in the past, a clear understanding of the principles governing bacterial invasion of the blood and the significance of a bacteremia in health and disease still need systematic and thorough investigation.

The wide variety of methods used in the blood culture technic by the various workers, undoubtedly accounts for some of the conflicting results. Some advocate the use of their own special type of enriched culture media, while others emphasize the need of dilution in eliminating the influence of the antibodies of the patient's blood, or they directly remove the antibodies by special procedures. Variations in methods of subculturing, length of incubation period, quantity of blood used, special treatment of the blood after removal from the patient, and many other factors, too numerous to be mentioned, bring about the wide differences in results in blood cultures reported in the literature.

It seems worth while, therefore, to establish some basic procedure in blood culture technic so as to obtain results that will or may be comparable in the study of disease conditions.

## II. METHODS FOR A ROUTINE BLOOD CULTURE

### A. EQUIPMENT NECESSARY FOR TAKING BLOOD CULTURES

20 ml. capacity Luer glass syringe
No. 18 gauge needle
The syringe and needle should be wrapped in a clean towel and then the towel wrapped in paper. This package should be sterilized in the autoclave for 30 minutes.
Tourniquet
Tincture of iodine and 70 per cent alcohol

Sterile gauze sponges
Sterile towels
Sterile test tube with citrate crystals
Culture media
Alcohol lamp

### B. PERSONNEL NECESSARY FOR COLLECTING BLOOD

Two persons should always be present for the technical procedure of obtaining the blood from the patient as this tends to reduce the chance for contamination of the blood culture. A majority of the hospital staffs require the interne and a nurse to collect the blood, but in some places a trained laboratory interne or technician is assigned to this responsibility for a definite period of time. Blood cultures on infants and young children should be taken by the pediatric interne who has had experience in handling infants.

The personnel of the bacteriological laboratory must consist of a competent and well trained bacteriologist and a technical staff sufficient to care for the work. An overburdened laboratory tends to inefficiency and unsatisfactory results.

### C. SITE FOR WITHDRAWING BLOOD

Blood for culturing is usually withdrawn from the median basilic vein of the arm. When this vein is not available because of previous use for therapeutic measures, the saphenous vein of the leg is next choice, or the external jugular vein. The jugular vein is usually the site most accessible in the infant. In special studies, other veins have been employed for blood cultures, especially those draining a particular focus of infection within the body.

Other sites have occasionally been used for blood culture studies, such as the arterial system [1,2] and the sternal bone marrow.[3] Blood cultures have been taken from the brachial, femoral, and other arteries of the body and their results compared with the venous blood. Likewise, a sternal bone marrow tap has been employed for bacterial studies. These methods, however, are not to be used as routine procedure for blood culture technique because of the associated danger.

### D. METHOD FOR WITHDRAWING BLOOD SAMPLE

An important step in the technic of sampling blood for bacteriological examination is the care with which this procedure is performed.

### 1. *Preparation of material at bedside*

The nurse or assistant should have the sterile supplies and culture media carefully arranged on the table beside the patient in order to facilitate the procedure. The operator (interne) should have clean, dry hands before handling the sterile material. A tourniquet is placed around the arm of the patient and tightened sufficiently to constrict the venous flow just before inserting the needle into the vein.

### 2. *Sterilization of skin*

The area of skin overlying the vein should be washed with green soap and water. Then it should be thoroughly painted with tincture of iodine, followed shortly by washing with 70 per cent alcohol. It is this part of the technic that is often carelessly and hastily performed so that sterilization of the skin is not adequate. It seems, however, that even with the iodine and alcohol method of sterilization of the skin, complete sterility is not obtained. Both Callow[4] and Wilson[5] have shown that the iodine-alcohol sterilized surface still will show bacterial growth in cultures from 30 per cent of the painted skin surfaces.

### 3. *Withdrawing of Blood*

The needle is first introduced through the sterilized area of the skin and then inserted into the vein. At least 10 to 15 ml. of venous blood is obtained for the blood cultures from adults, and not less than 5 ml. from infants or young children. When the required amount of blood is obtained, the tourniquet is quickly loosened, and the needle is withdrawn from the vein. Steady pressure is exerted over the needle puncture wound of the patient with a sterile sponge.

### 4. *Allocation of blood sample*

Upon removal of the needle from the syringe, the operator, with the assistance of the nurse, distributes the blood to the culture media and to the sterile test tube containing sodium citrate crystals. Needless to say, careful bacteriological technic is required in this process consisting of flaming the mouths of the flasks and test tubes over the alcohol lamp when they are opened.

## E. CULTURE MEDIA

The sample of blood in the syringe should be distributed at the time of collection into two containers. At least 5 ml. of the blood

should be introduced into the sterile test tube containing sufficient crystals of sodium citrate to prevent clotting. The remaining quantity of blood is then inoculated into a flask of meat infusion broth (C.M. No. 5). If the patient has received any of the sulfonamide drugs or their derivatives before taking the blood culture, para amino benzoic acid should be added to the medium. If the patient has received penicillin, penicillinase or clarase should be added to the medium.

### F. Transportation to Laboratory and Request Slips

It is important to have the blood and the flasks transported to the laboratory without delay. If the blood is collected at night or over the week-end, when the bacteriological laboratory is closed, an incubator should be available to keep the cultures warm. It is very essential to have a properly executed request slip accompany the specimens to the laboratory. This slip should record the patient's name, age, diagnosis, previous therapy (serum or chemotherapy), hour the culture was taken, and the signature of the operator or interne who is responsible for collecting the blood culture.

### G. Methods in the Bacteriological Laboratory

Two additional media are inoculated immediately in the laboratory from the blood in the citrate tube. Poured plates are prepared by inoculating 1 ml. and 2 ml. of the blood into 10 ml. of melted agar (C. M. No. 5a) cooled to 45° C. and thoroughly mixed in a sterile Petri plate. The remaining 2 ml. of the citrated blood are then inoculated into 25 ml. of thioglycollate medium (C. M. No. 58) or into cooked meat medium for anaerobic growth (C. M. No. 14).

All of the inoculated culture media are incubated at 37° C. for various periods of time. Daily inspection is made for growth, and those showing evidence of bacteria should have stained smears and subcultures prepared. When growth is obtained, the organism should be identified as rapidly and efficiently as possible. The culture media failing to show growth are maintained at 37° C. for 14 days before discarding. Daily inspection of all these negative culture flasks is necessary. The poured plates are examined after 24, 48, and 72 hours, and then discarded. If colonies are present, a total count is made of the number present per ml. of blood. Special anaerobic bacteriological procedures are utilized for identification of these organisms when present. Reports are given to the physician in charge of the

patient by telephone, and later verified by a complete detailed description of the findings. The all too frequent presence of contaminants in the blood culture is a true indictment of the care with which the bacteriologic technic is performed both at the time of collection of the blood and the manipulation of the cultures in the laboratory. Organisms commonly known to be contaminants are the diphtheroids, staphylococci, sarcinae, spore-forming bacilli, and molds.

## III. SPECIAL BACTERIOLOGICAL METHODS FOR BLOOD CULTURES

It is well known that the above routine method of culturing the blood will result in the growth of a large number of the important pathogens, but certain species require special methods in order to demonstrate their presence in the blood.

### A. Methods Requiring Special Treatment of Blood Samples

In attempting to grow bacteria from the blood stream of patients with rheumatic fever, chronic infectious arthritis or other chronic infections of unknown etiology, several technical procedures have been devised. These methods are somewhat complex and in a few instances require considerable handling of the blood and original cultures, which undoubtedly predisposes to contamination unless extremely careful bacteriological technic is employed. The methods described are chiefly modifications of Clawson's technic; a few investigators have used original procedures. These methods are of sufficient importance to warrant a brief description of them.

#### 1. *Rosenow's Method*[6]

Fifteen ml. of blood is put into a large tube containing 4 ml. of 2 per cent sodium citrate in NaCl solution. The citrated blood is then transferred by means of a sterile pipette to sterile distilled water contained in a bottle that also serves as a centrifuge tube. The proportion is 10 parts of water to 1 part of citrated blood. Centrifuged at once, the supernatant fluid is drawn off, the sediment removed with a sterile pipette and inoculated in 50 ml. of dextrose broth (pH 7.2). Infusion broth medium made from beef heart (pH 7.6) may be substituted for dextrose broth.

#### 2. *Clawson's Method*[7]

Fifty ml. of blood is the usual amount taken for cultural purposes. It is collected in two large sterile test tubes and allowed to clot. These clots, when firm,

are loosened and put into flasks containing 250 ml. of dextrose (0.2 per cent) beef infusion broth, pH 7.6 and incubated at 37° C. for 10 days or longer.

### 3. *Cecil, Nichols, and Stainsby's Method* [8]

The technic employed is an adaptation of Clawson's method. Twenty ml. of blood is divided into two sterile test tubes and allowed to clot. Each tube is treated separately. They are centrifuged and the serum drawn off by a sterile pipette. The clot is then broken up in the original tube with a sterile glass tube. Fragments of clot are then transferred to a 3 oz. bottle containing 50 ml. beef heart infusion broth, pH 7.6. The inoculated broth is then incubated at 37° C. and left unopened for 5 days. On the 5th day, 8 ml. of 1.5 per cent beef heart infusion agar is melted and cooled to 45° C. and 0.5 ml. of whole rabbit's blood is added. It is then inoculated with 0.1 ml. of broth from the original blood culture and pour plates are prepared. These plate cultures are incubated at 37° C. for 24 and 48 hours. Similar pour plates are made from the original broth culture every 3 to 5 days thereafter until the original culture has incubated at 37° C. for 30 days. Finally, the sediment of the centrifuged specimen of the original culture is similarly treated, and in addition a stained smear is prepared, if the previous studies have shown no growth.

### 4. *Margolis and Dorsey's Method* [9]

Fifteen to 20 ml. of blood is inoculated into 135 ml. of dextrose broth, pH 7.2–7.4, and also in similar broth plus human placental tissue. Subcultures are made by inoculating 0.5 ml. of the original broth into melted and cooled 2 per cent plain agar containing horse blood. Repeated subcultures are made every 4 to 5 days from the original cultures.

### 5. *Callow's Technic* [4]

Five ml. of blood is added to broth containing "double beef" infusion, pH 7.6–7.8. Agar plates are made with the same double beef infusion enriched with horse or rabbit blood. Duplicate cultures are made from the patient. All cultures are incubated at 37° C. for various lengths of time. Subcultures are made from the original after 48 hours' incubation and at weekly intervals thereafter.

## B. METHODS REQUIRING INCREASED $CO_2$ TENSION

Certain organisms require increased $CO_2$ tension in order to insure optimum growth. Many strains of the gonococcus, meningococcus and Brucella organisms will not grow without being placed in an environment of increased $CO_2$ tension. Several methods have been devised to maintain an increased $CO_2$ tension which are outlined briefly as follows:

### 1. *Jar Method*

Probably the best method for blood cultures is the use of an anaerobe jar or any satisfactorily sealed jar in which a 10 per cent concentration of $CO_2$ may be maintained during the period of growth of the organism. The jar, after receiving the cultures, is sealed and then attached to a suitable stopcock which is connected to a vacuum system, a mercury manometer and a tank of compressed $CO_2$. The air is removed from the jar until the mercury of the manometer is reduced by 5 to 10 mm. This space is then replaced by carbon dioxide so that there is from 10 to 12 per cent $CO_2$ in the air of the jar.

### 2. *Combustion Method*

A simpler and probably more practical method for many laboratories in producing increased $CO_2$ tension in the jar, is by burning a short candle in the sealed jar. When the oxygen is depleted, the candle will become extinguished and the $CO_2$ content of the jar will be suitable for the growth of these bacteria.

### 3. *Chemical Method*

Shaughnessy [10] has devised a method in which $CO_2$ tension may be supplied to the individual tubes and flasks. Sulfuric acid with a bicarbonate is used as the source of carbon dioxide.

a. *Blood cultures for meningococcus and gonococcus*—The meningococcus is not as difficult to grow as the gonococcus, so that it is not always necessary to use elaborate methods in order to obtain growth. About 10 ml. of blood is withdrawn from the patient and inoculated in 50 ml. of meat infusion broth (C. M. No. 5) with 0.1 to 0.2 per cent dextrose. The inoculated broth or blood agar plates may be placed in an atmosphere of 10 per cent $CO_2$.

A variety of media have been used for growing the gonococcus.* Tryptic digest broth and chocolate blood agar when placed under $CO_2$ tension will yield the best results after 48 hours' incubation at 37° C.

b. *Blood cultures for Brucella*—When attempts are made to isolate Brucella from the blood stream, both the aerobic and the increased $CO_2$ tension methods should be used simultaneously. An elaborate technic for the cultivation of Brucella from the blood has recently been described by Poston.[11]

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* See also chapter, The Gonococcus by Charles M. Carpenter.

*Poston Method*

Fifteen ml. of blood is introduced into a flask containing 4 ml. of 2.5 per cent sodium citrate solution; 2.0 ml. of the citrated blood is planted into each of 4 tubes containing 15 ml. of liver infusion agar (C. M. No. 7a), melted and cooled to 45° C., and poured into plates; 2 ml. of the citrated blood is put into each of two flasks containing 100 ml. of beef infusion broth pH 7.4 (C. M. No. 5). Half of the cultures are incubated at 37° C. in a 10 per cent $CO_2$ atmosphere, and the other half under aerobic conditions. After incubation for 48 hours, if no colonies are visible on the plates, 5 ml. amounts of the supernatant broth are transferred from the original flask to sterile centrifuge tubes. After centrifuging at high speed for 15 minutes, the supernatant fluid is discarded. A loopful of the sediment is streaked over the surface of the liver infusion blood agar plate and a stained smear is prepared. The original flasks are then shaken to mix the blood and broth and 10 ml. of this mixture is transferred to each of two flasks of fresh broth. Blood cultures for Brucella should be kept for 18 days before being reported as negative. Blood agar poured plates show colonies resembling *Streptococcus viridans*, while the colonies on the surface plates resemble *Salmonella typhosa*.

c. *Blood cultures for Fusobacteria and Spirochetes*—Dark-field illumination is rarely used in the examination of the blood or of blood cultures. Consequently, the demonstration of these organisms in cultures, as in the blood, is indeed unusual. Williams[12] recently reported the isolation of fusiform bacilli and spirochetes in blood cultures from two patients. The dark-field technic was used in the demonstration of these organisms and as a routine procedure the following method is briefly outlined:

*Method*

Twenty ml. of the patient's blood is introduced into 1.5 per cent solution of sodium citrate. After distributing 2.0 ml. to other types of media, 18 ml. is inoculated into a flask containing 300 ml. of dextrose-infusion broth (pH 7.7) which has been enriched with 20 ml. of sterile ascitic fluid. The broth is covered with melted petrolatum. The culture is at first incubated at 37° C. without increased $CO_2$ tension, but later the flasks are placed in a jar of $CO_2$ under 75 mm. pressure. Turbidity and hemolysis of the blood will appear.

Gram stains of the broth failed to show spirochetes, but the examination of wet preparations by dark-field illumination revealed the presence of many spirochetes, in the experience of Williams.

## C. METHODS REQUIRING REDUCED OXYGEN TENSION

Unfortunately, this procedure in the routine blood culture technic has been too much neglected. Not only the strictly anaerobic organisms require the reduced oxygen tension for growth, but there are many other strains in which the lower oxygen tension initiates growth. After several transfers on artificial media, these organisms are capable of growing on ordinary aerobic culture media without reduced oxygen tension. Weinberg and Sequin [13] have found *Clostridium perfringens* in the blood stream during life. Other anaerobic spore-forming bacilli are less frequently recovered from the blood stream.

Two kinds of culture media are particularly useful for routine anaerobic cultures. The cooked meat medium (C. M. No. 14) is especially practical for most laboratories as it is easily prepared. More recently, thioglycollate medium (C. M. No. 58) has been formulated which is an excellent culture medium for the growth of all anaerobic bacteria. Two ml. of blood are inoculated into 25 ml. of the thioglycollate medium and incubated without seal or vacuum jar.

The high degree of skill required in the technic for isolating anaerobic organisms as well as the numerous procedures necessary for purification and identification of these organisms, undoubtedly accounts for our meager information pertaining to their presence in the blood stream.

### 1. *Blood culture for Pasteurella tularensis*

There are only a few instances in which the isolation of *Pasteurella tularensis* by culture from the blood has been reported. Recently, Ransmeier and Schaub [14] have reported culturing *Pasteurella tularensis* from the blood of 3 cases by using blood-glucose-cystine agar (C. M. No. 35).

#### *Method of Blood Culture*

Blood was taken from the arm vein. Ten to 15 ml. of blood was introduced into about 6 ml. of a sterile 2.5 per cent solution of sodium citrate. One ml. of the citrated blood was then placed on each of several blood-glucose-cystine agar (C. M. No. 35) slants. The tubes were tilted several times before coagulation occurred, so that

the blood was distributed over the entire surface of the medium. *Pasteurella tularensis* was observed in 10 days.

Sodium thioglycollate may be substituted for the cystine in the blood-dextrose-cystine agar and will permit the growth of *Pasteurella tularensis*.

## D. Methods for Autopsy Blood Cultures

It is the opinion of many that after death bacteriological studies of the blood and tissues are of little value in the diagnosis and interpretation of disease. However, a systematic study of the relation of bacteria found in the blood and tissues of human cadavers,[15] together with an experimental study[16] of the factors controlling post-mortem invasion, revealed the fact that post-mortem invasion of saprophytic bacteria is not as rapid nor as common as was previously believed. Furthermore, a comparative study of blood cultures taken at autopsy and those obtained clinically[17] shows few striking differences. Therefore, the bacteriological examination of the blood at autopsy can be significant in the final interpretation of the clinical-pathological correlation, provided rigid bacteriological technic is strictly adhered to during the collection and treatment of the sample of blood at the autopsy table. Most of the unusual findings of the bacterial cultures obtained at post-mortem may be accounted for on the basis of carelessness in the technic at the autopsy table.

Two methods of taking blood at post-mortem have developed which depend upon the time the sample of blood is taken after the death of the individual. In both instances, the blood is obtained from the heart.

### 1. *Cardiac puncture immediately after death*[18]

The area of skin directly overlying the heart is painted with tincture of iodine and alcohol. A sterile large bore needle attached to a syringe is inserted through the chest wall at about the region of the 4th interspace slightly to the left of the sternum. The heart is penetrated, 5 to 10 ml. of blood withdrawn, and allocated to appropriate media. The media used are similar to those employed for routine clinical blood cultures.

### 2. *Blood culture at autopsy*

A sample of blood is withdrawn from the pulmonary artery (venous blood) after sterilizing the surface of the exposed pulmonary vessel

by searing with a red hot spatula. A sterile large bore needle is inserted through this area and 10 ml. of blood is obtained. Occasionally, small fragments of blood clots interfere with the removal of sufficient blood but, by proper manipulation and care, the blood may be obtained. The blood sample is then distributed to culture media that are similar to those used for clinical blood culture studies.

## E. BLOOD CULTURES FOR PUBLIC HEALTH LABORATORIES

The above described procedures for blood cultures are especially applicable to hospital laboratories and not entirely satisfactory for a routine public health laboratory. The public health laboratory usually cannot obtain samples of blood directly from the patient, but must resort to samples of clotted blood that are shipped to them from distant localities. This, therefore, necessitates a modified procedure in the allocation of blood to culture media.

### *Method*

Instead of immediately inoculating the culture media at the bedside of the patient, the sample of blood is introduced from the syringe into the sterile test tube and permitted to clot. After firm clotting, the sample is properly sealed and placed in a suitable container for shipment to the laboratory. The test tube of the clotted blood is centrifuged and the serum decanted. The clot is thoroughly macerated by a sterile glass rod and the macerated fragments are then inoculated into the routine aerobic and anaerobic culture media that are employed by the laboratory. The serum collected from the blood clots is then used for various agglutination and serological tests of diagnostic value. This method has been successfully employed by West and Borman [19] in a public health laboratory for the purpose of culturing the more sensitive type of organisms, such as the Brucella group. They isolated strains of Brucella from 42 of the 4,051 blood clots examined by them. Other pathogenic organisms were also obtained from the blood clots. Their method consisted of pouring off the serum and cells from the clot. Macerate the clot asceptically with a sterile glass tubing (7 mm). Transfer the macerated blood clots by means of this tubing to crystal violet tryptose broth in a screw vial. Incubate at 35° under $CO_2$ tension for 4 to 7 days before examining. After this incubation period, streak drop of inoculated broth culture onto crystal violet tryptose agar plates. Incubate the inoculated agar plates for 4 days under

$CO_2$ tension at 35° C. The broth to plate inoculation is repeated every week for 3 weeks before finally discarding the culture.

### F. ANIMAL INOCULATION OF BLOOD

In a few instances the ordinary and special culture technics are not sensitive enough or are otherwise not suited to the isolation of the bacterial agent in the blood. It then is necessary to inoculate animals with the blood of the patient. In order to demonstrate the presence of the organism, the animal employed for the test must be susceptible, so that signs of infection or death will develop. Guinea pigs, mice, and rabbits are the common laboratory animals employed in this procedure. Occasionally, a monkey is required to demonstrate some of the blood parasitic agents. For example, in Weil's disease it is necessary to inoculate from 3 to 6 ml. of whole blood into the peritoneal cavity of guinea pigs in order to demonstrate *Leptospira icterohaemorrhagiae* in the tissues of the animal. Francis[20] first isolated *Pasteurella tularensis* from the blood of a patient with the disease, by means of guinea pig inoculation. Some of the virus diseases may require the use of susceptible mice in order to demonstrate the agent in the blood. The tubercle bacillus has been demonstrated in the blood stream of chronic, pulmonary and miliary tuberculosis by animal inoculations.

### G. DIRECT EXAMINATION OF BLOOD

At times, the only method for demonstrating the parasite in the circulating blood is by direct examination either by wet preparations or by special stained smears of the blood. This method is routinely used for certain types of parasites. Bacteria are difficult to find in the blood unless there is an overwhelming infection such as occurs in anthrax. Dark-field illumination should be more frequently employed in blood examinations.

## IV. EVALUATION OF BLOOD CULTURES

In order to evaluate fully the result of blood cultures taken from individuals with disease and in health, it is necessary to be cognizant of some of the known biological facts concerning the presence of bacteria in the blood stream.

Bacteria have been recovered from the blood of healthy individuals as reported by Reith and Squier,[21] Callow,[4] and Wilson.[5] It has

been shown that certain physiological conditions of the body tend to induce a transient bacteremia. Samples of blood taken from individuals following a heavy meal may show bacteria. Kulka[22] has demonstrated organisms in the blood identical with those recovered from the vagina of healthy women during the first day of menstruation. The existence of a transient fetal bacteremia has been demonstrated by Kobok[23] who recovered organisms from the umbilical cord in 9 per cent of the 374 consecutive blood cultures. Dunham[18] reported bacterial growth in 34 per cent of 115 cultures taken from 49 normal new-born infants. The organisms isolated from the blood in the above investigations consisted usually of strains having slight, if any, pathogenicity.

Adverse physiological conditions or traumatic injuries to the body are known to permit a temporary bacteremia either from the normal bacterial reservoirs of the body or from small localized infections of the tissues. Richards[24] demonstrated that strenuous massage of various kinds of foci of infection may initiate a temporary invasion of the blood stream. It has been observed by Round and Kirkpatrick[25] and Murray and Moosnick[2] that the movement and pressure on the teeth by chewing a hard substance in individuals having a mouth infection will produce positive blood cultures. Minor surgical procedures, such as extraction of teeth, cause a transient bacteremia from the bacterial flora of the oral cavity. O'Kell and Elliott,[26] Round and Kirkpatrick,[25] and Burket and Burn[27] have demonstrated conclusively the source of the bacteria in the blood of dental patients following extraction of the teeth. A change of diet, as shown by Arnold,[28] will permit viable bacteria to pass through the wall of the intestinal tract of dogs. Any disturbance in the arterial supply of the small intestine has been revealed by Cirrincione and Francona[29] to increase the rate of absorption of bacteria from this site. Again, the kinds of bacteria recovered from the blood in these individuals were not of the highly pathogenic strains, but occasionally a demonstration in the blood of a real pathogen was reported. Furthermore, evidence of localization of bacteria in the tissues could not be demonstrated in these individuals, although it is believed that this temporary invasion into the blood may be responsible for the development of bacterial endocarditis in some patients.

Finally, the finding of the more highly pathogenic bacteria in the blood stream in acute infections constitutes, in most instances, a transfer of bacteria into the blood from a focus within the tissues. Very

few pathogenic bacteria are capable of multiplying actively and maintaining their numbers in the blood to the extent of producing a true septicemia. It is obvious from a diagnostic standpoint that the finding of the meningococcus, pneumococcus, gonococcus, typhoid bacillus, Brucella, and other pathogens in the blood usually means the presence of a lesion within the tissues. Only occasionally are organisms recovered from the blood before clinical evidence of localization has occurred, in which case the blood culture is of real diagnostic aid. Multiple invasions of the blood stream by bacteria have been demonstrated at post-mortem [17] as well as clinically which in the majority of the individuals studied were proved to be the result of primary and secondary involvement of the tissues.[30]

Bacteria are not always readily found within the blood even though definite primary foci exist in the tissues. In these instances, the negative blood cultures may be accounted for by the existence of a portal bacteremia or a thrombosis of cerebral vessels with infected emboli, or due to an infecting organism that is not of the ordinary bacterial type. Prognosis of the disease in the individual may be indicated by the number of bacteria in the blood, but the presence of a bacteremia does not necessarily mean an unfavorable outcome.

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# Technic of Rh Testing

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## I. INTRODUCTORY REMARKS

RECENT studies on the pathogenesis of erythroblastosis fetalis, a hemolytic disease of the fetus and the new-born, have brought about the rapid development of a new field of laboratory procedures. Simple serological tests are now available which will detect practically all mothers who deliver such infants because at least 93 per cent of these women lack the Rh blood factor known either as D or as $Rh_o$. Such individuals are called Rh-negative, while those who carry the D ($Rh_o$) factor in their red cells are called Rh-positive. The two contrasting types will be referred to hereinafter as Rh+ and Rh—. In the white population of the United States, 85 per cent of all individuals are Rh+ while 15 per cent are Rh—. The most important diag-

nostic serum, called anti-D (anti-$Rh_o$), is produced by Rh— individuals who become immunized against the D ($Rh_o$) factor.

The Rh blood factor is important clinically because it has the capacity to induce antibody formation when Rh+ blood is introduced into the circulation of an Rh— individual whether by transfusion, intramuscular injection or, most significantly, by means of fetal blood transfer across the placenta. More than 90 per cent of all intra-group transfusions reactions occur in Rh— individuals who receive repeated transfusions of Rh+ blood. In some Rh— women even the very first transfusion may result in a severe and perhaps fatal hemolytic reaction. These women have been stimulated to produce anti-Rh antibodies because in the course of their pregnancies, minute amounts of fetal Rh+ blood find their way into the maternal sinuses. It is assumed that this transplacental transfer occurs as a silent process

TABLE 1

| *Serum of Group* | *Antibodies in Serum* | *Reactions of Red Blood Cells of Group* | | | |
|---|---|---|---|---|---|
| | | *O* | *A* | *B* | *AB* |
| A | anti-B | o | o | + | + |
| B | anti-A | o | + | o | + |
| Incidence in white population (%) | | 45 | 41 | 10 | 4 |
| Incidence of Rh+ | | 38 | 35 | 8.5 | 3.4 |
| Incidence of Rh— | | 7 | 6 | 1.5 | 0.6 |

in the latter part of every normal pregnancy and is therefore a physiological phenomenon.

From the point of view of prevention of transfusion accidents in general, it is preferable to think in terms of at least eight blood differences instead of the classic four blood groups. Since the scheme of the four blood groups is genetically independent of the Rh factor, the incidence of Rh+ and Rh— individuals in any of the four blood groups is also 85 per cent and 15 per cent, respectively. These relationships are given in Table 1.

The A and B factors which form the basis of the four blood groups differ from the Rh factor since antibodies in the serum of Rh— individuals specific for Rh+ blood do not exist normally. Nevertheless, it is important to select group compatible Rh— donors for blood

transfusions to Rh— candidates in order to prevent (1) antibody formation and (2) serious or fatal intra-group hemolytic transfusion reactions if anti-Rh antibodies have already been produced by pregnancies or previous transfusions.

Rh testing should be carried out routinely on all pregnant women, all candidates for transfusion (or intramuscular injection of blood) and all blood donors. The preventive measure of giving only Rh— blood to all Rh— girls or women requiring blood transfusion will in itself reduce the incidence of erythroblastosis fetalis and the morbidity incidental to transfusion reactions.

## II. THE Rh-Hr SYSTEM

The subject is rendered more complex because there is not one Rh factor but a mosaic of several antigenic properties now called, D, C, and E ($Rh_o$, rh′, and rh″) each of which is genetically related to corresponding antigens known as Hr factors, namely, d, c, and e ($Hr_o$, hr′, and hr″).*[1-4] Accordingly, these new blood factors may be referred to as the Rh-Hr system. The blood of every individual must contain at least one member of each of the three pairs of Rh-Hr antigens and an individual who is heterozygous for each of the three pairs of factors will possess all six Rh-Hr blood factors.

In this chapter, preference is given to the alphabetic nomenclature (DCE) for both antigens and their specific antibodies as recommended by Fisher and Race. The earlier terminology based on variants of the terms "Rh" or "Hr" will frequently be given in parentheses in order to guide the reader who may be familiar with this system only.

The complexity of the Rh factor emerged from the observation that some of the Rh— mothers of erythroblastotic infants produced not only anti-D (anti-$Rh_o$) antibodies (85 per cent positive reaction) but also another antibody now known as anti-C (anti-rh′) which reacted with the blood of about 70 per cent of a random population in the New York area,[7] or occasionally anti-E (anti-rh″) which gave 30 per cent positive reactions.[8] Furthermore, Rh+ mothers were found who had produced antibodies specific for either of the two other Rh factors, C or E (rh′ or rh″),[9, 10] or for one of the three Hr factors (d, c, or e).[11-13] The first anti-Hr serum was observed in an Rh+ mother of an erythroblastotic infant and this antibody, anti-c (anti-hr′), described a new blood factor which was found to be genetically related to the Rh factor C (rh′).[7, 11]

* Actually, the situation is still more complex as indicated by the existence of variants of the C and D factors, called $C^w$, $C^u$, $c^v$ and $D^u$, respectively.[5, 6, 6a, 6b]

The relationship of the several antigens to each other is such that, for example, anti-D (anti-$Rh_o$) and anti-d (anti-$Hr_o$) differentiate three types of red cells, a blood which is agglutinated by both sera and two cross-specific types (Table 2).

TABLE 2

*Reaction of Red Blood Cells With*

| *Anti-D (anti-$Rh_0$)* | *Anti-d (anti-$Hr_0$)* | *Genotype* |
|---|---|---|
| + | + | *Dd* heterozygous |
| + | o | *DD* homozygous |
| o | + | *dd* homozygous |

A type of blood failing to react with either anti-D (anti-$Rh_o$) or anti-d (anti-$Hr_o$) does not exist and this relationship gives the clue to the genetic theory that factors D and d are allelemorphic to each other just as are factors M and N, or the genes for red and white in roses. Thus, the three genotypes *Dd, DD,* and *dd* are directly differentiated provided that the anti-d serum (anti-$Hr_o$) is also available.

Similarly, anti-C (anti-rh′) and anti-c (anti-hr′) differentiate three types of bloods and the same holds for anti-E (anti-rh″) and anti-e (anti-hr″). In each of the three instances, the capital letters D, C, E refer to Rh factors while the small letters d, c, and e refer to Hr factors.

The incidence of the various Rh-Hr factors in a random white United States population is given in Table 3.

TABLE 3

*Incidence of Positive and Negative Reactions*

| | *Per cent* | |
|---|---|---|
| | + | — |
| Anti-D | 85 | 15 |
| Anti-d | 63 | 37 |
| Anti-C | 73 | 27 |
| Anti-c | 80 | 20 |
| Anti-E | 30 | 70 |
| Anti-e | 97 | 3 |

A discussion of the interrelationship of the three Rh factors is given later in the text along with a description of procedures for the differentiation of individuals who are homozygous or heterozygous for the clinically most important D ($Rh_o$) factor.

## III. Rh-Hr ANTIBODIES

As mentioned above, there are no normally occurring anti-Rh or anti-Hr antibodies. These must be produced by antigenic stimulation of individuals lacking a given factor. It is important to note that the sera of most Rh— individuals who become immunized do not exhibit the usual agglutinins which specifically clump saline suspended red cells. Within the Rh— group, more than 50 per cent of those immunized, produce the so-called "blocking"[14] (incomplete,[15] inhibiting,[16] etc.) antibody which will agglutinate Rh+ cells suspended in any one of several special media, but not saline. Among these may be mentioned normal human serum or plasma,[17] bovine, or human albumin,[18] several varieties of gelatin and a group of non-protein materials such as acacia, polyvinyl alcohol, methyl cellulose, pectin, etc.[1, 19] At present, the reagent of choice is 20–30 per cent bovine albumin.

It is believed that in the course of isoimmunization agglutinins appear first and are then converted to the blocking antibody as immunization continues. However, there is some evidence to indicate that blocking antibodies may arise independently.

Occasionally, sera are found which contain a mixture of agglutinating and blocking antibodies. In these cases the action of the agglutinins is usually suppressed by the presence of the blocking antibodies and the agglutinins will only be detected upon titration, thus revealing a prozone.[14, 20] In other instances the action of the blocking antibody itself may be inhibited, possibly by a third order of antibody resulting in a prozone of the blocking antibody. It has been reported that a third order of antibodies may occur either by themselves or in the presence of agglutinins or blocking antibodies.[21] Prozone containing sera, and those containing third order antibodies alone, may be detected by the reaction of Coombs, Mourant, and Race,[22] to be discussed later (see Section XII).

The several differentiating features of agglutinins and blocking antibodies are tabulated below (Table 4).

A great deal of confusion has resulted from careless and indiscriminate application of terminology to the two contrasting varieties of antibodies. For the sake of completeness and in order to facilitate reference to the literature, a more or less comprehensive list of the terms used by workers in this field is given. However, there is no experimental evidence to justify the retention of many of the terms,

particularly "bivalent" and "univalent." The terms "conglutinin" and "conglutination" have already been assigned by Bordet to an entirely different phenomenon in beef blood, and their use should be discouraged. Initially, the term "blocking" was applied because of the interesting observation that union of the antibody with saline suspended cells rendered these cells resistant to the action of agglu-

TABLE 4

*Characteristics of Human Rh-Hr Agglutinins and Blocking Antibodies*

| | *Agglutinins* | *Blocking Antibodies* |
|---|---|---|
| other terms | saline agglutinins, early immune, complete, bivalent | albumin agglutinins, incomplete hyperimmune (late), univalent, inhibiting, glutinins (agglutinoids), coating |
| suspending media for test cells | saline | bovine or human albumin, serum or plasma, certain hydrophilic colloids |
| typing technic | test tube incubated at 37°-40° C. | warm slides, capillary tube, incubated test tube |
| indications for use | large scale work, generally more accurate, preferable for poor specimens. | rapid results in small scale work on slides |
| stability | destroyed at temperatures above 65° C. | generally more stable |
| incidence of anti-D (anti-$Rh_o$) | low | high |

tinins of identical specificity. This method for the detection of the blocking antibody known as the "blocking test," has now given way to more accurate procedures described below.

## IV. Rh TYPING WITH ANTI-D (ANTI-$Rh_O$) SALINE AGGLUTININS

### A. INTRODUCTION

Human anti-Rh agglutinins were the first reagents employed for clinical Rh typing. Agglutinating sera are preferable for large-scale work since the saline suspended cells are then also available for

simultaneous blood groupings. Agglutinins also give sharper differentiation of positive and negative reactions as compared with those given by blocking antibodies.*

The final product should meet the following requirements recently laid down by the National Institutes of Health[23] :

1. It must give a sharp and clear-cut differentiation of Rh+ and Rh− bloods, without the undesirable properties of rouleaux formation or hemolysis.
2. Specificity: It must react only with the D ($Rh_o$) factor, and no others.
3. Titer: It must have a titer of at least 1:32.
4. The normal anti-A and anti-B must be removed either by absorption with Rh− blood of groups A and/or B or by the addition of the soluble A and B specific substances.
5. Protein content: The final protein content of a diluted serum must not be below 25 per cent of its original protein content. Six per cent bovine albumin or normal serum of group AB may be used as diluents.
6. The serum should be sterile, and when stored at 5° C. or lower should retain its potency for at least one year.

## B. Method of Testing

### 1. *Preparation of cell suspension*

The cell suspension may be prepared either from clotted blood, oxalated blood, or from finger or ear lobe puncture. For large-scale work, clotted blood is recommended since suitable suspensions may be prepared from clots which have been stored in the refrigerator up to 4 or 5 days. (At the same time, serum will be available to test for the presence of antibodies.) Fresh suspensions should be made for tests carried out in any one day's run. Sufficient blood may be obtained by inserting several wooden applicators into the clot and then placing the applicators in test tubes filled with 0.85 per cent saline.

The suspensions are washed and the sediment is resuspended in saline to make an approximately 2 per cent cell suspension.

### 2. *Performance of test*

Using a sterile capillary pipette add one or two drops of the anti-D (anti-$Rh_o$) serum to a small test tube (75 x 10 mm.) placed in a metal rack. The tube then receives two drops of the washed cell suspension, is thoroughly shaken and incubated in a water bath at 37.5°–40° C.

* The use of experimental sera obtained by injection of rhesus blood into animals was abandoned soon after the description of the pathogenesis of erythroblastosis fetalis in 1940–1941.

for 1 hour.* The reaction is enhanced by occasional shaking during the course of the incubation.

### 3. *Readings*

The readings are made by noting the presence or absence of agglutination. However, before disturbing the contents of the tube, the character of the sedimented cells may be examined with the aid of a small hand lens (3x–3.5x–6x).† A round, smooth, and firm sediment indicates the absence of agglutination, while an irregular, crenated, and loose sediment signifies the presence of agglutination. The readings are further facilitated by gentle rotation of the tube just sufficient to disturb the sediment so that the character of the resuspended cells may be noted. Those mixtures in which no agglutination is visible may be examined microscopically, but this should not be necessary with the use of anti-Rh sera which have a titer of at least 1:32.

Readings may be made after shorter incubation (15–30 minutes) and negative or weak reactions may be confirmed by centrifuging the previously numbered test tubes for 2 minutes at low speed (500–1,000 r.p.m.). The quality of the sediment is noted and the cells gently resuspended as described above. Blood showing no agglutination are Rh—.

### 4. *Sources of error*

a. The blood to be tested must be such that the first or second washing gives a clear supernatant. If the blood is hemolyzed or contaminated, another specimen should be requested.
b. Contaminated, out-dated serum, and serum not stored at 5° C. or lower may give false readings.
c. It is well to plan the tests so that numerous bloods are tested at one time. In any event, it is always advisable to have a known Rh+ and Rh— blood to be run as controls.

A blood grouping with anti-A and anti-B sera may be carried out simultaneously using the same saline suspension. As will be shown later, the grouping can be confirmed by testing the normal agglutinin content of the serum with Rh— bloods of groups A and B.‡ The

* In testing numerous bloods, only one pipette need be used for the delivery of the cell suspensions provided that the pipette is rinsed each time in saline.

† With a little practice, the laboratory worker may readily train himself to detect even weak degrees of agglutination with the aid of this or similar type of hand lens, and a good source of light.

‡ Rh— bloods are used in order to avoid reactions attributable to the presence of anti-Rh agglutinins; however, should the serum contain anti-Hr or other agglutinins the results may not correspond with the agglutinogen content of the red cells.

recording on the patient's chart of the blood group as well as the Rh type will facilitate matters in case of emergency transfusion requirements.

*In this discussion the words "group" and "grouping" refer to the OAB system while "type" and "typing" are used to describe the Rh–Hr factors.*

## V. Rh TYPING WITH ANTI-D (ANTI-$Rh_o$) BLOCKING ANTIBODIES

### A. INTRODUCTION

In contrast to agglutinating sera, blocking antibodies must not be used with saline suspensions of red blood cells, nor with saline as a diluent. Typing with blocking antibodies may be carried out either on slides or in test tubes. Many workers prefer the slide test, at least for small-scale work, because positive reactions are obtained within 1 minute.[24]

The diagnostic blocking serum should meet the following requirements recently laid down by the National Institutes of Health.[23]

1. It must give sharp and clear-cut differentiation of Rh+ and Rh— bloods without the undesirable properties of rouleaux formation or hemolysis.
2. Specificity: The serum must react with only the D ($Rh_o$) factor and no others.
3. Titer: The serum should have a titer of at least 1:32.
4. Avidity: When used in the slide test, beginning agglutination on warmed slides should be seen within 1 minute, and clumps the size of 1 sq. mm. should appear within 3 minutes.
5. The normal anti-A and anti-B antibody must be removed either by absorption with Rh— blood of groups A and/or B, or by the addition of soluble A and B substances.
6. Blocking sera may be diluted only with 20 per cent bovine (or human) albumin, with normal human serum or plasma.
7. The serum should be sterile and when stored at 5° C. or lower, should retain its potency for at least 1 year.

### B. TECHNIC OF SLIDE TEST

#### 1. *Preparation of the cell suspension*

In order to carry out the slide test, heavy blood suspensions are required. Freshly oxalated (dry oxalate) blood may be used without further manipulation since it represents about a 40 per cent suspension of red blood cells in plasma.

If clotted blood is to be used, a sufficient number of free cells to

make about a 40 per cent suspension should be separated and resuspended in their own serum, or in serum or plasma of group AB.*

### 2. *Performance of the test*

Place one drop of the blocking serum on a previously warmed flat slide and adjacent to it one or two drops of fresh oxalated blood, or the 40 per cent suspension. Mix with the aid of an applicator, spreading the mixture over a rectangular area of about ¾ x 1 inch. The slides should be placed on a warm illuminated glass surface and rocked slowly from side to side for 2 minutes. Flat slides are preferable to "well" slides. Since blocking antibodies behave like "warm agglutinins" the slides should be preheated by placing them over a warm surface, the temperature of which should be about 40° C.

### 3. *Readings*

a. *Positive reaction*: Beginning agglutination should be seen within 30–60 seconds when sera are used which meet the requirements laid down by the National Institutes of Health. At the end of 2 minutes almost all red cells should be clumped with many large masses visible.
b. *Negative reaction*: Rh— bloods are characterized by the absence of clumps so that at the end of 2 minutes the cells remain smooth and uniform in appearance to the naked eye.†

It is recommended that Rh+ and Rh— control bloods always be included in any single run of tests.

### 4. *Sources of error*

a. The best results are obtained with freshly drawn oxalated blood. It is important not to use large amounts of oxalate since the reaction is inhibited as the salt concentration is increased.‡ [25]
b. The reactions are less distinct with stored blood, while contaminated blood will give entirely unreliable results.
c. Blocking sera which exhibit the phenomenon of pseudoagglutination (rouleaux formation) should not be used. However, the property may be inherent in the serum or plasma used for the suspension.

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* A drop of finger blood may also be tested directly on a slide provided that no anticoagulant is added to the mixture of cells and serum.

† Some producers of anti-D slide test serum are now recommending a final reading at the end of one minute.

‡ It is for this reason that the practice of adding a drop of "finger blood" to a few crystals of oxalate on a slide is not recommended.

d. Confusing effects may be produced by drying or prolonged standing of the test. This source of error will be eliminated if the slides are discarded at the end of the 2 minute reading.

e. False negative reactions may result from the use of too dilute a cell suspension, insufficient heat, or excessive amounts of oxalate or other anticoagulants.

f. On prolonged standing Rh— bloods of type dCe (rh′) may give weak reactions with potent anti-D (anti-$Rh_o$) slide test sera. Consequently, it is desirable to check all doubtful and negative reactions by testing saline suspended cells with an agglutinin serum in the test tube.

g. False-positive reactions may be obtained in those rare cases where an autoantibody "coats" the patient's red cells so that they are sensitized. This condition exists in acquired hemolytic anemias and possibly other diseases. When such sensitized red cells are mixed with 20 per cent bovine albumin, a component of most slide test sera, clumping may occur. Unless this sensitization is detected, Rh-negative blood may be wrongly classified as Rh-positive. The "coating" in these cases may be demonstrated by the use of anti-human serum for the Coombs test. To type properly such "coated" cells it is necessary to use the test tube test with Anti-D (Anti-$Rh_o$) saline agglutinins.

## C. Test Tube Typing Employing Blocking Antibodies

Rh typing with blocking antibodies may also be carried out in test tubes, but the blood suspension, as in the case of the slide test, must not be in saline.

### 1. *Suspending media*

Unpublished data indicate that clear-cut results are more readily obtained with bovine albumin as the red cell suspending medium if the blocking serum is used undiluted or diluted with serum of group AB. Should the serum be diluted with 20 per cent bovine albumin (as is the case with many commercial products) it is preferable to suspend the red cells in serum of group AB, or serum obtained from the blood to be tested. However, it is not probable that difficulties will be experienced in any of the combinations mentioned if fresh cells are tested with a blocking serum which has a titer of at least 1:32.

Although plasma suspended cells give good reactions, clot and rouleaux formation will frequently result when plasma and serum are mixed. This is true only in using the test tube method when fibrin has

been found to enclose most of the red cells, thus interfering with the reaction.

The use of the hydrophilic colloids (acacia, pectin, polyvinyl alcohol, methyl cellulose, gelatin, etc.) as media for suspending the red cells cannot be recommended because of their tendency to cause strong rouleaux formation which may be confused with true agglutination. It is sometimes possible to overcome this tendency by the addition of small amounts of saline at the completion of the incubation period. This usually results in the breaking up of the rouleaux formation only.[19]

### 2. *Technic of test and readings*

With the test tube method more dilute blood suspensions are used, preferably about 2 per cent. Equal amounts (2 drops) of the diagnostic blocking serum and cell suspensions are mixed in small test tubes (75 x 10 mm.) and incubated at 37.5°–40° C. for 30–60 minutes. Greater care must be taken in making the reading because the sediment in negative reactions differs from that in the tests with agglutinins. Less emphasis should be placed on the appearance of the sediment and more emphasis on the presence of clumps of agglutinated cells which persist on more vigorous resuspension. If centrifugation is employed after short incubation in order to accelerate the reaction, higher speeds are required, probably because of the greater viscosity of the reagents. At all times suitable control bloods should be included.

Chown [26] has described a sensitive method for Rh typing in which the blood suspension and the typing serum are mixed in small capillary tubes. This method, which may also be applied for detection of antibodies, is an economical procedure, which has been adopted by a number of workers.

## VI. THE SUBTYPES OF THE Rh SYSTEM

### A. INTRODUCTION

Prior to the introduction of the British terminology of **DCE** for the Rh factors, and **dce** for the corresponding Hr factors, a great deal of confusion existed regarding the subtypes of Rh because, in the historical development of the subject, the emphasis was wrongly placed on the serological and genetic relationship of the Rh factors to each other without any reference to the Hr factors. Another circumstance which added to the confusion was the rapid and unreasonable series of changes in the terminologies of both the antibodies and the antigens.

A discussion of the subtypes is intelligible only in terms of the DCE nomenclature since it indicates directly and concisely the antigenic components present in the red blood cells. In contrast to the series of earlier terminologies, which represent an abbreviated and inaccurate antigenic structure, the DCE system is rapidly gaining acceptance because " it is simple and direct, both typographically and genetically." [27]

### B. Value of Subtype Study

A study of the subtypes is necessary in a comparatively small group in which isoimmunization is brought about by factors other than D. The determination of the subtypes is also important for the differentiation of homozygous and heterozygous Rh+ husbands of Rh— mothers.

### C. Method for Determining Subtypes

When Rh+ and Rh— individuals are tested with anti-C serum they are divided into two unequal subtypes and further divided into a total of eight subtypes when tested with anti-E antibodies. These relationships, without reference to the further subdivisions resulting from tests with the three anti-Hr sera are given in Table 5. As required by the genetic theory, the corresponding Hr factor is also indicated for those types lacking a particular Rh antigen.

The subtypes can be demonstrated either with the aid of agglutinins or blocking antibodies. As in the case of anti-D agglutinins and blocking antibodies, the sera required for the finer analysis of the Rh–Hr complex should be properly standardized as to titer and specificity as given above.

Typing with an anti-C or anti-E agglutinin is carried out in the same manner as given for anti-D agglutinins. Similarly, if anti-C or anti-E blocking antibodies are available, either the slide or test tube method may be used, provided that the red cells to be tested are prepared as given in Section V, B, 1.

Sera containing only anti-C antibodies may be produced by Rh+ individuals of types DcE or Dce, but these are exceedingly rare. Most frequently, anti-C antibodies of the agglutinin variety will be found in the sera of Rh— mothers along with anti-D blocking antibodies. Such sera may safely be used with saline suspended cells in the test tube since blocking antibodies will not react under these conditions.

The clinical data indicate that the D factor is far more antigenic than the combined total of the other five Rh–Hr factors.[28] About 93 per

TABLE 5

| Reaction with anti-D | Antigen present | % Incidence | Reaction with anti-C | Antigens present | % Incidence | Reaction with anti-E | Antigens present (Phenotype) | Wiener Terminology | % Incidence | |
|---|---|---|---|---|---|---|---|---|---|---|
| + | D | 85 | + | DC | 68 | + | DCE | $Rh_1Rh_2$ | 14 | Rh+ |
| | | | | | | — | DCe | $Rh_1$ | 54 | |
| | | | — | Dc | 17 | + | DcE | $Rh_2$ | 15 | |
| | | | | | | — | Dce | $Rh_0$ | 2 | |
| — | d | 15 | + | dC | 1 | + | dCE | rh′rh″ | Rare | Rh— |
| | | | | | | — | dCe | rh′ | 1 | |
| | | | — | dc | 14 | + | dcE | rh″ | 1 | |
| | | | | | | — | dce | rh | 13 | |

cent of the individuals found to be immunized produce anti-D either alone or occasionally in combination with other antibodies (anti-C, anti-E, etc.). The remaining 7 per cent, who are Rh+, constitute the principal source of antisera other than anti-D and anti-C, so that the supply of such sera is necessarily limited and may not always be available. This state of affairs may be improved by the recent program of isoimmunization of volunteer donors [29, 30] or by future developments in experimental immunization of animals.*

## VII. REMARKS ON SELECTION OF DONORS

For transfusion purposes, it is not necessary to obtain a complete identity of antigenic structure of the bloods of patient and donor. The main objective is to prevent isoimmunization and hemolytic intra-group transfusion reactions attributable to the main antigenic factor D. Provided that a suitable compatibility test for detection of intra-group antibodies is carried out, it suffices in almost all instances to select Rh— donors for Rh— patients and Rh+ donors for Rh+ patients.

If anti-C and anti-E sera are available, it is desirable to screen out from among the Rh— donors those individuals whose blood contains the factors C and E. This will eliminate the possibility of isoimmunization involving these factors.

When a cross-matching test discloses an incompatibility within the Rh— group, it usually involves the C factor. Assuming the patient to be of type cde who produced anti-C, in addition to anti-D, an Rh— donor whose blood contains the C factor is not compatible. The same rule holds for transfusing the affected infant whose mother produced anti-D and anti-C. Such compatible donors may be selected with the aid of the anti-C serum or one which contains anti-C as well as anti-D.

The use of an Rh typing serum which contains both anti-D and anti-C (87 per cent positive reactions) for selection of donors for Rh— patients is an expedient measure since it will exclude the 2 per cent of Rh— individuals who are of type dC (rh′). But *only* prospective donors may be tested with the 87 per cent serum.† All patients (pregnant women or candidates for transfusion) should be typed with anti-D

* In contrast to A, B, or M and N, the Rh-Hr factors are poorly antigenic in animals. The only variety produced experimentally in rabbits and guinea pigs is probably anti-D,[31] but its use for routine testing is not recommended since the experimental serum falls far short of meeting the specifications of N. I. H.

† The routine practice in some blood banks is first to screen out all bloods which are negative with anti-D (85 per cent). These Rh— bloods are then tested with anti-DC (87 per cent), which detects the C factor if present, and checks the result of the test for D.

(85 per cent), to avoid wrongly classifying the 2 per cent of type dC as Rh+.

## VIII. ISOIMMUNIZATION OF Rh-POSITIVE MOTHERS

A finer antigenic analysis is indicated in the study of bloods of Rh+ mothers of erythroblastotic infants. Most frequently, the specific factor involved will be revealed in a comparative study of the bloods of the husband, mother, and affected infant. These findings will serve as a guide to the specificity of the antibody the mother is apt to produce.

In rare instances it will not be possible to demonstrate a specific difference involving any of the six Rh–Hr factors, in which case additional tests should be carried out to detect antibodies specific for a special and probably rare factor which will be found in the blood of the husband and affected infant. Agglutination by the mother's serum of the husband's cells suspended in his serum, serum of group AB or bovine albumin is proof that the mother has been immunized by a dominant hereditary factor transmitted to the offspring. It is, of course, assumed that an incompatibility of the A or B factors is excluded.* Once an immune antibody is demonstrated, its nature, whether agglutinin or blocking antibody, its specificity and titer can then be determined.

When all intra-group differences are excluded, one may suspect that an incompatibility of the factors A or B is responsible for erythroblastosis. In such cases, the infant should be a non-secretor and titration of the mother's serum should reveal a specific increase of the corresponding antibody.[32] It is recommended that these infants be transfused with group O blood to which the soluble A and B Witebsky substances[33] have been added.

## IX. DIFFERENTIATION OF HOMOZYGOUS AND HETEROZYGOUS Rh-POSITIVE INDIVIDUALS

### A. IMPORTANCE

From a clinical viewpoint, it is important to determine the genotype of the Rh+ husband of an Rh− wife (genotype *dd*). If he is

* The presence of an atypical antibody where there is an incompatibility of the A or B factors can be detected by first neutralizing the mother's serum with soluble A and B substances. The completely neutralized serum may then be studied both qualitatively and quantitatively with the husband's blood and suitable controls.

homozygous (*DD*), i.e., he inherited from each of his parents a gene *D* which determines the Rh+ reaction, all their offspring must be Rh+, and each pregnancy offers an opportunity for immunization. If the husband is heterozygous, having inherited a gene *D* from one of his parents, and from the other parent a gene *d*, which determines the Rh— reaction, his genotype is given as *Dd*. In a mating of *Dd* x *dd*, 50 per cent of the offspring will be Rh— and the outlook for future pregnancies is more favorable. These two contrasting matings are shown in Table 6.

TABLE 6

*Incompatible Matings*

| | *Homozygous Father* | *Heterozygous Father* |
|---|---|---|
| | ♂ ♀ | ♂ ♀ |
| Genotypes | *DD* x *dd* | *Dd* x *dd* |
| Genes in Gametes | *D* ←→ *d* | *D* ←→ *d*<br>or ↗<br>*d* ↙ |
| Offspring | 100% *Dd* Rh+ | 50% *Dd* Rh+<br>50% *dd* Rh— |

## B. PRESUMPTIVE METHOD

Reference to Table 2 shows that Rh+ genotypes *DD* and *Dd* can be differentiated since the former blood will fail to agglutinate with anti-d. Unfortunately, the test with anti-d serum cannot be routinely performed since this antibody is exceedingly rare. However, with the aid of the more common anti-c (anti-hr′) serum, a presumptive diagnosis of the genotype may be made on the 54 per cent of individuals who are of type DCe ($Rh_1$). Those DCe individuals whose blood fails to react with anti-c, and are therefore homozygous for C (*CC*), are presumed to be also homozygous for D (*DD*). This assumption is based on the probability that DCe individuals inherit from each of their two parents the same gene combination, namely, *DCe* which is the most frequent combination in the white population. Such indviduals therefore have the genotype *DCe/DCe*. Those DCe individuals whose blood reacts with anti-c are presumed to be heterozygous also for D (*Dd*), since they very likely inherited from one of their parents the common gene combination *dce*, and have, therefore, the genotype *dce/DCe*.

However, an individual of type DCe may inherit the rare combination of *Dce* from one parent and the frequent *DCe* from the other parent. His genotype is then *Dce/DCe,* and although heterozygous for C he is obviously homozygous for the clinically more important D factor. This rare genotype is calculated to occur in about 2 per cent of the population. In other rare instances he may inherit the infrequent combination *dCe* from one parent and have the genotype *dCe/DCe* in which case he is heterozygous for D but homozygous for C. Accordingly, the use of anti-c serum can give only a presumptive diagnosis of the homozygous or heterozygous nature of the D factor.

For the 17 per cent of Rh+ individuals who are of types Dce or DcE, the presumptive method of determining the genotype involves a greater error, but the majority of them are heterozygous. A fuller discussion of this subject is given by Mollison, Mourant and Race.[44]

Almost all bloods of type DCE ($Rh_1Rh_2$) occur as genotype *DCe/DcE* and are heterozygous for C but homozygous for D. This rule is not applicable in the rarely occurring genotypes *DCe/dcE* or *cDe/CdE,* both of which are heterozygous for C and D.

### C. FAMILY STUDY METHOD

Without the aid of anti-Hr sera, the genotype may be sometimes determined by studying the blood of the children or the parents of the Rh+ individual. He is heterozygous for the clinically most important D factor if any one of his children, or if one of his parents, is Rh—.

## X. DETECTION OF INTRA-GROUP ISOIMMUNIZATION BY THE Rh-Hr BLOOD FACTORS

### A. INTRODUCTION

In a complete Rh testing laboratory it is important not only to screen out the Rh— pregnant women and Rh— patients but also to determine whether or not they have been immunized by Rh+ fetal blood, transfusion, or a combination of both factors. In the early work on erythroblastosis fetalis, a panel of at least 7 group O, Rh+ and one Rh— blood was used in the tests to detect antibodies.[7, 34] It is now possible to test the patient's serum with only one carefully

selected blood which contains the three Rh factors, D, C, and E, and two of the three Hr factors, c and e. The test is so devised that with the aid of two test tubes (10 x 75 mm.) each serum is tested for the presence of agglutinins and blocking antibodies specific for any one of the five antigens mentioned. Once an immune isoantibody is detected its titer and specificity (anti-D, anti-C, and anti-E, anti-c, anti-e, or a combination of these several antibodies, or still other antibodies) can then be determined.

### 1. *Selection of the test blood*

A freshly drawn DCE blood group O should be used. This blood will probably contain also the antigens c and e. In the absence of a blood of this type, two group O bloods of types DCe ($Rh_1$) and DcE ($Rh_2$) may be pooled. In using the mixture of these two bloods, the antigen d will also be included. Since freshly drawn bloods vary in their agglutinability, the test blood should be selected on the basis of good reactivity.

If a random group O, Rh+ blood is to be used it may be possible to detect only those Rh— patients who produced anti-D antibodies. Under these conditions patients who produce anti-C or anti-E, or any of the three varieties of anti-Hr antibodies may be missed. Actually, every Rh+ blood must have at least two other antigenic components, and may have as many as five others.

### 2. *Preparation of cell suspensions*

a. *For detection of agglutinins*: The selected group O test blood is washed several times in 0.85 per cent saline. A part of the sediment is suspended in the saline solution to make about a 2 per cent cell suspension.

b. *For detection of blocking antibodies*: Another portion of the above mentioned sediment, from which all saline has been carefully removed by aspiration, is suspended in 20–30 per cent bovine albumin to make a 2 per cent suspension. For the sake of economy, it is advisable to prepare only small amounts of this suspension, sufficient for the day's run. Bovine albumin is the reagent of choice, but, if it is not available, a pool of normal, preferably male, adult serum may be employed. As previously mentioned, a mixture of plasma and serum frequently results in formation of rouleaux and fibrin. For this reason also a mixture of plasma and albumin is not recommended.

### 3. *The test*

To each of two tubes add two or three drops of the patient's serum.* One tube then receives two drops of the saline suspended cells and the second receives two drops of the albumin suspended cells. The racks containing the test tubes are shaken and incubated at 37.5°–40° C. for 60 minutes, at the end of which time readings may be made. Reincubation for an additional hour or two is desirable for the detection of weak reactions.†

### 4. *Readings and interpretation*

Readings are made as given in the sections on Rh typing with agglutinins and blocking antibodies. The presence of agglutinating antibodies is indicated by a positive reaction with the saline suspended cells. Invariably, this serum will also agglutinate the albumin suspended cells. When blocking antibodies alone are present, only the albumin suspended cells will be clumped.

Again, a word of caution is necessary in the reading of the sediment of albumin suspended cells, since it may appear irregular and crenated, in the absence of agglutination.

In all cases in which the test for agglutinins is negative, the same test tube mixtures may be saved and subjected to the indirect Coombs test (Section XII, D, 2).

### 5. *Titration*

a. *Agglutinins*: The potency of an agglutinating antibody is determined by making progressively double dilutions, in 0.1 ml. amounts, of the serum in saline and testing each dilution with 2 drops of the saline suspended test cells.

b. *Blocking antibodies*: In these tests, pooled normal male serum is used as a diluent instead of saline. Progressively double dilutions are made in 0.1 ml. amounts and 2 drops of the albumin suspended cells are added.

In both cases, incubation and readings are made as in the respective qualitative tests. The titer is the reciprocal of the highest dilution in which agglutination is still present.

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* An additional two test tubes are required if the serum is tested for its content of anti-A or anti-B (confirmation of blood groupings). As already mentioned, Rh— bloods of groups A and B are preferable.

† Hattersley[86] reported that immediate, intensive centrifugation with no incubation, when testing for blocking antibodies with albumin suspended cells, will give excellent results. All negative tests are then checked by incubation for the usual period of 1 hour or more.

### B. THE PROZONE PHENOMENON

Occasionally, sera are observed which, although giving negative or weak reactions in the qualitative test, give strong and distinct reactions when titrated. Such sera exhibit the prozone phenomenon. This will not be a source of error in the case of agglutinins which exhibit a prozone, since the blocking antibody responsible for this prozone will be readily detected in its qualitative test.

When a blocking antibody exhibits a prozone, a serious error may result if additional tests are not made routinely.[35, 36] If the history suggests isoimmunization such sera should be titrated in spite of a negative qualitative test. In any Rh testing service, however, it is highly desirable to detect all instances of isoimmunization in a qualitative test, particularly in the absence of histories. This objective can be attained by routinely testing with albumin suspended cells a 1:8 dilution (pooled normal male serum as a diluent) of the patient's serum or preferably by carrying out the test of Coombs, Mourant, and Race which will detect all instances of prozone.

Another method to overcome the effect of a prozone in blocking sera is by immediate intensive centrifugation of the mixture without any incubation as suggested by Hattersley.[36] It is remarkable that the agglutination cannot be demonstrated if the centrifugation is carried out at the end of the usual incubation period of 1 hour, or even shorter intervals.

### C. TESTS TO DETERMINE SPECIFICITY OF ANTI-RH-HR ANTIBODIES

Once a serum containing antibodies is found, tests should be carried out to determine the variety of antibody or antibodies produced. By and large, it is not profitable to carry out such tests unless the serum has a titer of 1:4 or higher, because some of the test bloods may be poorly reactive with weak antibodies. As mentioned above, about 93 per cent of all sera will contain anti-D either alone or, occasionally, in association with anti-C, anti-E, or rarely still others. The antigenic content of the husband's blood, or the blood of the affected infant, will serve as a guide to the specificity of the antibody to be expected.* In the case of Rh+ mothers, a comparison of the antigenic content of both parents' bloods may immediately disclose, for instance, only one incompatibility. Thus, in a mating where the father is type DcE and

* Rarely, exceptions have been noted as indicated by the production of anti-C as well as anti-D, although the C antigen was lacking in the immunizing blood.[37]

the mother is type DCec, the latter may be expected to produce only anti-E. If the mother were homozygous for the C factor, she might, perhaps, produce anti-c as well as anti-E. In all cases of this sort, it is advisable to determine the genotype of both parents, but at present one is limited because some of the Hr sera (anti-d and anti-e) are not generally available.

### 1. *Determination of specificity by panel of test cells*

In practice, it is necessary to test the patient's serum with carefully selected group O or group compatible bloods of different Rh–Hr patterns. By analyzing the reactions obtained, the specificity of the antibody may be readily determined in the vast majority of cases. Illustrative examples of tests to determine the specificity are given in Table 7. These should be confirmed in tests of numerous bloods of all types.

TABLE 7

*Determination of Specificity of Antibodies in Human Sera*

| | *Test Cells* | | | *Reactions with Antiserum* | | | | |
|---|---|---|---|---|---|---|---|---|
| | *Fisher-Race* | | *Wiener* | | | | | |
| *No.* | *Phenotype* † | *Genotype* * | *System* | *I* | *II* | *III* | *IV* | *V* |
| 1 | DCEc | *DCe/DcE* | $Rh_1Rh_2$ | + | + | + | + | + |
| 2 | DCe | *DCe/DCe* | $Rh_1$ | + | + | + | 0 | 0 |
| 3 | DcE | *DcE/dce* | $Rh_2$ | + | + | 0 | + | + |
| 4 | Dce | *Dce/dce* | $Rh_o$ | + | + | 0 | 0 | + |
| 5 | dCec | *dCe/dce* | rh′ | 0 | + | + | 0 | + |
| 6 | dCEc | *dcE/dCe* | rh′rh″ | 0 | + | + | + | + |
| 7 | dcE | *dcE/dce* | rh″ | 0 | 0 | 0 | + | + |
| 8 | dce | *dce/dce* | rh | 0 | 0 | 0 | 0 | + |
| | | | Specificity: | anti-D | anti-D plus anti-C | anti-C | anti-E | anti-c |

† Incomplete—based on tests with available sera.
* Based on the most frequent combinations of genes.

In some instances, where more than one antibody may have been produced, it will be difficult to determine the specificity by a panel of test cells. This has been observed, for example, in Rh+ patients of genotype *DCe/DCe* who have received multiple transfusions. Individuals of this type may produce both anti-c and anti-E. If the specific

antibodies produced cannot be tested separately, one as an agglutinin and the other as a blocking antibody, a panel of test cells will not distinguish between anti-c and a mixture of anti-c and anti-E except with the aid of the rarest of all bloods, genotype *dCE/dCE*. In these cases one must resort to quantitative studies. Thus, if cells of type dce reveal consistently lower titers than cells of type DcE, one may assume the presence of a weak anti-c along with a stronger anti-E. In the absence of differences in titer, and also as a further check, a selective absorption with cells of type dce may be carried out. This may be expected to unmask the anti-E antibody if present.

If the anti-E were an agglutinin and the anti-c a blocking antibody, the specificity would have been more easily determined by a double panel of test cells suspended in saline and in 20 per cent bovine albumin.

Specificity tests may be carried out either for agglutinins or blocking antibodies provided that the proper suspending medium is used. One should be prepared to find sera which may contain both sorts of antibodies of the same or different specificities. The most common example of the latter occurs in the serum of an Rh— mother which contains anti-C agglutinins and invariably also anti-D blocking antibodies.

### 2. *Determination of specificity by frequency method*

Table 7 includes the several varieties of antibodies most commonly found. Because anti-d and anti-e sera are not available for selection of standard test bloods, their presence in any given serum must be determined by other means, principally the incidence of positive and negative reactions in a random white population (see Table 3). In addition, an anti-d serum will always give positive and frequently stronger reactions on the homozygous Rh— individuals (*dd*) and will split the 85 per cent Rh+ group into about 48 per cent positive for d (heterozygous *Dd*) and about 37 per cent negative who must be homozygous for D (*DD*). A final confirmation may be had by tests on selected rare bloods whose genotype has previously been determined by family studies. For example, a blood of genotype *DcE/dcE* must not react with a suspected anti-e serum.

### 3. *Johannsen formula*

In the absence of the antisera for d, c, and e, the incidence of these factors was first determined by the Johannsen formula which is based on the application of the binomial theorem to the gene frequencies.[1, 28] This formula is useful in any system in which there are two allelic

genes and three types (phenotypes) corresponding to the three genotypes. For example, gene $d$ is equal to the square root of the percentage of non-reactors with anti-D:

$$
\begin{array}{lcll}
\text{Frequency of gene } d & = & \sqrt{15} & = 3.87 \\
\text{Frequency of gene } D & = & 10 - 3.87 & = 6.13 \\
(D + d)^2 & = & DD + 2Dd + dd & = 100 \\
DD & = & (6.13)^2 & = 37.6 \\
Dd & = & 2 \times (6.13 \times 3.87) & = 47.4 \\
dd & = & (3.87)^2 & = 15
\end{array}
$$

($DD + Dd$ = 85%; $Dd + dd$ = 62.4%)

Similarly, an anti-e serum should split the 30 per cent of bloods containing the factor E into 27 per cent positive for e (genotype $Ee$) and 3 per cent negative for e (genotype $EE$). Accordingly, the incidence of positive reactions with anti-e = 70 per cent ($ee$) + 27 per cent ($Ee$) = 97 per cent. The incidence of the genotypes $CC$, $Cc$ and $cc$ may be determined by similar application of Johannsen formula.

## XI. MODIFIED COMPATIBILITY TEST (CROSS-MATCHING) FOR TRANSFUSIONS

Compatibility tests have been carried out in the past by testing the patient's serum with group-compatible donors' cells suspended in *saline*. If agglutination occurs with this method, it may indicate either (1) an error in blood grouping, or (2) the presence of atypical, normal, or immune isoagglutinins in the patient's serum. In view of recent knowledge of the existence of antibodies which fail to agglutinate saline suspended cells (blocking antibodies), the time-honored method of handling the donor's cell suspension must now be supplemented.

In order to exclude an incompatibility attributable to the presence of blocking antibodies in the patient's serum, one of the previously described methods for their detection should be applied. Accordingly, the slide test may be used in which one drop of the prospective donor's oxalated blood (or a 40 per cent suspension in albumin or serum) is added to one drop of the patient's serum on a warmed slide. The readings are made as for the slide test.

An objection to the slide test is the presence of pseudoagglutination associated with rapid sedimentation in the blood of many patients requiring transfusions. Accordingly, preference should be given to the procedures mentioned below.

In an alternative method, the donor's cells are suspended in the donor's own serum, in normal serum of group AB, or in 20–30 per cent

bovine albumin to make a 2 per cent cell suspension. The test is carried out exactly as given in the section on the detection of blocking antibodies using the test tube technic.

In all cases, the indirect Coombs test may also be used as a final check on the compatibility test. This test is extremely useful in the compatibility test where a pilot tube blood preserved for 2–3 weeks is used, since the agglutinability of such blood in the direct test for blocking antibodies may be diminished.

When using Rh— donors exclusively for Rh— patients and Rh+ donors for Rh+ patients, agglutination under the above conditions will indicate an incompatibility attributable to isoimmunization by finer differences within the Rh— or Rh+ groups. If the test of patient's serum and donor's cells prepared as given above is negative, the donor can be declared compatible.

## XII. THE TEST OF COOMBS, MOURANT, AND RACE

### A. Introduction

A valuable aid in (1) the diagnosis of erythroblastosis of the newborn, and (2) the detection of isoimmunization, is the anti-human globulin test of Coombs, Mourant, and Race. This test is based on the specific reaction of a rabbit anti-human globulin (precipitin) serum with red cells which have absorbed on their surface a human immune globulin fraction. The globulin fraction involved is almost always the blocking antibody, which although combined with its specific antigen in the red blood cells, fails to produce clumping. Such sensitized red cells react with the precipitin serum to produce the visible effect of agglutination.

The use of the phrase "anti-human globulin test" is too unwieldy for either written or oral purposes. Hill and Haberman [37] suggested the name "developing test" because of the analogy to the development of a sensitized photographic film. More recently it has been referred to as the direct or indirect Coombs test, depending on its mode of application.

### B. Preparation of Rabbit Anti-Human Serum

#### 1. *Inoculation of Rabbits*

Rabbits are immunized by a series of injections of human serum until a titer of about 1:10,000 or higher is developed. It is not necessary nor desirable to inject a processed globulin fraction. For this

purpose, group O serum is employed since it avoids the possibility of immunization against the soluble group-specific A and B antigens which may be present in other sera.

A course of immunization which has proved satisfactory consists of six intravenous injections of 0.5 ml. serum given over a period of 2–3 weeks. One week after the last injection small test bleedings are made. Rabbits having a sufficiently high titer (at least 1:10,000 by the antigen dilution method) will provide a satisfactory precipitin serum. These rabbits are then submitted to a large bleeding and the blood is allowed to clot. After the serum is separated, it is sterilized by filtration and may now be stored at 5° C. or lower. The precipitin serum is not yet ready for use, however, since it usually contains antibodies for human erythrocytes which must be removed by absorption.

### 2. *Absorption of the precipitin serum*

Portions of the serum, inactivated at 56° C. for 20 minutes are diluted about 1:10 in saline and are absorbed with thoroughly washed packed red cells of groups O, A, and B in order to remove agglutinins for all human red cells. Because it is essential to remove all traces of serum from the absorbing bloods, they should be washed at least four or five times with 10 volumes of saline. It is obvious that the presence of any residual serum in the absorbing bloods will result in a diminution of the specific antibody involved in the reaction.

As a rule, one volume of the 1:10 saline dilution of the rabbit serum is absorbed with one-half volume of the washed packed mixture of blood sediment. The absorption is carried out either at room temperature or at 37.5° C. for 1 hour. The treated serum is then separated by centrifugation and tested against a panel of thoroughly washed groups O, A, and B cells, including the three absorbing bloods, in order to determine the completeness of the absorption for all human erythrocytes. At least thirty fresh bloods should be used in this test. If necessary, another absorption with ¼ volume of the washed packed blood sediment of those cells which still react, is performed.

### 3. *Standardization of the absorbed precipitin serum*

To check the sensitivity of the absorbed serum and to determine the working dilution at which it may be used, a cross-titration is set up in which various saline dilutions of the absorbed precipitin serum are tested with red cells which have been deliberately sensitized with de-

creasing amounts of a known blocking antibody. This is done by serially diluting a fairly high titered blocking antibody in saline and adding to it saline suspended red cells. For this purpose, the saline suspended cells and dilutions of the blocking antibody are mixed in quantities of about 1.0 ml. each. After 1 hour's incubation at 37.5° C., the cells are washed three times with tubesful (volume 8 ml.) of saline and then brought up to their original 1.0 ml. volume. Two drops of each of the red cell suspensions, now sensitized with decreasing amounts of blocking antibody, are then added to 2 drops of each of the dilutions of the absorbed precipitin serum. The tests may be incubated either at room temperature or at 37.5° C. for 15–30 minutes, and the readings for agglutination are made as described in Section XII, C, 2.

The results of a typical cross-titration of a precipitin serum with Rh+ sensitized cells are given in Table 8.

It is evident that the Coombs test is somewhat more sensitive than the direct reaction with albumin suspended cells. The results also indicate that the complete titer of the blocking antibody can be demonstrated with a dilution of 1:8 of this absorbed precipitin serum. Accordingly, it is safe to use the serum in a working dilution of 1:2 which represents a 1:20 dilution of the original rabbit immune serum.

## C. Diagnosis of Erythroblastosis Fetalis by Testing Cord Blood

### 1. *Importance*

In practically all cases of erythroblastosis fetalis due to blocking antibodies, the diagnosis can be confirmed or established by a serological study of the cord blood obtained at delivery.[22] The most important tool for this study is the Coombs test which has the capacity to detect the intrauterine sensitization of the cord cells by maternal blocking antibodies. Such cells, which have escaped hemolysis before birth, are labelled for possible destruction soon after delivery. A similar intrauterine sensitization or coating apparently does not occur when the mother has agglutinating antibodies alone. In instances in which the mother's serum appears to contain only agglutinins, a positive Coombs test on the infant's cells indicates the presence also of blocking antibodies which were masked by a higher titer of agglutinins.

The serum of the cord blood should also be tested for the presence of passively transferred maternal antibodies. This will usually, but not always, confirm the diagnosis of erythroblastosis fetalis. In some instances, the complete titer of maternal antibodies may be demon-

**TABLE 8**

**A. CONTROL TITRATION OF BLOCKING ANTIBODY USED IN B**

*Diluted in Pooled Male Serum and Tested with Albumin Suspended Cells*

| Diluted 1: | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 |
| ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++ | + | ± | 0 |

**B. CROSS-TITRATION OF PRECIPITIN SERUM AGAINST CELLS SENSITIZED WITH DECREASING AMOUNTS OF BLOCKING ANTIBODY**

| *Dilutions of Absorbed Precipitin Serum in Saline 1:* | *Saline Suspensions of Cells Coated with Blocking Antibody Diluted in Saline 1:* | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 |
| 1 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++ | + | 0 |
| 2 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++ | + | 0 |
| 4 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++ | + | 0 |
| 8 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | +± | ± | 0 |
| 16 | ++++ | ++++ | ++++ | ++++ | ++++ | +++± | ++± | + | 0 | 0 |
| 32 | ++++ | ++++ | ++++ | ++++ | +++ | ++± | + | ± | 0 | 0 |
| 64 | ++++ | ++++ | +++ | ++± | ++ | + | ± | 0 | 0 | 0 |
| 128 | +++ | ++± | ++ | + | + | 0 | 0 | 0 | 0 | 0 |
| 256 | +++ | ++ | ++ | + | 0 | 0 | 0 | 0 | 0 | 0 |
| 512 | +± | + | + | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

strable in the cord serum, but the cells are not sensitized and the infant will be normal because it is genetically Rh— like the mother. This may occur in cases where the father is heterozygous, and the maternal antibodies are residual from an earlier immunization (pregnancies with affected infants or transfusions).* In other cases, no, or only a low titer of maternal antibodies will be found in the cord serum, but nevertheless, the cord cells may be so strongly coated with maternal blocking antibodies that all receptors are saturated and the blood cells will fail to agglutinate with anti-D agglutinating serum. In the differentiation of these strikingly different cases the Coombs reaction is exceedingly useful (see Table 9).

Demonstration of cord cell sensitization is also most valuable in those cases in which the infant, apparently normal at birth, develops symptoms after several days. In cases of this sort, the onset of the disease may be anticipated so that the proper therapeutic measures may be instituted immediately. For this reason, a Coombs test on the cord blood may well be carried out in all incompatible matings at delivery, regardless of the clinical condition of the infant. This measure will also serve to detect unsuspected cases in which antibodies in the mother, for one or another reason, were overlooked. The test should be carried out on the cord blood as soon as it is obtained, along with an Rh typing, blood grouping, tests for antibody content of the cord serum and hematologic studies.

### 2. *Technic and readings*

A saline suspension of cord cells is washed thoroughly (three or more times) in an excess of saline in order to remove all traces of serum and 2 drops of a 2 per cent suspension of the washed cells is added to 2 drops of the anti-human serum.

With strongly sensitized cells, agglutination will appear almost immediately when the tube (10 x 75 mm.) is examined with the aid of a hand lens, or the contents placed on a slide and rocked to and fro. However, occasional shaking plus incubation at room temperature, or 37.5° C. for 15–30 minutes will aid in the detection of cells which are only weakly sensitized. Final readings are made after light centrifugation (500–1,000 r.p.m.) for 1 minute and gentle resuspension of the sediment.

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* Rarely, nonspecific increases in titer have been observed due to the anamnestic stimulus of an Rh— fetus[41] or due to the inoculation of antigens such as pertussis or typhoid vaccines.[42]

TABLE 9

*Correlated Studies on Cord Blood*

| Case | Red Blood Cells Tested with: Anti-$Rh_o$ Agglutinin | Red Blood Cells Tested with: Coombs Reagent | Serum, Test Cells Suspended in: Saline | Serum, Test Cells Suspended in: Albumin | Coombs Reagent | Condition to be Expected in Infant |
|---|---|---|---|---|---|---|
| 1 | 0 | ++++ | 0 | ++++ | ++++ | severe symptoms |
| 2 | +± | ++++ | 0 | ± | ++++ | " " |
| 3 | +++ | ++++ | 0 | 0 | +± or 0 | moderate symptoms |
| 4 | ++++ | +± | 0 | 0 | 0 | may have mild disease |
| 5 | ++++ | 0 | 0 | 0 | 0 | should be free from symptoms |
| 6 | 0 | 0 | 0 | +++ | ++++ | normal Rh− child; antibodies residual from preceding pregnancy |
| 7 | 0 | 0 | 0 | 0 | 0 | normal Rh− child |

TABLE 10

*Detection of Sera Which Give Prozones with Albumin Suspended Cells*

| Series | Test Blood Suspended in | Diluent | Serum Diluted 1: 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 | 2048 | 4096 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Bovine albumin (20%) | Pooled normal male serum | 0 | ± | + | ++ | +++ | ++++ | ++++ | ++++ | ++++ | ++ | + | 0 | 0 |
| 2 | Saline | Saline | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | Cells in series 2, washed 3 times in saline and absorbed precipitin serum added | | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++ | ± |

Other methods described for the detection of coated cord or infants' cells are somewhat less sensitive than the Coombs test. Witebsky [39] recommends the addition of pooled group compatible adult serum to packed unwashed blood cells, either in test tubes or, preferably, on slides.

Diamond and Abelson [40] had previously employed their so-called "reagglutination test." One drop of a concentrated suspension of the infants' washed cells is placed on a slide and mixed with a somewhat larger drop of group compatible Rh+ whole oxalated blood. The mixture is then rocked slowly from side to side for 2 minutes. A positive reaction indicates the presence of sensitizing antibodies on the infants' cells which are released and agglutinate the Rh+ test cells.

### 3. *Comments*

In some cases, a confusing picture is presented when the erythroblastotic infant of an Rh— mother appears to be Rh— upon typing its cells with anti-Rh agglutinating serum. As mentioned above, the infant is actually Rh+ but its cells were completely saturated *in vivo* with maternal blocking antibodies. This is demonstrated by a strongly positive Coombs reaction. More frequently, the anti-Rh agglutinating serum will give varying degrees of agglutination of the cord blood, but a positive reaction in the test for coating, nevertheless, reveals that the red cells are sensitized and are, therefore, subject to destruction. In these cases, the *in vivo* sensitization with maternal blocking antibodies is incomplete.

A correlated study of the cord blood should include (1) the Coombs reaction, (2) the reaction with anti-Rh agglutinating serum, and (3) a test for the antibody content of the cord serum. This will establish the diagnosis and will indicate, in the vast majority of cases, the severity of the disease and the measures necessary for treatment (Table 9).

The data presented in Table 9 are based on cases in which the mother's serum contains blocking antibodies. The Coombs reaction cannot be elicited if the mother's serum contains pure agglutinins.

Exceptional instances have been observed in which the symptoms were unusually severe in spite of comparatively low titers in the mother and weak coating of the infant's red blood cells. It is therefore important to take into account also the obstetrical and transfusion history and, more specifically, the physical condition of the infant at delivery.[43]

The Coombs test is also useful in studying the replacement of sensitized cells by the exchange transfusion and also their rate of disappearance in all cases, mild or severe.

## D. DETECTION OF ISOIMMUNIZATION BY THE INDIRECT COOMBS TEST

### 1. *Value of method*

The Coombs test on the cells, exposed to the patient's serum in the qualitative test for detection of agglutinins, should be positive in all instances where the test for blocking antibodies was positive.* In this respect, the Coombs reaction serves to confirm the presence of blocking antibodies. It will also detect sera having only weak blocking antibodies which may otherwise be missed. Its greatest value, however, is in a group of cases which are negative in the qualitative test with albumin suspended cells, because of a prozone demonstrable on titration (Table 10). In these instances the test gives an intense reaction which is indicative of a strongly sensitized red cell.

As mentioned earlier, the effect of the prozone may be caused by a different variety of antibody, apart from the agglutinating and blocking antibody, which is characterized by a failure to clump albumin suspended cells. It has been reported that such antibodies may exist alone and in high titer, so that the Coombs reaction is the only means of their detection.

Occasionally, one may be misled by a negative Coombs test where a weak, but positive, reaction was obtained in the test with albumin suspended cells. In such cases there is also a weak reaction with the saline suspended cells which may be overlooked. Since the washed cells may not remain clumped and agglutinins do not coat the red blood cells, a negative Coombs reaction is obtained.

### 2. *Technic and readings*

The Coombs test is performed with all sera which failed to give direct reactions with saline suspended cells. The tubes in which the test for agglutinins were carried out (containing 2 drops of patient's serum and 2 drops of the saline suspended test cells) are saved for this purpose (Section X, A, 4). The cells, which had been exposed to the patient's serum at 37.5°–40° C. for 1 hour without agglutination, are washed thoroughly (at least three times) by filling the tube with saline, centrifuging heavily, and decanting. At the end of the last washing, the supernatant is poured off as completely as possible so that not more than a drop or two of saline is left for resuspension of the sediment. Two drops of the working dilution of the reagent are then added to the

* Rare exceptions have been observed, possibly due to variations in human globulin.

washed cells and the mixture is shaken. The incubation and readings are made as described above for cord cells.

### E. CONTROLS

#### 1. *Positive Control*

A positive control for the Coombs test may be prepared by adding two or three drops of a 2 per cent suspension of Rh+ cells in saline to an equal volume of a known anti-Rh blocking antibody. A convenient source of such an antibody is Rh typing serum for the slide test. No agglutination of saline suspended cells should occur with such a serum after incubation at 37.5°–40° C. but the cells become coated with blocking antibody and serve as a positive control.

#### 2. *A Negative Control*

Prepare a 2 per cent cell suspension in saline from a fresh unrefrigerated normal blood.

Wash and treat the positive and negative control as for the unknowns.

### F. COLD AGGLUTININS AS A SOURCE OF ERROR

It should be emphasized that the Coombs test is not specific for Rh-Hr antibodies, but will clump red cells which are sensitized with any antibody-bearing fraction of human serum. Experience has shown that cold sensitizing antibodies, some of which may produce only weak agglutination when examined after 2 hours' incubation at 5° C. will in many cases be readily detected by this reagent, as indicated by strong clumping of the sensitized cells. It has been shown [41] that some of these cold sensitizing fractions of human serum remain attached to the red cells even after warming at 37.5° C. for 1 hour, and continue to give weak but distinctly positive reactions in the Coombs test. For this reason, red cell suspensions mixed with human serum should never be stored in the icebox to await later performance of the test. All sera showing a discrepancy between the Coombs test and the other tests for isoimmunization should be studied for the presence of strong specific or nonspecific "cold" agglutinins which may sensitize at room temperature, and in rare instances even at 37.5° C. If all types of bloods, including the patient's own cells, are sensitized in the same way, the serum may be assumed to contain a nonspecific antibody of the "cold" variety.[41]

## G. PRECAUTIONS

a. Use only thoroughly washed glassware and fresh saline. Contamination with traces of human serum will materially affect the outcome of the test. For this reason, all saline washings indicated above must be carefully carried out.

b. Cell suspensions from cord blood previously kept at low temperatures should be warmed at 37.5° C. for 1 hour or longer before testing, and then washed in warm saline in order to remove cold sensitizing antibodies, if present.

c. The saline suspended cells which were negative in the test for agglutinins should be washed without further delay. Storage of cell and serum mixtures in the cold will result in false-positive reactions.

d. A cause of false-positive reactions is heavy centrifugation when making the final readings. A low speed of 500–1,000 r.p.m. for 1 minute should not be exceeded.

e. It is advisable to become familiar with the approximate color of a 2 per cent red cell suspension, since tests with more dilute suspensions may give reactions which are difficult to read.

f. As in the case of all diagnostic reagents, the serum should be used under sterile conditions, and stored at 5° C. or lower.

## ADDENDUM

Rarely other blood factors, independent of the Rh–Hr system, may be responsible for isoimmunization and erythroblastosis fetalis. The most important of these, the Kell-Cellano factors described recently, form a genetic system analogous to M and N or Rh and Hr.[45, 46, 47] These factors are generally identified by the name of the first patient producing the antibody. Other blood factors (Lutheran, Levay, Lewis and S) have been described with the aid of antibodies produced generally by Rh— patients along with anti-Rh antibodies.[5, 48, 49] By and large, these are less important clinically also because the antibodies behave more or less like "cold" agglutinins. This does not apply to the hemolysin of Levine and Polayes[50] held to be responsible for an intra-group transfusion reaction. Rarely anti-M and anti-N antibodies can be demonstrated in human serum. From a theoretical standpoint the various permutations and combinations of all these factors along with the A, B, and Rh-Hr factors provide proof for the concept that no two human bloods are alike in their antigenic composition.

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# Antimicrobial Assays: Bacterial Response and Level Determinations in Body Fluids

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# I. INTRODUCTION

## ANTIMICROBIAL AGENTS CONSIDERED

ANTIMICROBIAL agents have the property of inhibiting the growth of and even destroying bacteria and other microörganisms. Their action is selective. Some organisms are affected, while others are not at all or to a limited degree.[1] In general, similar problems are encountered in the use of any one of these substances. While these considerations are general, the application to any one drug or organism may have considerable variation.

When antibiotics are used in the treatment of infectious diseases, it is desirable that curative doses be established. *In vitro* tests are fairly reliable as a preliminary guide in determining the sensitivity of the infecting organism as well as deciding whether adequate levels are being achieved at the site of infection. However, since a number of exceptions have been reported,[2-5] it is clearly indicated that these tests cannot be considered a substitute for *in vivo* findings.

Only those antimicrobial agents which are most frequently used in the public health field at the present time will be considered in this chapter. These include: penicillin, streptomycin and the sulfonamides.

### 1. *Penicillin*

Penicillin exists as a dibasic organic acid. It is extremely unstable and loses its activity on exposure to air and when heated. It is also unstable when treated with dilute acids, alkalies, and primary alcohols, oxidizing agents and heavy metals. It is unstable below pH 5 and above pH 7. The sodium and calcium salts have been most widely investigated, particularly the calcium salt which is not hygroscopic.

Several antibiotics of the penicillin class are known and have the empirical formula $C_{14}H_{22}O_4N_2S$. Commercial penicillin has consisted of varying mixtures of one or more of the five known individual frac-

tions: F, G, X, K, and dihydro F. These different types have different antibiotic activities.[6] They respond differently to three bacteria, *Staphylococcus aureus*, strain 209P; *Bacillus brevis*, and an unidentified spore-forming lactic called E. These organisms have been standardized on pure penicillin standard* and suitable equations have been set up for calculating the composition of the mixtures. Penicillin K is apparently rapidly destroyed and eliminated in the body, and therapeutic levels are not achieved or maintained in body fluids following ordinary dosage.[7] Commercial penicillin now available is predominantly G which is known to be effective. A purified form known as crystalline G is also widely used.

Penicillin activity is expressed in terms of units. The original Oxford unit was defined as the least amount of penicillin which when dissolved in 1 ml. of water will inhibit the growth of Staphylococcus on an agar plate for a diameter of 2.5 cm.[8] In 1944 the International Unit was proposed to establish the same dosage all over the world.[9] This was pure sodium salt produced by U. S. and British manufacturers, which is kept in the National Institute for Medical Research in London. The International Unit is approximately equivalent to the Oxford Unit. Because of the tremendous potency of penicillin this is very small, 0.6 micrograms. For purposes of comparison, an International Working Standard is distributed from London. This consists of a sodium salt. The potency of any penicillin sample is obtained by direct comparison with a primary standard, or indirectly by comparison with standards the potencies of which have been previously established by the primary Oxford standard. In this country a master standard in the form of sodium penicillin G is kept in the Food and Drug Administration of the Federal Security Administration in Washington, D. C. This agency distributes a reference working standard, which has been compared with the master standard, that may be used in determining the potency of test samples of penicillin. The action of penicillin may be counteracted by the use of either penicillinase or clarase. One unit of penicillinase will inactivate 50 units of penicillin.

## 2. *Streptomycin*

Streptomycin[10] is an organic base, soluble in water and insoluble in most organic solvents. It may be considered related to sugars in con-

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* Obtained from the Food and Drug Administration, Federal Security Agency, Washington, D. C.[48]

trast to penicillin which is related to amino acids. It has been possible to prepare three crystalline salts from partially purified hydrochloride or sulfate salts. The calculated formula of streptomycin is $C_{21}H_{39}N_7O_{12}$. A weakly alkaline environment pH 7.8 to 8.2 is favorable to the action of streptomycin, while acidic conditions are injurious. The antibacterial activity of streptomycin can be neutralized by such agents as glucose, an anaerobic environment, and certain sulfhydryl and ketone reagents.[11] It is also antagonized by the use of thioglycolate broth as a culture medium[12] and the presence of high concentration of NaCl.[13] A derivative, dihydrostreptomycin, has also been prepared.[14] This derivative is more stable in alkaline solution than streptomycin and has the antibiotic activity of the order of streptomycin.[15] The unitage was originally expressed as " S " units. As described by Waksman,[16] it was the amount of streptomycin diluted in 1 ml. that would cause the complete inhibition of *Escherichia coli.* Recently, however, to insure uniformity in an expression of streptomycin values and to facilitate accurate comparisons of reported dosages, the Food and Drug Administration recommended that streptomycin be referred to only in terms of pure streptomycin base, one gram of streptomycin base being equivalent to one million " S " units. Similarly one microgram or gamma is equivalent to one unit. As in the case of penicillin a reference standard to be used in comparison with test specimens is distributed by the F.D.A.[48]

### 3. *Sulfonamides*

Sulfanilamide,[17] the amide of sulfanilic acid, exists as a white, practically odorless crystalline substance, with a taste usually described as "bitter with a sweet after taste." The empirical formula is $C_6H_8N_2O_2S$. The solubility of sulfanilamide in water varies greatly with the temperature. It is also soluble in hot alcohol, and cold acetone, is only slightly soluble in cold alcohol, and is insoluble in ether, benzene, and chloroform. It is not affected by light or temperature below 100° C. and is neutral to litmus in aqueous solution. Numerous sulfanilamide derivatives are in use. The most important of these are: sulfadiazine, sulfaguanidine, sulfathiazole, sulfamerazine and sulfapyridine.

The concentration of sulfonamides is expressed in mg. per cent. The action of these substances is neutralized by the use of para-aminobenzoic acid.[18]

## B. NEED FOR SENSITIVITY TESTS AND LEVEL DETERMINATIONS

The success of an antibiotic in the treatment of infectious disease is dependent on the maintenance of appropriate levels at the site of infection. This can best be determined by testing suitable body fluids at appropriate intervals following drug administration. The increased use of antimicrobial agents for therapeutic and prophylactic purposes has developed the need for carrying out these tests in public health laboratories.

Accurate, simple, chemical methods have not yet been devised[19] except in the case of the sulfonamides. The antibiotics are still best assayed by biological methods in which the test fluid is compared with similar concentrations of an antibiotic standard distributed by the F.D.A. Similarly, the sensitivity of an organism is determined by testing it against graded concentration of antimicrobial standard solution.

## C. LIMITATIONS

Many technics have been suggested for these assays including diffusion,[20–26] turbidimetric[27–31] and dilution[32–38] methods. None of these methods gives precise accurate quantitative assays. The accuracies of the tests vary from 20 to 100 per cent and have all the drawbacks inherent in any *in vitro* biological test.

## D. FACTORS INFLUENCING RESULTS

Numerous workers in the field have discussed the qualifications of the ideal method.[28, 29, 40, 41] This method should show reproduceable results in the same and different laboratories; should not require any unusual or complicated apparatus; should require a minimum of test fluid; should not require pre-treatment of specimens to make them suitable for assay; must be adaptable to large numbers of tests; should give results in as short a time as possible; should give unequivocal readings and express results of antimicrobial activity in standard units; and finally should be adaptable with slight modifications to the several types of antimicrobials used.

Other factors influencing the results of assays are (1) the period of time that has elapsed between drug administration and collection of the test specimen; (2) the method of collection of the test specimen; (3)

the temperature of storage of the specimen until it reaches the laboratory; (4) the time that elapses between the collection and the assay of the specimen; (5) other medication given simultaneously with the drug for which the assay is being made; (6) other physiological conditions affecting the individual under study; and (7) the chemical constituents of the particular lot of drugs used in treatment. Unless all these factors are controlled, laboratory results may be difficult to evaluate.

The most practical method for assaying antibiotics at the present time appears to be some form of the dilution method.[42] This is particularly true when large numbers of specimens are to be tested routinely. For purposes of screening, diffusion methods may be advantageous and furnish an additional check. In fact, this method may be the only one applicable in cases where less than 0.1 ml. of specimen is sent to the laboratory for test purposes or in instances when the sample is contaminated and insufficient in volume to be filtered.

The following methods outlined for the testing of antimicrobials are those currently in use in the Bureau of Laboratories of the Department of Health of the City of New York. These have been used over a five year period, during which more than 10,000 miscellaneous specimens have been examined.

## II. SENSITIVITY TESTS

The susceptibility of organisms to antimicrobial agents varies not only between different species, but shows wide variation between different strains of the same species.[43] When treatment fails it may often be ascribed to one of the following reasons: the organism was not sensitive to the drug; the organism became resistant during treatment; the predominant species of infecting organism changed during treatment; or the dosage was inadequate. Bacteriologic studies are therefore necessary to direct prompt attention to the cause of failure.[10]

### A. SELECTION OF MEDIUM TO BE USED FOR TESTS

The choice of medium for sensitivity tests is of the utmost importance in the interpretation of the test. It is desirable that the medium be as

simple as possible, but must contain the necessary growth factors for the test organism. If a "starvation" medium is used, absence of growth may be attributed to the medium and not necessarily to the action of the drug. Furthermore the ingredients of the medium must be such that they will not of themselves antagonize the action of the antimicrobial agent. In most instances, a liquid medium seems most practical; however, a solid medium may be employed in such cases as it seems expedient. The media commonly used for test organisms are listed below:

| *Medium* | *Test organism* |
|---|---|
| 1. Beef heart broth | *Bacillus subtilis, Escherichia coli,* Staphylococci, *Pseudomonas aeruginosa,* miscellaneous organisms isolated from the intestinal tract |
| 2. Beef heart broth—1 per cent erythrocytes | Hemolytic streptococci, *Klebsiella pneumoniae,* Non-hemolytic streptococci, pneumococci, *Streptococcus viridans* |
| 3. Levinthal broth | *Hemophilus influenzae* |
| 4. Bordet-Gengou agar | *Hemophilus pertussis* |
| 5. Miller's buffered cornstarch agar | *Neisseria meningitidis* |
| 6. Dubos or Youngman and Karlson modification of Proskauer and Beck medium | *Mycobacterium tuberculosis* |

It is advisable to determine before a test is made whether the test organism will grow on the medium selected.

## B. CONCENTRATION OF ANTIMICROBIAL AGENT

The concentrations of antimicrobial agent used depend on the specific agent to be tested. The range of units which include sensitive strains is given below:

| *Antimicrobial agent* | *Range of units used in test* |
|---|---|
| Penicillin | 0.001 to 10 |
| Streptomycin | 0.02 to 500 |
| Sulfonamides | 1 mg. per cent to 25 mg. per cent |

### C. CONTROLS

1. Culture control : tube of medium to which culture but no drug has been added.
2. Medium control : tube of medium incubated without the addition of culture.
3. Drug control : duplicate set of antibiotic dilutions run with organism of known susceptibility.

### D. INCUBATION

Readings are usually made after 18 hours' incubation at 37° C. In the case of slowly growing organisms such as tubercle bacilli, the first reading is made on the third to fourth day following inoculation, and subsequent readings made at weekly intervals for six weeks.

### E. FACTORS INFLUENCING RESULTS

Factors that influence the results of susceptibility tests include: (1) the size of the inoculum, less than 100 or over 1,000,000 organisms per ml. will give unreliable results; (2) the pH of the medium must be such that it will not antagonize the action of the drug or the growth of the organism; (3) the presence of inhibitors in the culture medium or produced by the growth of the test organism; (4) the age of the test organism—since the action of the drugs is most effective in the actively growing stage, or logarithmic phase, it is essential that actively growing young cultures be used.

### F. ORGANISMS SUSCEPTIBLE

Organisms ordinarily susceptible to penicillin, streptomycin, or the sulfonamides are listed below:

*1. Penicillin*

*Diplococcus pneumoniae*
*Streptococcus hemolyticus*
*Staphylococcus, albus* or *aureus*
Anaerobic streptococci
*Streptococcus viridans*
*Neisseria meningitidis*
*Neisseria gonorrhoeae*
Clostridia
*Bacillus subtilis*
*Bacillus anthracis*
Organisms of Vincent's angina
*Streptobacillus moniliformis*
*Actinomyces bovis*
*Treponema pallidum*

*2. Streptomycin*

*Escherichia coli*
*Salmonella typhosa*
*Bacillus proteus*
*Bacillus lactis aerogenes*
*Pseudomonas aeruginosa*
*Vibrio cholerae*
*Pasteurella pestis*
*Pasteurella tularensis*
*Klebsiella pneumoniae*
Salmonella
*Brucella abortus*
*Brucella melitensis*
*Brucella suis*
*Hemophilus influenza*
*Hemophilus pertussis*
*Mycobacterium tuberculosis*

*3. Sulphonamides*

*Streptococcus hemolyticus*
*Diplococcus pneumoniae*
*Neisseria meningitidis*
*Neisseria gonorrhoeae*
*Clostridium welchii*
*Brucella melitensis*
*Klebsiella pneumoniae*
Staphylococci and many Gram-negative bacilli found in the urinary tract

## G. ACQUIRED RESISTANCE

Individual strains of infecting organisms may show decided changes in sensitivity during the course of treatment. When the infecting agent becomes resistant to one type of antimicrobial agent, it may still be sensitive to another type of drug. Furthermore, high dosages of drug may act as a growth factor for the infecting agent and thus produce variants which are stimulated by its presence.[44]

## H. TECHNIC OF SENSITIVITY TESTS

### 1. *Penicillin sensitivity*

| *Tube No.* | *Dilution of 20 unit/ml. Working Standard* | *ml. Standard dil.* | *ml. diluent* | *Units of penicillin* |
|---|---|---|---|---|
| 1 | Undilute | 0.5 undil. | – | 10 |
| 2 | Undilute | 0.4 undil. | – | 8 |
| 3 | Undilute | 0.2 undil. | – | 4 |
| 4 | 1–2 | 0.2 undil. | 0.2 mix | 2 |
| 5 | 1–4 | 0.2 of 1–2 dil. | 0.2 " | 1 |
| 6 | 1–8 | 0.2 of 1–4 " | 0.2 " | 0.5 |
| 7 | 1–16 | 0.2 of 1–8 " | 0.2 " | 0.25 |
| 8 | 1–32 | 0.2 of 1–16 " | 0.2 " | 0.125 |
| 9 | 1–64 | 0.2 of 1–32 " | 0.2 " | 0.062 |
| 10 | 1–128 | 0.2 of 1–64 " | 0.2 " | 0.031 |
| 11 | 1–256 | 0.2 of 1–128 " | 0.2 " | 0.0156 |
| 12 | 1–512 | 0.2 of 1–256 " | 0.2 " | 0.0078 |
| 13 | 1–1024 | 0.2 of 1–512 " | 0.2 " | 0.0039 |
| 14 | 1–2048 | 0.2 of 1–1024 " | 0.2 " | 0.0019 |
| 15 | 1–4096 | 0.2 of 1–2048 " | 0.2 " | 0.00095 |
| 16 | 1–8192 | 0.2 of 1–4096 " | 0.2 " | 0.00047 |
| | | | Discard 0.2 " | |
| Control | – | – | 0.2 " | 0 |

Add 0.5 ml. of a $10^{-5}$ dilution of a 4–5 hour broth culture to each of the tubes. Dilute culture to $10^{-7}$ and make poured plates for colony count. Incubate overnight at 36° C. Then streak last four tubes with no visible growth, and also first tubes showing growth. Incubate streaked plates 36° C. overnight and read streaks for the presence of growth. The sensitivity of the organisms is the number of units in the last tube showing complete sterility.

### 2. *Streptomycin sensitivity*

This test follows the same general pattern as the penicillin test except that a standard with a different unitage is used.

| *Tube No.* | *Dilution of 1000 unit/ml. Working Standard* | *ml. of standard diluted* | *ml. of diluent* | *Units or µg. streptomycin* |
|---|---|---|---|---|
| 1 | Undilute | 0.5 undil. | – | 500 |
| 2 | Undilute | 0.4 undil. | – | 400 |
| 3 | Undilute | 0.2 undil. | – | 200 |
| 4 | 1–2 | 0.2 undil. | 0.2 mix | 100 |
| 5 | 1–4 | 0.2 of 1–2 dil. | 0.2 " | 50 |
| 6 | 1–8 | 0.2 of 1–4 " | 0.2 " | 25 |
| 7 | 1–16 | 0.2 of 1–8 " | 0.2 " | 12.5 |
| 8 | 1–32 | 0.2 of 1–16 " | 0.2 " | 6.25 |
| 9 | 1–64 | 0.2 of 1–32 " | 0.2 " | 3.125 |
| 10 | 1–128 | 0.2 of 1–64 " | 0.2 " | 1.56 |
| 11 | 1–256 | 0.2 of 1–128 " | 0.2 " | 0.78 |
| 12 | 1–512 | 0.2 of 1–256 " | 0.2 " | 0.39 |
| 13 | 1–1024 | 0.2 of 1–512 " | 0.2 " | 0.19 |
| 14 | 1–2048 | 0.2 of 1–1024 " | 0.2 " | 0.09 |
| 15 | 1–4096 | 0.2 of 1–2048 " | 0.2 " | 0.045 |
| 16 | 1–8192 | 0.2 of 1–4096 " | 0.2 " | 0.022 |
| | | | Discard 0.2 " | |
| Control | – | – | 0.2 " | 0 |

Add 0.5 ml. culture diluted $10^{-5}$ to each tube and proceed as for penicillin sensitivity tests, i.e., incubate and streak endpoints as described above.

Exceptions to the above procedures:

a. *H. Influenza* cultures are grown in Levinthal broth.* The 4 hour growth is diluted 1–200 in the same medium and the colony count is omitted.

b. *M. tuberculosis* cultures are grown in Dubos medium for 4 to 7 days. The density of the culture should be slightly less than a No. 1 barium sulfate nephelometer; 0.1 ml. of this culture is then added to tubes previously prepared as follows:

Using 6 x 1 inch metal capped tubes containing 4.5 ml. sterile Dubos medium add 0.5 ml. sterile glucose albumin mixture containing the following amounts of streptomycin:

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* *Levinthal Broth*

Boil a mixture of 10 per cent blood in saline for 15 minutes. Centrifuge until clear. Add the clear supernatant to an equal volume of infusion broth (C.M. No. 5) and incubate to test sterility. *H. influenzae* should show growth in the medium in two hours.

| *Tube No.* | *Streptomycin in albumin-glucose* | *Final concentration of streptomycin* |
|---|---|---|
| 1 | 0 | Culture Control |
| 2 | 1 μg/ml. | 0.1 μg/ml. |
| 3 | 10 " | 1.0 " |
| 4 | 100 " | 10. " |
| 5 | 1000 " | 100. " |
| 6 | 10000 " | 1000. " |

Tubes are observed for growth for a period of 42 days at which time smears are made to determine the purity of the culture in tubes showing growth.

### 3. *Sulfonamide sensitivity*

No completely satisfactory test has been devised. A rough idea of the susceptibility of an organism can be obtained by incorporating known amounts of the drug into solid medium. The surfaces of plates containing 1 mg. per cent, 5 mg. per cent, 10 mg. per cent of drugs are inoculated with the strain or strains to be tested.[45]

## III. LEVEL DETERMINATIONS IN BODY FLUIDS

### A. KINDS OF FLUIDS TESTED

Antimicrobial level assays may be carried out on blood, either clotted or citrated; on urine; on spinal fluid or bile.

### B. HANDLING OF THE FLUIDS

To obtain satisfactory results, it is necessary that care be taken in handling the test fluids. Since antimicrobial agents are affected by temperature, fluids should be kept in the refrigerator from the time of collection until they are to be tested. Changes may also occur due to the length of time the specimen has been stored. For this reason the interval of storage should be as short as possible. Any contaminated fluids must be filtered through a Seitz filter before testing, as the contaminant will obscure the results. In instances where more than one antimicrobial agent is present, the media used in the test should contain an inhibitor for the agent which is not being assayed. For example, penicillin and sulfonamides are frequently used in combination. When tests are made for penicillin the medium should contain para-aminobenzoic acid, which neutralizes the action of the sulfonamide.

### C. AMOUNT OF FLUID REQUIRED

A minimum of 2 ml. of fluid is required to carry out a complete test in duplicate. It is desirable that at least 5 ml. be available, so that replicate tests may be carried out if necessary.

### D. SELECTION OF THE TEST ORGANISM

The amount of active antibiotic in body fluids is measured by its ability to effect bacteriostasis and bactericidal action. The sensitivity of the test is therefore dependent on the choice of test organism. The selected organism must be sensitive to small amounts of antibiotic; capable of maintaining this property after long periods of laboratory cultivation; should grow rapidly in simple medium; and not be stimulated by the addition of body fluids used in the test. Several types of organism have been selected by different laboratories. The choice has been made in accordance with the practicability for the particular laboratory making the assays. For penicillin determinations, these organisms include: staphylococci,[20, 24, 28] *B. subtilis*,[41] and *S. hemolyticus*.[32] Each organism has advantages as well as drawbacks. Staphylococci show considerable variability on continued cultivation, tend to be stimulated by the presence of serum, and do not have as great a sensitivity as hemolytic streptococci or *B. subtilis*. *B. subtilis* grows luxuriantly and maintains its sensitivity over long periods of time without the necessity of repeated transfers.[41] However, this organism is impractical for use in any laboratory where the presence of spores is undesirable. Hemolytic streptococci show approximately the same sensitivity as *B. subtilis* but require frequent transfer, the use of freshly prepared cultures and a source of red blood cells. In our experience we have not found these facts a serious drawback.

For streptomycin level determinations similarly numerous organisms have been used. These include: staphylococci,[46] *Bacillus prodigiosus*,[36] *E. coli*,[16] *Ps. aeruginosa*,[16] *K. pneumoniae*,[37, 47] and *Bacillus circulans*.[38] *K. pneumoniae* and *B. circulans* appear to be the most sensitive and satisfactory organisms. In our hands, *K. pneumoniae*, grown in the presence of red blood cells do not seem to be stimulated by the presence of the test body fluids and have given regular and reproducible results. This organism, however, requires frequent transfers, freshly prepared culture for the test, and a source of red blood cells. On the other hand, *B. circulans* may be stored in the refrigerator in broth culture for at least a month without loss of sensitivity.[38] Again as in the case of *B. subtilis*, this organism can only be used in laboratories where spores offer no embarrassment.

## E. Controls on Antimicrobial Level Determinations

These should include: (1) determinations run with a standard antimicrobial agents of definitely known potency; (2) tests for the sterility of the test fluid; (3) tests for the sterility of the test medium; (4) tests for the growth of the test organism; and (5) inactivation of the antimicrobial agents by the use of penicillinase or clarase in the case of penicillin, thioglycolate broth for streptomycin and para-aminobenzoic acid for sulfonamides.

## F. Methods of Testing Body Fluids for Antimicrobial Levels

### 1. *Penicillin*

*a. Determination by the Dilution method (Rammelkamp)*[32]

Materials required:

a. Laboratory stock penicillin standard—This is a commercial penicillin preparation that has been checked against the reference working standard supplied by the F.D.A.[48] It is diluted in chilled buffer solution pH 6.0 so that each ml. contains 20,000 units. When stored in the ice compartment of the refrigerator this standard will maintain its potency for at least one month.

b. Laboratory work standard—Diluted in chilled buffer pH 6.0 so that each ml. contains 20 units. A fresh standard is prepared at least once a week, and is also stored in the ice compartment of the refrigerator.

c. Sterile plugged 3 x ½ inch test tubes

d. Sterile pipettes:

0.2 ml. graduated 1/100
1.0 ml. graduated 1/100
5.0 ml. graduated 1/10

e. Beef heart broth—pH 6.9 to which 50 mg. per liter para-aminobenzoic acid (P.A.B.) has been added. (C.M. No. 5)

f. Beef heart agar—2 per cent agar pH 6.6. (C.M. No. 5a)

g. Washed red blood cells (rabbit or horse).

h. A four to five hour broth culture of *S. hemolyticus*, strain 98 Rammelkamp. This culture is consistently sensitive to from 0.0019 to 0.0078 units of penicillin per ml. Stock cultures of this strain are transplanted weekly on 5 per cent blood agar. A preliminary transplant is made the afternoon preceding the test in beef heart broth pH 6.9, and incubated at 37° C. overnight. This culture is then used to seed the transplant used for the test. A reserve stock of the culture is kept lyophilized.

i. Chopped ice—All specimens and tubes of medium are kept in an ice bath during the execution of the test.
j. Test fluids.

*Procedure of test*

a. Dilution of test fluid—Set up required number of 3 x ½ inch test tubes and make dilutions as follows:

| *Tube No.* | *Amount of test fluid* | *Amount of chilled beef heart broth pH 6.9+ P.A.B.* | *Final Dilution* |
|---|---|---|---|
| 1 | 0.4 ml. undil. | – | Undil. |
| 2 | 0.2 ml.undil. | – | Undil. |
| 3 | 0.2 ml. undil. | 0.2 ml. Mix well | 1–2 |
| 4 | 0.2 ml. of 1–2 dil. | 0.2 ml. " " | 1–4 |
| 5 | 0.2 ml. of 1–4 dil. | 0.2 ml. " " | 1–8 |

Continue as above for the desired number of dilutions and discard 0.2 ml. from the last dilution tube. Each tube, with the exception of the first tube that contains 0.4 ml., now should contain 0.2 ml. of the various dilutions of the test fluid.
b. Immediately upon completion of the dilutions, place the racks of tubes in the refrigerator until the entire test is completed.
c. Dilution of culture—Make a $10^{-5}$ dilution of the test culture in beef heart broth, pH 6.9 + P.A.B., to which 1 per cent washed red cells have been added. This dilution should contain between 1,000 and 100,000 organisms per ml. The number of organisms used is checked by making poured blood agar plates of a $10^{-7}$ dilution of the culture.
d. Remove racks from the refrigerator, two at a time and add 0.5 ml. of culture diluted $10^{-5}$ and shake well as soon as the culture is added.
e. Controls on test:

(1) Sensitivity of the test culture. Use 20 unit per ml. laboratory working standard. Start with a 1-2 dilution and continue serial dilutions for 15 tubes.

(2) Culture control: 0.5 ml. of culture diluted $10^{-5}$ is added to a tube containing 0.2 ml. broth.

(3) Media Control: 0.2 ml. broth + 0.5 ml. sterile blood broth.

(4) Sterility test of fluid: 0.2 ml. undiluted specimen + 0.5 ml. sterile blood broth.

f. Incubate tests overnight 16 to 18 hours at 37° C.
g. Examine tubes for hemolysis and growth.
h. A 3 mm. loop of the culture near the endpoint is streaked on a blood

agar plate as a check of sterility. Approximately 30 streaks can be made on a plate marked off in 10 mm. squares.

i. Calculation of unitage: The concentration of penicillin in the sample fluid is calculated by multiplying the dilution factor (the dilution of fluid which was completely sterile) by the number of units required to sterilize a culture of the organism used. For example:

| *Sample* | *Culture* | *Serial Dilutions in beef heart broth pH 6.9+ P.A.B.* | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | *0.4 undil.* | *0.2 undil.* | *1–2* | *1–8* | *1–64* | *1–128* | *1–512* | *1–1024* |
| Standard penicillin 20 U/ml. | Hemolytic Strepto-coccus No. 98 | | | 0 | 0 | 0 | 0 | 0 | ++ |
| Test fluid | | 0 | 0 | 0 | 0 | 0 | ++ | ++ | ++ |

Standard penicillin is diluted in buffer solution pH 6.0 to contain 20 units per ml. or 4 units per 0.2 ml.

4 units ÷ 512 = 0.0078 units required to sterilize culture 98. 0.0078 × 64 = 0.5 unit in 0.2 ml. or 2.5 units in 1 ml.

When large numbers of tests are to be run it is convenient to read the results directly from the following table:

| *Dilution of the test sample* | *Dilution of 20 Unit/ml. Penicillin Standard Required to Kill the Test Organism* | | |
|---|---|---|---|
| | *1–512* | *1–1,024* | *1–2,048* |
| | *Units Contained in the Above Dilutions* | | |
| | *0.0078* | *0.0039* | *0.0019* |
| 0.4 ml. undil. | 0.02 | 0.01 | 0.005 |
| 0.2 ml. undil. | 0.04 | 0.02 | 0.01 |
| 1–2 | 0.08 | 0.04 | 0.02 |
| 1–4 | 0.16 | 0.08 | 0.04 |
| 1–8 | 0.32 | 0.16 | 0.08 |
| 1–16 | 0.64 | 0.32 | 0.16 |
| 1–32 | 1.28 | 0.64 | 0.32 |
| 1–64 | 2.56 | 1.28 | 0.64 |
| 1–128 | 5.12 | 2.56 | 1.28 |
| 1–256 | 10.24 | 5.12 | 2.56 |
| 1–512 | 20.48 | 10.24 | 5.12 |
| 1–1024 | 40.96 | 20.49 | 10.24 |
| 1–2048 | 81.92 | 40.96 | 20.48 |
| 1–4096 | 163.84 | 81.92 | 40.96 |
| 1–8192 | 327.68 | 163.84 | 81.92 |

### *b. Penicillin level determination by the filter paper disc diffusion method (Vincent and Vincent[24])*

a. Transfer *Staphylococcus aureus H* from an agar slant twice through peptone broth for 24 hour growth periods at 37° C. The second transfer is held at 5° C. for 16 to 18 hours.

b. 10 ml. of fresh nutrient agar is pipetted into uniform flat bottomed Petri dishes and incubated for 16 to 18 hours at 37° C. These plates are held in the refrigerator for at least 1 hour before use.

c. Flood each plate with 1 ml. of refrigerated culture. Remove excess moisture with a capillary pipette and dry plates for 1 hour at 37° C. (Use wooden racks which support the top half of the Petri dish above the bottom half with about ½ inch clearance.) Store plates, inverted, in the refrigerator for at least 1 hour before use.

d. Sterilize 10 mm. filter paper discs by dry heat. Fifty or more may be sterilized in a Petri dish at one time.

e. Using a sterile forceps, immerse disc in test fluid until saturated (30 seconds). Remove from fluid, and after removing the excess fluid by shaking it off gently, place on seeded plate. Special care should be taken to set the discs in place without smearing the surface of the agar. Three to four discs may be placed on each plate. Two of these discs should be immersed in standard penicillin control, one in a solution containing 1 unit per ml. and the other in a solution containing 0.25 unit per ml. For accuracy plates should be run in triplicate.

f. Incubate plates, without inverting, at 37° C. for 14 to 18 hours.

g. If the test fluid contains more than 0.125 units per ml., a clear, well defined zone will appear around each disc when held against a diffuse light.

h. The concentration of penicillin may be determined by comparing the diameter of the zones of the test fluid with that of the known concentrations of the penicillin standard solution. Less than 0.12 unit or more than 4 units cannot be measured by this method.

### *2. Streptomycin*

#### *Determination by the dilution method*

Follow the procedure used in the penicillin dilution method using the following materials:

a. Laboratory stock streptomycin standard—A commercial streptomycin preparation that has been checked against the reference working standard supplied by the F.D.A.[48] It is diluted in chilled sterile distilled water so that each ml. will contain 100,000 units. When stored in the ice compartment of the refrigerator, this standard will maintain its potency for 2 months or more.

b. Laboratory working streptomycin standard—Diluted in chilled sterile distilled water from the laboratory stock standard so that each ml. will contain 1,000 units. A fresh standard is prepared at least once a week and is also stored in the ice compartment of the refrigerator.

c. Beef heart broth with a NaCl content of 0.25 per cent and a pH of 7.8, to which 50 mg. per liter of para-amino-benzoic acid (P.A.B.) has been added.

d. A four to five hour broth culture of *K. pneumoniae* (New York City Health Department Strain 1142) which is regularly sensitive to from 0.19 to 0.39 units of streptomycin. Stock cultures of this strain are transplanted weekly on 5 per cent blood agar. A preliminary transplant is made to 1 per cent blood broth on the afternoon preceding the test and incubated at 36° C. overnight. This culture is then used to seed a second tube of blood broth to be used in the test. A reserve stock of the culture is kept lyophylized.

e. *Calculation of the unitage*—The concentration of streptomycin in the test sample of fluid is calculated by multiplying the dilution factor by the number of units required to sterilize a culture of the test organism. For example:

| | | Serial Dilutions in Broth | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Sample* | *Culture* | *0.4 undil.* | *0.2 undil.* | *1–2* | *1–4* | *1–8* | *1–512* | *1–1024* |
| Standard Streptomycin 1,000 units/ml. | *K. pneumoniae* No. 1142 | .. | .. | 0 | 0 | 0 | 0 | ++ |
| Test Fluid | | 0 | 0 | 0 | 0 | ++ | ++ | ++ |

Standard streptomycin solution in sterile distilled water contains 1,000 units per ml. or 200 units per 0.2 ml.

200 units ÷ 512 = 0.39 units, which are required to sterilize culture 1142.
0.39 × 4 = 1.56 units in 0.2 ml. or 7.8 units in 1 ml.

When large numbers of tests are run, it is convenient to read the streptomycin unitage of a test fluid directly from the following table:

| | *Dilution of 1,000 Unit Streptomycin Standard Required to Kill the Test Organism* | | | |
|---|---|---|---|---|
| | *1–256* | *1–512* | *1–1,024* | *1–2,048* |
| | *Units Contained in the Above Dilutions* | | | |
| ***Dilution of Test Fluid*** | *0.78* | *0.39* | *0.19* | *0.09* |
| 0.4 ml. undil. | 2 | 1 | 0.5 | 0.25 |
| 0.2 ml. undil. | 4 | 2 | 1 | 0.50 |
| 1–2 | 8 | 4 | 2 | 1 |
| 1–4 | 16 | 8 | 4 | 2 |
| 1–8 | 32 | 16 | 8 | 4 |
| 1–16 | 64 | 32 | 16 | 8 |
| 1–32 | 128 | 64 | 32 | 16 |
| 1–64 | 256 | 128 | 64 | 32 |
| 1–128 | 512 | 256 | 128 | 64 |
| 1–256 | 1,024 | 516 | 256 | 128 |
| 1–512 | 2,048 | 1,024 | 512 | 256 |
| 1–1024 | 4,096 | 2,048 | 1,024 | 512 |
| 1–2048 | 8,192 | 4,096 | 2,048 | 1,024 |
| 1–4096 | 16,384 | 8,192 | 4,096 | 2,048 |

## 3. *Sulfanilamide*

### *Determination in Blood and Urine*[49]

### REAGENTS *

1. A solution of trichloroacetic acid containing 15 gm. dissolved in water diluted to 100 ml.

2. A 0.1 per cent solution of sodium nitrite.

3. An aqueous solution of N-(1-naphthyl) ethylenediamine dihydrochloride containing 100 mg. per 100 ml. This solution should be kept in a dark colored bottle.

4. A solution of saponin containing 0.5 gm. per liter.

5. 4 N hydrochloric acid.

6. A solution of ammonium sulfamate containing 0.5 gm. per 100 ml.

7. A stock solution of sulfanilamide in water containing 200 mg. per liter. This solution can be kept for several months in the icebox. The most convenient standards to prepare from the stock solution are 1, 0.5 and 0.2 mg. per cent. To prepare these 5, 2.5 and 1.0 ml. of the

* Obtainable from La Motte Chemical Products Company, Baltimore.

stock solution plus 18 ml. of the 15 per cent solution of trichloroacetic acid are diluted to 100 ml.

### PROCEDURE FOR BLOOD

(Serum and reagent volumes can be proportionally reduced to give the minimum amount of filtrate necessary for accurate color comparison).

1. 2 ml. of oxalated blood are measured into a flask and diluted with 30 ml. saponin solution.

2. Allowed to stand 1–2 minutes.

3. Precipitate with 8 ml. of the trichloroacetic acid solution.

4. The *free sulfanilamide is determined* in the filtrate as follows:

a. 1 ml. of sodium nitrite solution is added to 10 ml. of filtrate.
b. Allow to stand 3 minutes.
c. Add 1 ml. of sulfamate solution.
d. Allow to stand 2 minutes.
e. Add 1 ml. of N-(1-naphthyl) ethylenediamine dihydrochloride solution.
f. The unknown is compared with an appropriate standard which has been treated as above. This comparison can be made immediately and no color change is observed for 1 hour or more.

5. The *total sulfanilamide is determined* as follows:

a. Add 0.5 ml. 4N hydrochloric acid to 10 ml. of filtrate.
b. Heat in boiling water bath 1 hour.
c. Cool.
d. Adjust volume to 10 ml.
e. Subsequent procedure is the same as stated above for free sulfanilamide.

### PROCEDURES FOR URINE

1. Method for protein free urine.

a. Dilute to contain about 1 to 2 mg. per cent of sulfanilamide.
b. Add 5 ml. of 4 N hydrochloric acid to 50 ml. of diluted urine.
c. Dilute to 100 ml.
d. 10 ml. of the product of this second dilution are treated as a blood filtrate for free sulfanilamide and 10 ml. heated without further addition of acid for total sulfanilamide.

2. Method for urine containing protein.

a. Dilute and treat by the procedure used for blood.

## PHOTOELECTRIC COLORIMETER METHOD

When a photoelectric colorimeter is available, dilutions of blood 1–50 or 1–100 can be used.

1. Blood is diluted with water (saponin is unnecessary).
2. Allow to stand a few minutes.
3. Precipitated with trichloroacetic acid with a volume that is 1/5 of the final mixture.

This allows the use of 0.1 or 0.2 ml. samples of blood which are measured with washout pipettes. Determinations on urine or other body fluids are easily made after appropriate dilution. The reagent blank of distilled water is quite low, but increases with time if the solution is left in the light. For this reason such solutions should be protected from the light unless the reading is made immediately. Some reaction occurs between the trichloroacetic acid and N-(1-naphthyl)-ethylenediamine; since solutions acidified with hydrochloric acid do not show an increased color on exposure to light. The blood blank is extremely low and negligible for most purposes. With a 1–50 dilution of human blood the correction due to the blood blank varies from 0 to 0.03 mg. per cent. This blank can be easily determined by performing an analysis as usual, except that water is substituted for the sodium nitrite solution. When small concentrations of sulfanilamide are to be determined or when a foreign dye such as prontisil or neoprontisil is present, this procedure is quite useful. The color of the normal urinary pigments can be conveniently corrected by the same procedure.

When only a very small amount of blood is available, as in the case of small animals such as mice, a determination can be made with considerable accuracy on 0.02 ml. This adaptation is essentially that described by Marshall and Cullen.[60] (For these dilutions a photoelectric colorimeter of high sensitivity must be used.)

In using a photoelectric colorimeter a filter is essential. With dimethyl-a-naphthylamine the peak of absorption of the azo dye formed occurs at 530 mμ.[57] Where N-(1-naphthyl) ethylenediamine is used the peak of absorption is shifted to 545 mμ. The dyes formed by sulfapyridine and N-ethanolsulfanilamide show the same absorption peak.

## BODY FLUIDS OTHER THAN BLOOD AND URINE

No difficulty has arisen in estimating sulfanilamide and its acetyl derivatives in other body fluids by the same procedure as used for

blood. When tissues are to be analyzed it appears desirable to extract the ground tissue in a Soxhlet apparatus with a limited amount of alcohol, dilute with an aliquot portion of the extract with water and proceed as in blood, with a photoelectric colorimeter.

### SULFANILAMIDE DERIVATIVES

This method can be used for determining diazotizable primary aryl amines containing either a free amino group or a blocked amino group which can be freed by hydrolysis. Three points of importance must be mentioned in applying this method to the estimation of compounds other than sulfanilamide.

1. With a difficultly soluble substance the recovery in the blood filtrate is generally not quantitative unless high dilutions (1–100 or greater) are used, so that high dilutions are necessary or the original alcohol precipitation method [52] must be resorted to. In the latter case, ammonium sulfamate is used to destroy excess nitrite and N-(1-naphthyl) ethylenediamine dihydrochloride is used as the coupling component.

2. With certain derivatives, the azo dyes formed will not be acid-soluble and a certain amount of alcohol must be added with or just before the coupling component.

3. With some substances buffering is necessary to obtain sufficient speed of coupling (e.g., aniline).

In the determination of sulfapyridine (2-'sulfanilamido')—pyridine with the present method incomplete recovery in blood samples is obtained unless a dilution of 1–50 or greater is used.

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15 SEP 1955

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