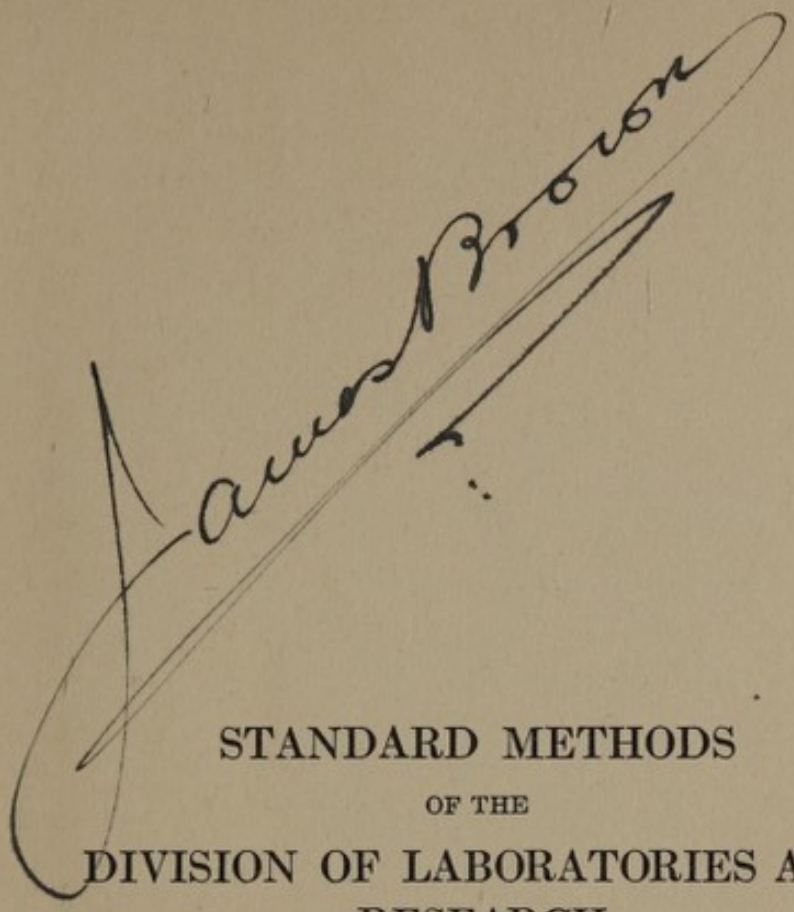
**Standard methods of the Division of Laboratories and Research of the New York State Department of Health : General laboratory procedures and methods used ... / Augustus B. Wadsworth, director.**



# STANDARD METHODS

OF THE

# DIVISION OF LABORATORIES AND RESEARCH

OF THE

# NEW YORK STATE DEPARTMENT OF HEALTH

THE MAIN BUILDING AND EAST WING

# STANDARD METHODS

OF THE

# DIVISION OF LABORATORIES AND RESEARCH

OF THE

# NEW YORK STATE DEPARTMENT OF HEALTH

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*GENERAL LABORATORY PROCEDURES AND THE METHODS USED IN*

THE DEPARTMENT FOR THE PREPARATION OF MEDIA AND GLASSWARE
THE LABORATORIES FOR SANITARY AND ANALYTICAL CHEMISTRY
THE RESEARCH, PUBLICATIONS, AND LIBRARY DEPARTMENT
THE ANTITOXIN, SERUM, AND VACCINE LABORATORIES
THE DIAGNOSTIC LABORATORIES
THE EXECUTIVE OFFICES

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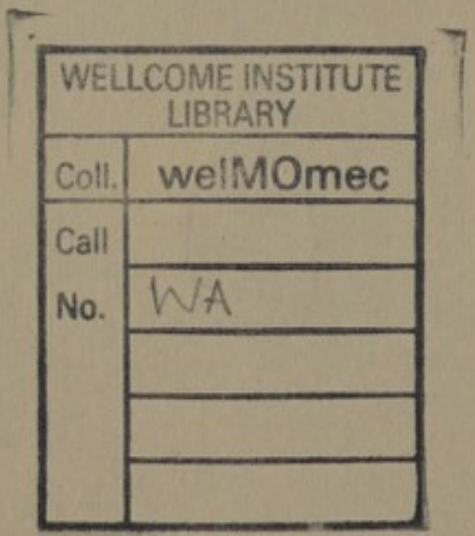


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*Dedicated*
*To the Memory of*
*T. MITCHELL PRUDDEN*
*and*
*HERMANN M. BIGGS*
*Pioneers and Leaders in*
*Public Health*

# CONTENTS

## METHODS USED IN THE LABORATORIES FOR SANITARY AND ANALYTICAL CHEMISTRY

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## METHODS USED IN THE RESEARCH, PUBLICATIONS, AND LIBRARY DEPARTMENT

# PREFACE

The reorganization in 1914, and subsequent development of the laboratory and its present staff as a branch of a large department of the state government was possible only with the sympathetic and appreciative support of Dr. Hermann M. Biggs, Commissioner of Health for nearly ten years, and of Dr. Matthias Nicoll, Jr., Commissioner for the past three years. The governors of the state and the leaders of the legislature, during this period, have also contributed in no small measure the means with which the work has been done. The laboratory has thus been free to concentrate upon its scientific pursuits without political interference. Finally, the Civil Service Commission has always coöperated to advance standards in personnel.

Laboratory service for all districts of the state outside the Greater City of New York was early considered in the scheme of organization and has proceeded, step by step, according to a preconceived plan. The establishment of branches of the central state laboratory was considered, on the one hand, and on the other, the development of local laboratories supported and operated independently but under supervision. The one promised immediate results but involved a large expenditure on the part of the state, and a service which would never become adequate in scope for all rural districts, whereas the other, although beset with many pitfalls and difficulties at every turn, relieved the state budget, stimulated local initiative, interest, and pride, and offered the opportunity in its successful development of providing an adequate service for all districts. Thus it was that only one branch of the main central laboratory was established, in New York City, for the twofold purpose of covering an inaccessible, unserved district and, at the same time, bringing the state laboratory work into close touch with the educational and hospital facilities of the great medical center in New York City.

Attention was therefore directed to the consolidation of the existing local laboratories, the advancement of standards in personnel and methods of work, and at the same time, to the problem of, so to speak, weaving these heterogeneous laboratory units into a homogeneous laboratory service for the state as a whole with uniform standards of work.

The advancement of standards started with the issuance of an ap-

proval after inspection, and has now reached the stage of advanced requirements as to the qualifications of the personnel, inspection of facilities and equipment, agreement as to methods of technical and office procedure, periodic testing of the results of the work—thus, complete supervision as to the fulfillment of minimum requirements of approved laboratories. The confidence and coöperation of these local laboratories were secured by embracing every opportunity of rendering valuable service to them, and recognizing and supporting, in every way possible, their independence, with only the one provision that standards of work were maintained. Owing to the whole-hearted support of the local laboratories, this program has progressed with the most gratifying results, almost on its own initiative, because it eliminated incompetent and unreliable laboratories and is now culminating most successfully, through the development of the activities of the New York State Association of Public Health Laboratories, in which organization every laboratory, including the state laboratory, has only one authorized vote on questions of policy; and through the increased facilities which have become available since grants of state aid have been made to the municipal and county laboratories. Thus it is that there are now 105 approved laboratories serving the state, and the standardization of laboratory procedure has become one of the paramount interests of the central state laboratory.

These standard methods, however, are not established by law but are subject to constant revision. Whatever official significance they may connote pertains only to laboratory activities in the state exclusive of the Greater City of New York. Federal standards promulgated by the United States Government rest upon authority to control interstate commerce and relate chiefly, if not exclusively, to the sale of biologic products.

The methods of this laboratory are the result of long experience in dealing with a large heterogeneous staff whose qualifications, on entering the organization, have ranged from mere high-school training to thorough preparation in scientific subjects following fundamental academic education. Early in the history of the laboratory, it became evident that a high standard of work could only be maintained by providing each worker with precise, detailed directions for his routine technical work. These directions, constantly improved throughout more than a decade by contributions from the staff, constitute this volume.

Quite apart from immediate practical aims, however, the development of these standard methods has served a much deeper and broader

purpose. The laboratory is engaged in performing daily a large volume of routine work; but it is also a laboratory for research, since research is essential to the advancement of routine standards. How then to develop in the staff the capacities, the vision, and the genius so essential in research, early became a problem in advanced education in an institution primarily without educational scope or purpose. Whatever measure of success has been achieved in solving this problem must be attributed fundamentally to the working out in detail of these standard methods. It has been required that the literature bearing on all proposed scientific investigations should be carefully reviewed, and the purpose, scope, and plan of study of such investigations should be approved before any work might be undertaken, the result of which might form the basis for changes in the methods. In this way, the necessary stimulus has been provided which has led to the inception and growth of the spirit of research. The results have been so fruitful that the laboratory now has in its scientific file a collection of data of incalculable value to its work, while the educational effect of the policy adopted has been immediate and cumulative.

Any criticism of the material as a whole, or of any of its sections, will be received with appreciation. The methods, which incidentally are a record of the laboratory's progress up to the present time in formulating precise procedures for the conduct of the work, are published for a threefold purpose: to keep uniform the established methods; to instruct the new worker and prescribe exact procedures for him and for those who are unable to take independent responsibility; and to serve as a guide to the policy which the trained, responsible worker may follow or depart from as the immediate situation demands.

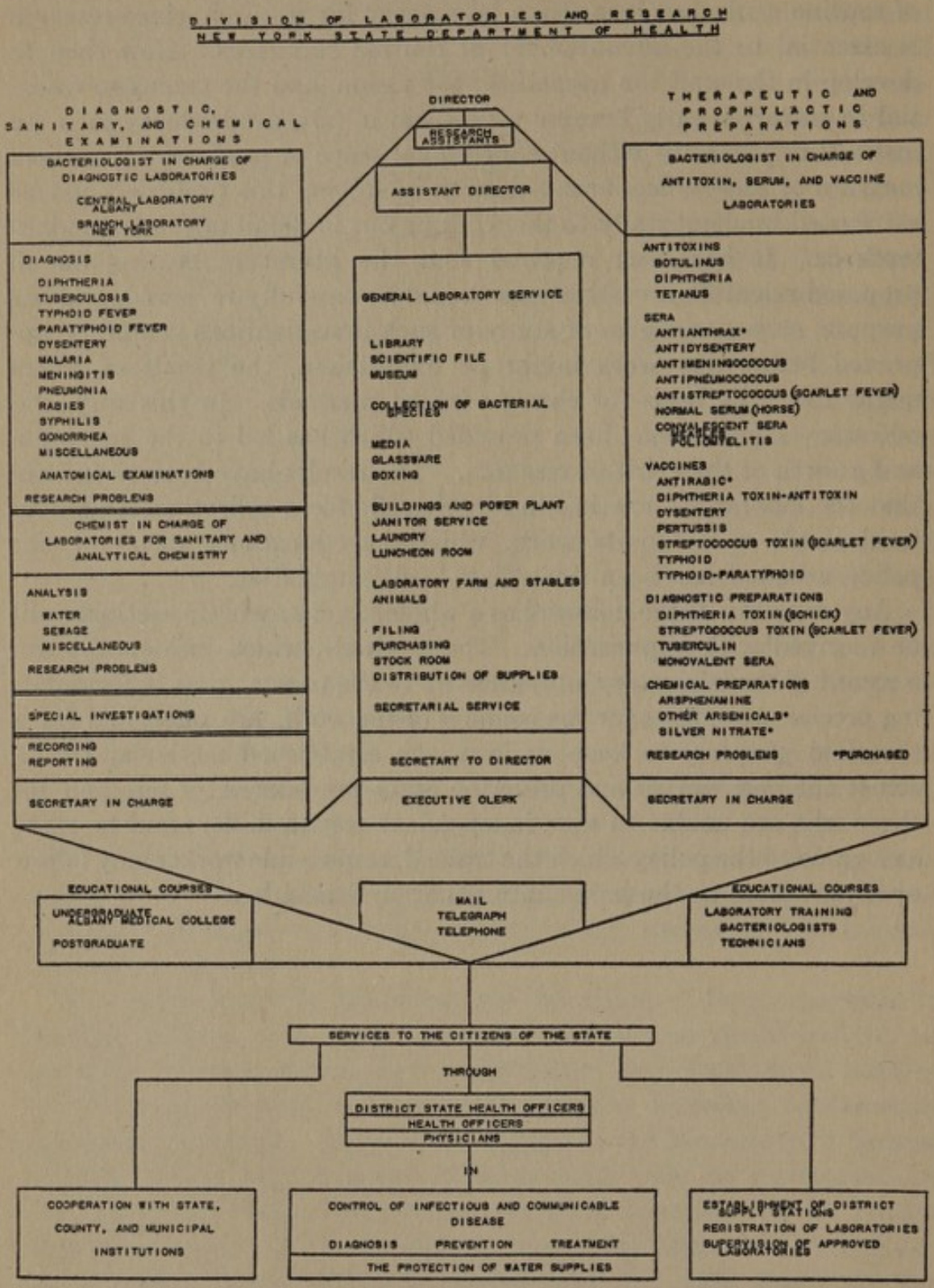



FIG 1. CHART OF ORGANIZATION

# INTRODUCTION

During the past ten years, these methods, which have been in typewritten form, have been so continually revised and improved that printed copies are now necessary not only for further revision but to meet the requests for copies from other laboratories engaged in similar work. The extent of the detail is the result of experience in guarding against error. The arduous task of preparation for publication has been shared by the entire staff although the chief responsibility has been assumed by the heads of the laboratory departments. Each series of methods is a unit in itself, and does not necessarily conform to the others in arrangement or in the amount of detail it contains.

The instructions and directions for the media and glassware groups, for instance, are precise and somewhat elementary because the methods are intended for workers who, at first, as a rule, are unfamiliar with laboratory regulations. The diagnostic methods are equally precise but they are focused differently, being intended for workers with some experience in laboratory technic. The methods for the examination of water, sewage, ice, and milk in the laboratories for sanitary and analytical chemistry are practically the same as those prepared by the American Public Health Association, and have merely been slightly modified to meet special conditions. They are included with the other methods principally because any presentation of the work done in this laboratory would be incomplete without them, and because they serve as a guide to the newcomer. The formulation of the methods used in the antitoxin, serum, and vaccine laboratories has presented a series of problems. Owing to the lack of definite knowledge concerning the nature and interaction of the bacterial poisons and their antitoxins or antisera, there is a great deal of empiricism in laboratory practice in this field; and the results of research, which has everywhere been directed to the study of prophylactic and therapeutic agents, have necessitated repeated modifications of the methods prescribed.

The administrative and the secretarial and library groups have been organized and developed in accordance with the best office and library practice. In the present volume, their functions in the laboratory organization are briefly but definitely outlined, and information which it is essential for the scientific staff to have concerning the work of these groups is given in detail.

The organization of the Division of Laboratories and Research is best presented in a diagram which shows how the laboratory is ad-

ministered and the various activities are correlated. Apart from the cleaners, laborers, and other general workers required to maintain any institution, the strictly scientific and technical personnel is separated into two main divisions: one making the necessary examinations and analyses; and the other preparing biologic and medicinal products. The general service needed by these two is supplied by a third division composed of several groups. They prepare the media, glassware, and reagents and carry out the work connected with sterilization, and with certain laboratory tests.

This third division of general service groups includes besides, a number of secretaries, stenographers, and clerks in a main office under the direction of a competent executive who not only coördinates and correlates the general work of the laboratory, but whose department is also, to a large extent, the channel of communication between the laboratory and those outside. Finally, as part of the general service available for members of the two main divisions, the laboratory has a well-trained library staff who not only administer the library material, but also file and index all outlines and reports on research written by members of the staff (the Scientific File). Likewise, they help workers in every way to prepare their papers for publication.

The work of a public-health laboratory is thus not only scientific and technical, but business-like. The clerical staff serves the professional workers, and at the same time exercises a most important control, insuring accuracy, promptness, order, and system. It may be added that while the clerical staff is distinct from the scientific staff, yet whatever the educational background of its members may be, they require special training and experience in a public-health laboratory in order to do the work well and quickly, and they must be infused with the spirit of research and responsibility to maintain standards of clerical service in keeping with the high aims of scientific work. Every facility in the way of standard methods, adequate clerical, secretarial, and library service is thus provided for the members of the scientific staff to the end that they may devote their best energies to the continual improvement of their work.

Besides the director and the assistant director, the staff includes at present 5 bacteriologists, 1 pathologist, 5 chemists, 1 veterinarian-bacteriologist, 15 assistant bacteriologists, 2 assistant serologists, 2 assistant pathologists, 9 assistant chemists, 12 laboratory assistants, 4 laboratory apprentices, 52 technical assistants and helpers.

The appropriations for 1926–1927 include $368,660.00 for personal service, $166,220.00 for maintenance and operation, and $100,000.00 for state aid for approved laboratories.

## LABORATORY BUILDING

The building is constructed of brick and reinforced concrete, and is fireproof throughout. It consists of three stories and basement, each floor having a separate corridor extending the length of the building with rooms on each side. The outside dimensions of the building are approximately 150 by 50 feet.

The laboratories have been planned to consist of a central building with two wings, one of which has been completed and the other, it is hoped, will be started during the following year. The main building is designed and equipped for research; the east wing, for diagnostic work; and the west, for the preparation and standardization of antitoxins, sera, and vaccines. The floor space of the two wings equals that of the main building.

Each floor has a refrigerator and an incubator room. Space has also been assigned for miscellaneous purposes, such as X-ray work, photography, animal operating room, a rest room for women, a luncheon room, a laundry, a machine and a carpenter shop.

Ventilation is accomplished by exhaust fans driven by motors located at the top of the building. There are two separate systems of ventilating which can be operated individually, or cross-connected so that, when desirable, either fan can be used to exhaust both systems.

Steam pressure is available in all laboratory rooms, as are vacuum, air under pressure, gas, and electrical outlets. The refrigerating system pumps brine to the cold rooms, to the refrigerators, and to the antitoxin dialyzing tanks. Fume hoods are provided in the various laboratories and are equipped with gas, vacuum, air pressure, hot and cold water, porcelain sinks, steam baths in certain cases, and electrical outlets.

The permanent work tables, large sinks, and vats are constructed of alberene. The new surfaces of the alberene are treated with a paraffin dressing before use (see p. 106). The treatment is repeated when the surface becomes dull, worn, or corroded locally by chemicals. The black, acid-proof finish, wooden, table tops are kept bright and the finish is protected by a thin coating of Butcher's wax. Only a small quantity of the wax is applied and then well rubbed in.

The floor covering of all the laboratories is of battleship linoleum. The building from top to bottom is thoroughly cleaned every night, and the floors are protected by frequent polishing with wax.

The construction of the draft-free rooms where bacterial transfers are made is of special interest. Such rooms must be practically free from dust. This has been accomplished in the following manner: The walls of the rooms are of metal, forming frames for windows, down to a point level with the table top in the room. Below this point, they are constructed of expanded metal, plastered flush with the window frames. The doors are metal-covered, suspended from above on rollers. In order to assure ventilation and at the same time reduce the amount of air contamination, space is left between the top of the side walls and the ceiling, this space being protected with metal aprons.

The buildings and grounds are maintained by the staffs of the engineer and the janitor. The engineer and the janitor coöperate to avoid duplication of work and the responsibility for it. The janitor is responsible for the care of the laboratory buildings and grounds—order, cleanliness, and the disposal of waste materials. A complete inspection is made once every month. The engineer is responsible for the maintenance and operation of sewers, drains, plumbing, gas, heating, ventilating, electric lighting, electric power, the clock system, vacuum, air-pressure and cooling systems, the steam plant, and all machinery and equipment.

# GENERAL LABORATORY PROCEDURES

# CHAPTER 1

## GENERAL BACTERIOLOGICAL TECHNIC

### CULTURAL EXAMINATION

Handle all specimens and cultures with aseptic precautions to avoid contamination of the material and to prevent infection of the worker.

Examinations for evidence of unusually infectious microörganisms or those that are resistant to the routine methods of disinfection, should be made by a responsible person especially assigned to do the work by the bacteriologist in charge of the department. Another worker should always be present to assist in the procedures.

Make the complete examination in a room set aside for the purpose.

Wear a special gown, labeled so that no other worker will use it by mistake and, when it is not in use, hang it in the room where the examination is made. When the examination is completed, wrap the gown in heavy paper and place it in an autoclave to be sterilized.

Carry all cultures and specimens to and from the incubator and the cold room in pails.

Discard all materials used, other than instruments and syringes, in a tightly covered pail which must be left nearby on the work table. Label this conspicuously with a red pencil, stating the name of the organism, and mark "Do not touch." Personally place this pail in the autoclave at the end of the day. If no autoclave is available, leave the pail on the work table until the following day.

Place all instruments and syringes in a pan containing soda solution and boil for thirty minutes, or sterilize them in the autoclave.

Wash the work table with 10-per-cent crude cresol.

For precautions in the use of animals, see: precautions against highly pathogenic microörganisms.

Since these examinations are seldom made, confirm all indefinite reactions by a parallel study of known strains of the organism under investigation.

Keep for one month all cultures isolated so that confirmatory tests may be made if desired.

When preparing material for cultural or microscopic examination, transfer it by means of a wire needle or loop held lightly, as a pencil is held (See: fig. 2). Sterilize the needle or loop immediately before and after making the transfer by first passing the wire slowly through the flame of a Bunsen burner until it turns a dull red, then, more rapidly, the end of the handle to which the wire is attached.

The prompt flaming of needles and loops in transferring cultures should become a habit.

When a large amount of fluid material is to be transferred, use a sterile pipette containing a cotton plug. When material is very infectious, use a Pasteur pipette fitted with a rubber bulb. As soon as the culture has been dispensed, place the pipette in a jar containing disinfectant.

If the material to be transferred is contained in a test tube, hold the tube in a well-slanted position between the thumb and the three fingers of the left hand. Remove the plug by grasping it between the third and fourth fingers of the right hand, flame the mouth of the tube, introduce the needle (or pipette), and collect the culture. Then withdraw the needle, distribute the culture as desired, flame the mouth of the tube again, and replace the plug. (When making stained preparations, replace the plug before spreading the material on the slide.)

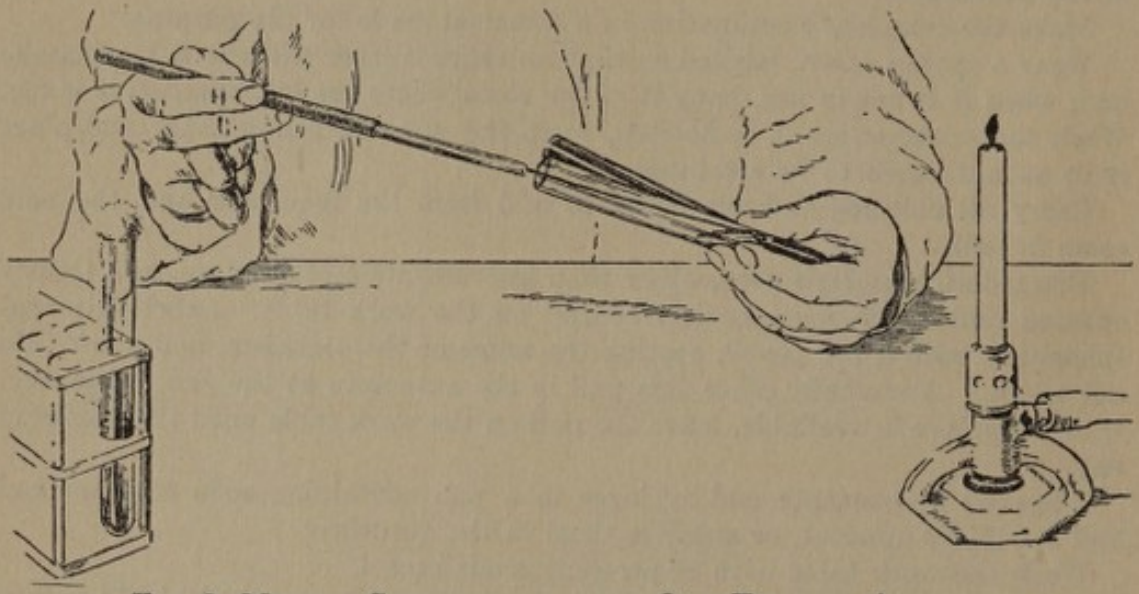


FIG. 2. MAKING SUBCULTURES FROM ONE TUBE TO ANOTHER

If the material to be transferred is contained in a Petri plate, raise the cover of the plate at one side just high enough to admit the needle, keeping the plate as completely covered as possible to prevent contamination from the air. Perform work of this type in a room free from drafts, preferably in a sterile room.

Discard all contaminated material in special cans, which are collected once a day for sterilization.

## SUBCULTURES

When making subcultures from one tube to another, hold the two together, the seed tube on the inside, and make the transfer as previously described (See: fig. 2).

Immediately after transferring the material, replace the plugs in the tubes. Be sure that the plugs are inserted so deeply that they will not become loosened.

Write legibly on the paper label of each tube, the number or name of the culture and the date of inoculation. Never use a wax pencil for marking culture tubes. Incubate gelatin cultures at 20°C. and all other routine cultures at from 35 to 37°C. for from eighteen to twenty-four hours, unless otherwise indicated.

Various methods of inoculation are used according to the nature of the medium.

**For Aerobic and Facultative Anaerobic Growth.**—When the culture medium is solid and slanted, either spread the material along the slant with a wire needle or loop, or puncture the butt and streak along the slant with a needle.

When the culture medium contains gelatin, make a deep puncture with a needle. Because medium containing gelatin that has been stored, often becomes somewhat desiccated, especially at the surface of the medium, and may crack when inoculated, always melt the medium immediately before inoculating it and allow it to resolidify.

When the culture medium is semisolid, make a deep puncture with a pipette and expel the material slowly as the pipette is withdrawn. (A loop may also be used.)

When the culture medium is liquid, suspend the growth in it with a needle, loop, or pipette, depending on the amount required and the character of the inoculum.

When the culture medium is liquid and contained in fermentation tubes, inoculate as described above, taking care in handling the tubes to avoid the entrance of air.

**For Anaerobic Growth.**—Whenever any medium, liquid, semisolid, or solid, is to be used for anaerobic cultures, expel the oxygen by heating in boiling water for from fifteen to twenty minutes and then cooling to from 40 to 42°C. before inoculating. Inoculate the medium with the material to be examined, using either a wire loop or a pipette. To avoid the entrance of air after inoculation, cool the tubes rapidly by immersing them in cold water.

When the culture medium is liquid and sealed with paraffin, heat the sides of the tube slightly until the layer of paraffin becomes loosened. Immediately after inoculating, place the tubes in an upright position so that the paraffin will again seal the tubes in an even layer. If this medium has been prepared for more than three weeks, heat, to expel the oxygen before inoculating.

For microörganisms that require fresh tissue, use the tissue semi-solid medium of Smith and Noguchi.

Use, when available, an anaerobic jar similar to the McIntosh and Fildes (1) hydrogen jar for cultivation of anaerobes on the surface of media.

This method, which is one of the most satisfactory devised for obtaining complete anaerobic conditions, depends on the displacement of the air by hydrogen, and the subsequent removal of any residual oxygen by oxidation of the hydrogen by the catalytic action of palladium or platinized asbestos electrically heated by means of a small resistance coil suspended just beneath the cover of the jar.

Place the plates (prepared as for aerobic growth) in the jar on a piece of wire gauze at the bottom to prevent breakage. Clean the opposing surfaces of the lid and the jar carefully with xylol and smear lightly with a grease composed of beeswax and vaseline. Grease also the tip and the threads of the needle valves of the gas inlet and outlet on the cover. Prepare an indicator by mixing in a test tube equal quantities of the following stock solutions: (*a*) N/160 NaOH; (*b*) 0.015-per-cent aqueous, methylene blue; (*c*) 6-per-cent aqueous solution dextrose containing a few drops of chloroform or other preservative. Boil thoroughly until the methylene blue is reduced, as shown by the disappearance of color. Place the tube of indicator, while still colorless, in the jar, close immediately, and screw on the lid. Avoid screwing down the lid too tightly as the pressure may break the jar. Attach the tube from the outlet of the hydrogen tank or generator to the gas-inlet valve on the cover of the jar. (In this laboratory, a tank of compressed hydrogen is used as the source of supply, the flow being regulated by a reducing valve of the diaphragm type.)

First, open the main valve on the hydrogen tank, then, screw down the reducing valve until the pressure on the small gauge registers between one and two pounds. With both the inlet and outlet valves on the jar open, flood the jar with hydrogen at this pressure for one minute. Close the outlet valve on the cover of the jar, then immediately the inlet valve, and then the reducing valve on the hydrogen tank. Disconnect the tube from the inlet valve on the jar. Connect the heating coil in the jar with the electric current through the required outside resistance,[1] attaching the wires to the terminal posts on the cover of the jar before plugging into the source of current. Leave the current on for ten minutes. (There is no flow of hydrogen during this period. In order to insure complete removal of any oxygen still

[1] With the particular jars used in this laboratory, with a 110-volt-current supply, a vitrohme resistance unit of 125 ohmes has been found satisfactory.

present and to equalize the pressure, more hydrogen is now allowed to enter the jar.) Place one finger tightly over the tube from the hydrogen tank and open the reducing valve until the flow of hydrogen is just perceptible. The small gauge should barely register. Attach the tube to the gas-inlet valve on the jar and open this valve, the outlet valve being kept closed. Continue the flow of hydrogen at this very slight pressure for from ten to fifteen minutes. During this period, allow the electric current to remain on as long as the top of the jar is cool. If the top becomes warm, turn the current off. Avoid excessive heating or prolonged heating of the coil in the jar as it shortens the life of the coil. Close the gas-inlet valve on the jar tightly and remove the tube connecting the hydrogen tank. Close the reducing valve and then the large valve on the hydrogen tank. Remove the plug connecting the outside resistance with the source of electric current and then the wires from the terminal posts on the cover of the jar.

The methylene-blue indicator remains colorless as long as perfect anaerobic conditions are maintained.

**For Increased Carbon Dioxide Tension.**—To produce an atmosphere containing approximately 10 per cent of $CO_2$ (2), use a museum jar approximately 5 inches in diameter and 8 inches in height (inside measurements), which can be tightly sealed. Put the plates or tubes in the jar, then place 0.6 gram of $Na_2CO_3$ in the bottom of the jar. Pour over it a mixture of 1 cc. of $H_2SO_4$ and 10 cc. of water.[2] When the reaction begins to subside, place an air-tight cover on the jar.

## ISOLATION OF PURE CULTURES

### *Plating*

To obtain a pure culture of a microörganism, make Petri-plate cultures by one of the following methods. Mark the plates legibly with a wax pencil just before using, giving the name or number of the culture, the date of inoculation, and the number of the plate, if it is one of a series. If large numbers of organisms are present, make a preliminary dilution.

**Surface Inoculation.**—Make surface inoculations in one of the following ways:

1. Place from one to five loopfuls of the material to be examined on the surface of the medium in the first of a series of two or three plates. Regulate the amount of inoculum according to the relative number of organisms in the material and the kind of medium used. With a sterile

[2] These amounts are calculated for a jar of approximately 2½ liters.

glass or wire plating rod, spread the material over the medium in each plate in succession, taking care to avoid breaking the medium. In this way, discrete colonies should be obtained on one of the plates (See: fig. 3).

2. Streak one loopful of the material, in parallel rows, over the surface of one or two plates.

3. Deposit material obtained on a swab on one small area of medium and streak with a wire loop in fan-like radiations, going back each time to the original area. Then, streak a second plate as described above, to insure the presence of isolated colonies.

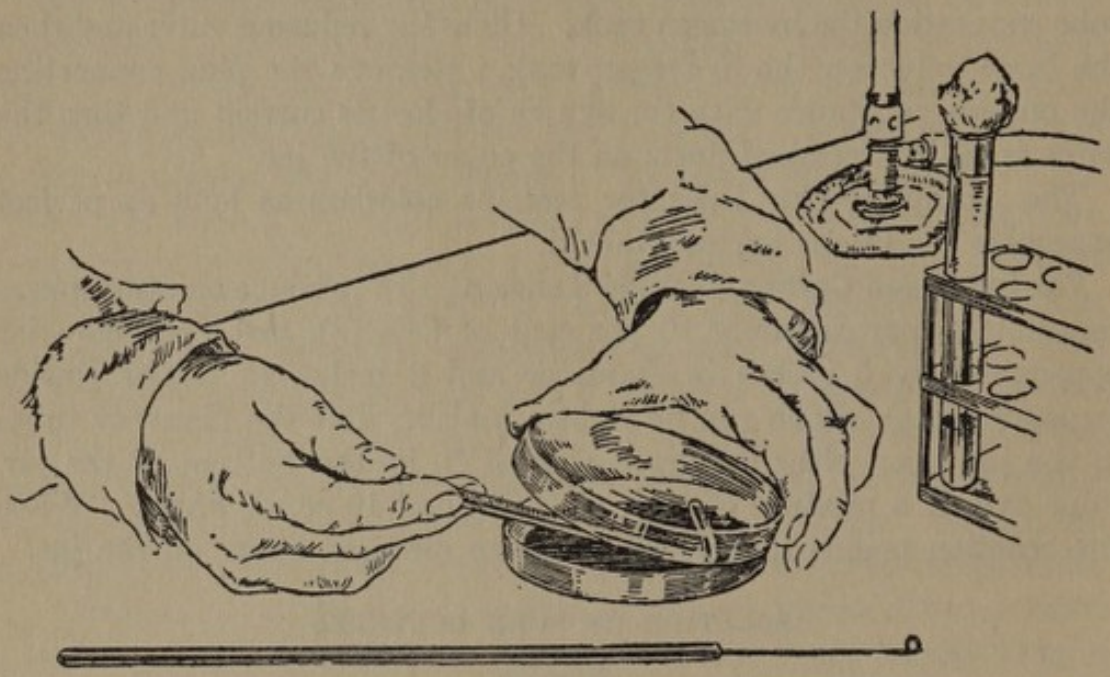


FIG. 3. METHOD USED IN SPREADING A DROP OF INOCULUM OVER THE SURFACE OF MEDIUM IN A PETRI PLATE BY MEANS OF A STERILE GLASS ROD

**Pour Plates.**—If gelatin medium is to be used, melt it by immersing the tubes in water at from 35 to 40°C. If agar is to be used, melt the medium in boiling water and cool to from 40 to 42°C. before inoculating. Be sure that all of the medium is melted before cooling. Transfer to a tube of melted medium one loopful of the material to be examined and mix thoroughly by tilting the tube back and forth. Take care to prevent the formation of bubbles and contact of the medium with the plug. After flaming the needle, transfer three loopfuls from this tube to a second, and mix. Then, flame the needle again and transfer five loopfuls from the second tube to a third, and mix. Pour the contents of each tube into a Petri plate, taking care to flame the mouth of each and to lift the lids of the plates just enough to admit the end of the

tube. Give a slight turn to the tube as the contents flow out to aid in distributing the medium, and tilt the plate gently to spread it evenly over the bottom. (See: fig. 4.)

### *Pour Plates for Bacterial Count*

To estimate the number of bacteria, dilute the material with sterile water, salt solution, or broth as desired, place accurately measured amounts of the dilution in Petri plates, and pour the melted medium on them. Mix the two by tilting the plates back and forth very gently.

The above methods of obtaining isolated colonies are usually satisfactory for securing pure cultures. In some instances, however, it is desirable to carry the procedure further. This may be done by one of the following special methods:

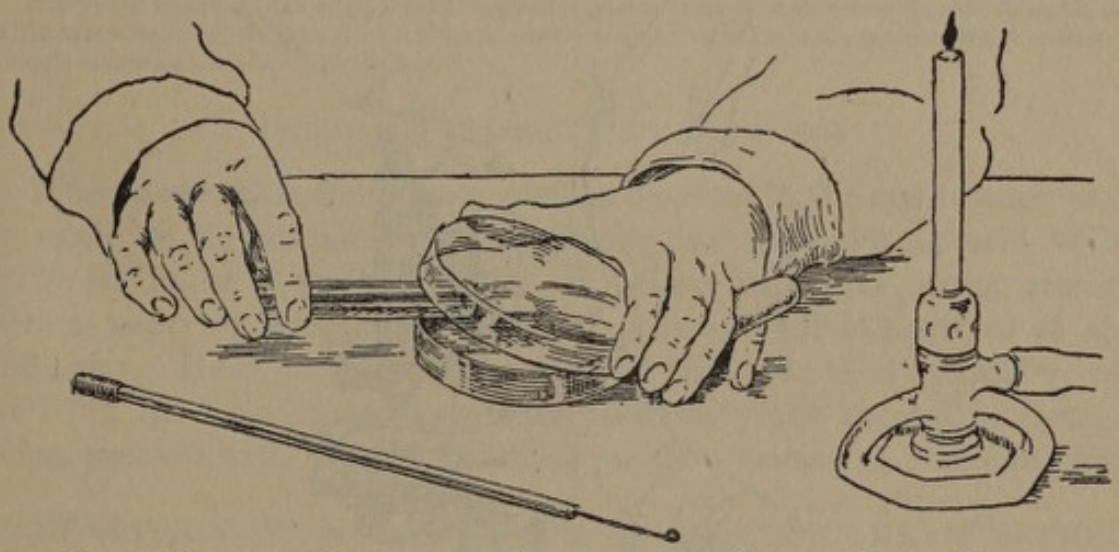


FIG. 4. POURING INOCULATED MEDIUM INTO A STERILE PETRI PLATE

### *Colony to Plate Method*

After the colonies have grown on the original plates, transfer some of the growth from a single colony, if possible, to from 0.5 to 2 cc. of broth, emulsify thoroughly and make pour or streak plates from the suspension. After these plates have been incubated, repeat the process. When the organisms have been transferred from colony to plate in this manner three times, the culture in most cases is pure.

### *Single-Cell Method*

If it is desired to obtain a culture from a single cell, Ørskov's method has been found to be reliable and easily executed (3).

*Hydrogen-Jar Method: (for Anaerobic Organisms)*

Inoculate plates by any of the methods described above and incubate in the hydrogen jar for anaerobic growth.

*Deep-Agar Method: (for Anaerobic Organisms)*

Inoculate tubes of deep, dextrose, beef-infusion agar (See: anaerobes, cultivation of). After incubation, flame the cotton plug thoroughly,

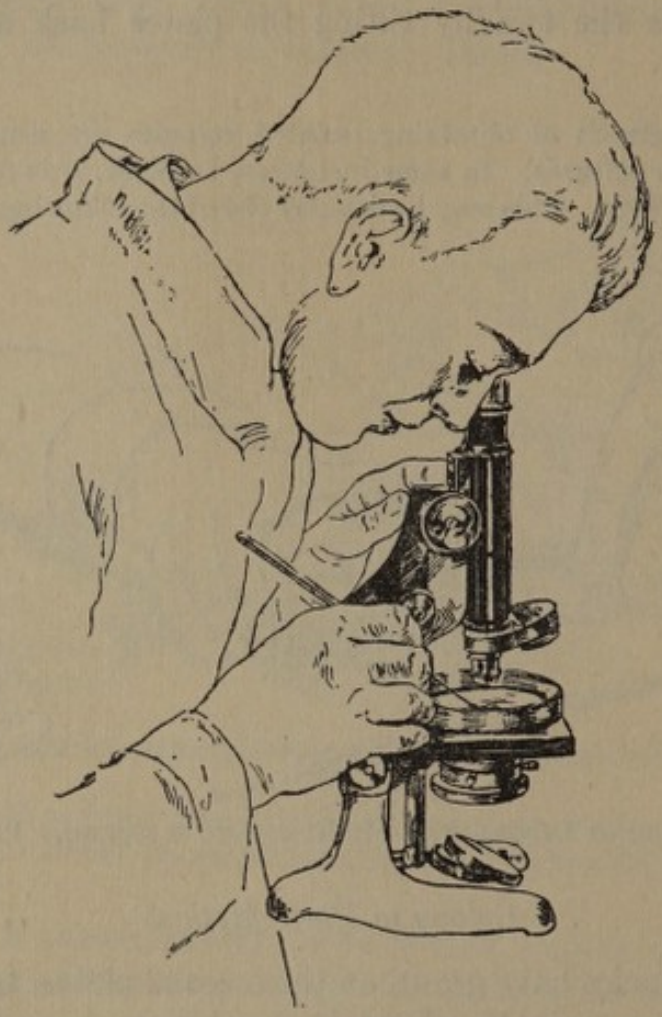


FIG. 5. FISHING A COLONY WITH THE AID OF A LOW-POWER LENS

heat the entire tube in a small flame just enough to soften the agar in contact with the glass, and then apply the flame to the bottom of the tube, expelling the cylinder of agar containing the colonies into a Petri plate. With a sterile knife or rod, cut the agar in sections so that the isolated colonies can be easily studied.

### *Fishing*

The transferring of microörganisms from a colony to fresh medium is called fishing.

Remove the cover of the Petri plate and place it right side up on the table. If water of condensation has collected on the inside of the cover, place it on a surface that has been wet with disinfectant. If the medium is translucent, select the colony and guide the needle under the low power of the microscope (See: fig. 5). Use a hand lens if preferred. If the medium is opaque, examine the plate with an unaided eye or hand lens and select an isolated colony.

If the colonies are not well isolated, fish several with a blunt needle directly to another plate, after suspending the growth in broth as previously described. Then, after incubation, fish colonies as desired.

In order to insure growth when fishing colonies of pneumococci or streptococci directly to broth, it is sometimes necessary to cut out with a sterile wire loop a small portion of the medium which contains a single colony and transfer it to the broth.

### *Isolation of Spore-Forming Organisms*

When material is thought to contain bacteria in the spore stage and is likely to be contaminated, neutralize the specimen in acid with N/20 NaOH or a similar dilution of sodium carbonate, using sterile litmus paper or a drop of 1-per-cent phenolphthalein solution as an indicator. Heat part of the material at 80°C. for thirty minutes, or at 70°C. for from one to two hours to destroy the vegetative forms. Then proceed with the usual method for the isolation of pure cultures.

## PREPARATION OF MATERIAL FOR MICROSCOPIC EXAMINATION

### MOIST PREPARATION (HANGING DROP)

To make the hanging drop, transfer a loopful of the culture suspension to the center of a clean cover glass. Invert over this a hollow, ground slide, the depression of which has been ringed with vaseline so that the cover glass will adhere to it. (The slides are ringed by dipping into vaseline the end of a rubber stopper that has been hollowed out, and applying it to the slides.) With a quick motion, turn the slide over and press the edges of the cover glass into the vaseline to seal the chamber formed, thus preventing drying of the contents.

### DRIED PREPARATION

To study the morphology of bacteria in a stained preparation, transfer some of the material to a clean glass slide that has been passed

through a flame. When the material is liquid, transfer it to the slide with a wire loop or with a soda straw. When the culture to be examined has been grown on solid medium, transfer a loopful of sterile, distilled water to a slide and emulsify in it a small amount of the growth. Spread the material in a thin, even film, allow it to dry in the air, and then fix it to the slide by passing the latter slowly, film side down, through the flame of a Bunsen burner.

### *Staining*

For routine morphologic examination, observe these general rules:

Do not use the stains and reagents more than once.

Do not use either side of the blotting paper more than once.

Do not allow the film sides of the slides to come in contact with each other.

Do not touch the films with forceps used in handling the slides.

## BIOCHEMICAL REACTIONS

### DETERMINATION OF THE REDUCTION OF NITRATES TO NITRITES

Inoculate tubes of nitrate solution with the organism to be tested and incubate them for four days. For controls, incubate at the same time one uninoculated tube and one tube inoculated with a known nitrate-reducing organism such as *B. coli communis*. After incubation, place two or three drops of reagents A and B in each tube (See: stains and reagents; reagents for determining presence of nitrites). The presence of nitrites is indicated by the appearance of a red color.

### DETERMINATION OF INDOL PRODUCTION

**Ehrlich's Test.**—Inoculate tubes of Dunham's peptone solution with the organisms to be tested and also with an organism known to produce indol, e.g., *B. coli communis*, and one known not to produce indol, e.g., *B. typhosus*, for controls. Incubate the cultures for from four to six days. Then add 1 cc. of ether and shake. Allow the ether to rise to the surface, and superimpose a few drops of Ehrlich's reagent. (See: stains and reagents.) A pink color at the point of contact between the two fluids indicates the presence of indol.

### FERMENTATION TESTS

Inoculate carbohydrate medium and incubate at the desired temperature. As a control, incubate at the same time an uninoculated tube of medium. Read and record the reactions after twenty-four

hours, forty-eight hours, and at longer intervals up to three weeks, if desired. If no change in the medium is visible after forty-eight hours, make stained preparations to determine whether the culture has grown.

## THE MICROSCOPE

### DESCRIPTION

A compound microscope consists primarily of a magnifier or simple microscope, known as an ocular, and a lens or combination of lenses, known as an objective.

The eyepieces, or oculars, in general use are those which magnify 5 × and 10 ×. The higher the magnification afforded by the ocular, the smaller the microscopic field. Less light is required with a low-power than with a high-power ocular.

The objectives most frequently used in a bacteriological laboratory are an oil-immersion, a high-power dry, and a low-power dry which, when used with a high-power ocular, magnify approximately 1000 ×, 500 ×, and 100 ×, respectively.

For bacteriological work, objects are usually illuminated by transmitted light which is concentrated by means of a system of lenses, called a condenser, situated beneath the central opening of the stage. The light is adapted to the objective and to the object by means of a diaphragm below the condenser.

A mirror is used to direct the rays of light through the condenser. This is double-faced, one side being plane and the other concave. The plane surface is used with the condenser. The light is best when reflected from a white cloud or blue sky. A northern exposure is preferred for microscopic work. Direct sunlight is unsatisfactory. When sufficient light cannot be obtained from these natural sources, a daylight lamp is used which consists of an incandescent bulb arranged so that the rays pass through a blue glass before striking the mirror.

### USE

Before attempting to bring any object into focus, adjust the tube length, which is determined for each microscope by the manufacturer. This is the distance between the objective below and the ocular above (usually 160 mm.). To focus the microscope, slowly lower the tube by means of the coarse adjustment, watching from the side with the eyes on a level with the stage, taking care that the objective never touches the slide or object. Never lower the tube with the coarse adjustment while looking through the ocular. Lower the low-power

objective to within one-quarter inch of the object to be examined, or the high-power dry lens until it very nearly touches the object. If the examination is to be made with the oil-immersion lens, place a small drop of cedar oil on the preparation and lower the objective until the lens rests in the oil but does not touch the slide. Look through the ocular with the left eye and adjust the mirror and diaphragm so that the illumination is central, even, and not too bright. Raise the tube very slowly, using the coarse adjustment, and watch for shadows in the field, which indicate that the object is nearly in focus. Then use the fine adjustment until a clear definition is obtained.

Much time can often be saved if the film is examined under the low power and satisfactory portions are selected for study.

For the examination of stained preparations of bacteria, blood cells, etc., use an oil-immersion lens with either a low- or a high-power ocular. When sharp differentiation of the object is desired, use a high-power ocular. Use the low-power ocular in the search for tubercle bacilli and gonococci and in the examination of blood films, changing to one of higher power when an object of interest is found. After using the oil-immersion objective, always remove the oil from the lens with lens paper, which may be slightly moistened with xylol if necessary. Use a rotary motion to lessen the danger of scratching the lens.

For moist preparations, use the high-power dry objective with either the high- or low-power ocular. Find the field with the low-power objective but for a final examination greater magnification is necessary.

For counting red blood cells, use the high-power dry objective with the low-power ocular.

For counting white cells and cells in spinal fluids, a moderately low-power objective with a high-power ocular is most satisfactory.

For fishing, use the lowest-power objective and either ocular.

## USE OF THE DARK FIELD[3]

For examination over a dark field a special condenser, with a very oblique beam, and a so-called funnel stop, to cut down the aperture in the objective, are necessary. The light must be very strong and is best provided by a small arc lamp but if this is not available an incandescent, nitrogen-filled lamp of 100 or more watts may be used.

[3] For description of technic used in examining material over a dark field, see: The Microscope by S. H. Gage, Ed. 12, p. 70–76 (4).

## ACCESSORIES

### *Mechanical Stage*

Use a mechanical stage whenever it is desired to examine systematically the entire preparation. Have the slide fitted exactly into the stage so that an accurate reading may be made on the vernier scale if desired. For the same reason, always work with the labeled end of the slide in the same position, preferably at the left. In recording the position of an object by means of this scale, designate the position of the slide, the number of the microscope, and the mechanical stage used.

### *Object Marker*

It is sometimes more convenient to mark an object by scratching the slide upon which it is mounted than to record the position with the mechanical stage. This is done with a diamond-pointed object marker which screws into the nose piece of a microscope.

Clamp the slide securely to the stage and, after bringing the object to be marked into the center of the field, replace the objective by the marker, and lower the tube until the diamond point presses firmly upon the slide. Make a complete revolution of the marker by means of the side screw. Raise the marker from the slide, and examine with the low-power objective. The circle made by the marker should be plainly visible with this lens and no difficulty should be experienced in locating the object.

### *Micrometers*

Micrometers are instruments used in determining the size of objects. There are two kinds which may be used with a microscope, stage, and ocular. A stage micrometer consists of an accurate scale in 0.1- and 0.01-mm. divisions. The object is placed upon the scale, examined with the most desirable objective, and measured directly, as with a ruler. The ocular micrometer, as the name indicates, is situated in an eyepiece. It is a graduated scale, the value of which must be determined for each objective and a given tube length. This value is determined by the aid of a stage micrometer, the scales being superimposed. The main advantage of the ocular micrometer is that no matter what the position of the object in the field, the eyepiece can be turned so that the object will lie perpendicular to the lines of the measurement. A filar-ocular micrometer is provided with one movable line which passes across the scale and which is operated by means of a screw at the side. The screw is divided into 100 small divisions whose value is

determined also by means of the stage micrometer. With this type of instrument, accurate measurements can be made to a tenth of a micron.

### *Warm Stage*

A warm stage, as the name indicates, is a device for holding material which is being examined with the microscope, at a temperature above that of the room. It is most commonly used in the examination of feces for protozoa and to study development of bacterial or other cells. The warm stage fits on the stage of a microscope leaving a hole in the center to admit the light. It is usually heated by means of electricity but warm water is used in some of the older types.

Never, under any circumstances, attempt to repair a microscope. Report any injury or difficulty to the bacteriologist in charge of the group.

## PREPARATION OF STAINS AND REAGENTS

### GENERAL

**Acid Alcohol.—**

| | |
|---|---|
| Hydrochloric acid, C.P | 3.00 cc. |
| Alcohol, 95 per cent | 97.00 cc. |

**Albert's Solution No. 1.—**

| | |
|---|---|
| Toluidin blue | 0.15 gram |
| Methyl green | 0.20 gram |
| Glacial acetic acid | 1.00 cc. |
| Alcohol, 95 per cent | 2.00 cc. |
| Distilled water | 100.00 cc. |

Mix well, let stand 24 hours and filter.

**Albert's Solution No. 2.—**

| | |
|---|---|
| Iodine | 2.00 grams |
| Potassium iodide | 3.00 grams |
| Distilled water | 300.00 cc. |

Grind the iodine crystals and the potassium iodide in a mortar until thoroughly mixed. Add the water slowly.

**Ammonium Ferric Sulfate Solution (Iron Alum).—**

| | |
|---|---|
| Ammonium ferric sulfate | 2.00 grams |
| Distilled water | 98.00 cc. |

**Anilin Gentian Violet.—**

| | |
|---|---|
| Anilin oil | 1.00 cc. |
| Distilled water | 25.00 cc. |

Shake well in a bottle and filter through hard filter paper, moistened

before use. To 18 cc. of this anilin water add 2 cc. of saturated alcoholic solution of gentian violet. Filter through soft filter paper. Prepare a new supply at least once a week and filter daily if necessary.

**Benedict's Solution (Qualitative).—**

Copper sulfate ..... 17.30 grams
Sodium citrate ..... 173.00 grams
Sodium carbonate (crystallized) ..... 200.00 grams
(or 100.00 grams of the anhydrous salt)
Distilled water to make ..... 1000.00 cc.

With the aid of heat, dissolve the sodium citrate and carbonate in about 600 cc. of water. Filter into a glass graduate and make up to 850 cc. with distilled water. Dissolve the copper sulfate in about 100 cc. of water and make up to 150 cc. with water. Pour the carbonate-citrate solution into a large beaker or casserole and add the copper-sulfate solution slowly with constant stirring. After mixing, the solution is ready for use.

**Benedict's Solution (Quantitative).—**

Copper sulfate (pure crystallized) ..... 18.00 grams
Sodium carbonate (crystallized) ..... 200.00 grams
(or 100 grams of the anhydrous salt)
Sodium or potassium citrate ..... 200.00 grams
Potassium sulfocyanate ..... 125.00 grams
Potassium ferrocyanide solution, 5 per cent ..... 5.00 cc.
Distilled water to make ..... 1000.00 cc.

With the aid of heat, dissolve the carbonate, citrate, and sulfocyanate in about 700 cc. of water and filter. Dissolve the copper sulfate in 100 cc. of water and pour slowly into the other fluid, stirring constantly. Add the ferrocyanide solution, cool, and dilute to 1000 cc. Weigh the copper sulfate on analytical balances. This solution keeps well and is of such strength that 25 cc. are reduced by 0.05 gram of glucose.

**Benzidin Reagent.—**

Prepare a saturated solution of benzidin (C.P.) (Harmer) in glacial acetic acid. This reagent should be made up in small quantities just before using.

**Carbolfuchsin.—**

Absolute alcohol ..... 10.00 cc.
Basic fuchsin ..... 1.00 gram
5-per-cent aqueous solution of phenol ..... 90.00 cc.

Dissolve the fuchsin in the alcohol. Add the phenol solution and allow to stand overnight. Filter.

**Copper Sulfate, 20-per-cent Solution.—**

Copper-sulfate crystals.....20.00 grams
Distilled water.....80.00 cc.
Dissolve the crystals in the water with the aid of heat, and filter.

**Diazo Reagent No. 1.—**

Sulfanilic acid..... 1.00 gram
Concentrated hydrochloric acid..... 10.00 cc.
Distilled water.....200.00 cc.
Keep in a dark glass bottle.

**Diazo Reagent No. 2.—**

Sodium nitrite..... 0.50 gram
Distilled water.....100.00 cc.
Keep in a dark glass bottle.

**Dilution Fluid for White-Blood-Cell Counts.—**

Glacial acetic acid..... 0.50 cc.
Distilled water.....99.50 cc.

**Ehrlich's Reagent for Determining the Presence of Indol.—**

Paradimethylaminobenzaldehyde..... 4.00 grams
Absolute alcohol.....380.00 cc.
Concentrated hydrochloric acid..... 80.00 cc.

**Eosin, 1-per-cent Alcoholic Solution.—**

Eosin..... 1.00 gram
Alcohol, 95 per cent.....99.00 cc.

**Eosin, 0.1-per-cent Aqueous Solution.—**

Eosin..... 0.10 gram
Distilled water.....100.00 cc.

**Esbach's Reagent.—**

Picric acid..... 1.00 gram
Citric acid..... 2.00 grams
Distilled water to make.....100.00 cc.

**Fehling's Solution No. 1.—**

Copper sulfate..... 34.65 grams
Distilled water to make.....500.00 cc.

**Fehling's Solution No. 2.—**

Potassium hydroxide.....125.00 grams
Sodium potassium tartrate.....173.00 grams
Distilled water to make.....500.00 cc.

**Gentian Violet, 5-per-cent Aqueous Solution.—**

Gentian violet, saturated alcoholic solution..... 5.00 cc.
Distilled water.....95.00 cc.
Mix and filter.

**Goodpasture's Peroxidase Stain.—**

Alcohol, 95 per cent ..... 100.00 cc.
Sodium nitroprusside ..... 0.05 gram
Benzidin (C.P.) (Harmer) ..... 0.05 gram
Basic fuchsin ..... 0.05 gram

Dissolve the sodium nitroprusside in as little water as possible (1 or 2 cc.) and add to the alcohol. Add the other ingredients. This staining solution will keep indefinitely. A slight flocculent precipitate may appear in a few days but does no harm.

**Gram's Iodine Solution.—**

Iodine crystals ..... 1.00 gram
Potassium iodide ..... 2.00 grams
Distilled water ..... 300.00 cc.

Grind the iodine crystals and the potassium iodide in a mortar until thoroughly mixed. Add water slowly.

**Hematoxylin, 0.5-per-cent Aqueous Solution.—**

Hematoxylin ..... 0.50 gram
Distilled water ..... 99.50 cc.

**Hayem's Solution.—**

Mercuric chloride ..... 0.50 gram
Sodium sulfate ..... 5.00 grams
Sodium chloride ..... 1.00 gram
Distilled water to make ..... 200.00 cc.

**Iodine Protozoan Stain.—**

Potassium iodide ..... 5.00 grams
Iodine ..... 2.50 grams
Distilled water ..... 92.50 cc.

Prepare in the same way as Gram's iodine. For use, add an equal amount of distilled water.

**Methylene Blue, 0.1-per-cent Aqueous Solution.—**

Methylene blue ..... 0.10 gram
Distilled water ..... 100.00 cc.

Mix and filter.

**Methylene Blue, Loeffler's Alkaline.—**

Saturated alcoholic solution methylene blue ..... 30.00 cc.
Solution of KOH in water 1:10,000 ..... 100.00 cc.

Mix and filter.

The 1:10,000 solution of KOH is prepared as follows:

10-per-cent solution KOH ..... 0.10 cc.
Distilled water ..... 100.00 cc.

**Mordant for Casares-Gil's Flagella Stain.—**

Tannic acid ..... 10.00 grams
Aluminum chloride (hydrated) ..... 18.00 grams
Rosanilin hydrochloride ..... 1.50 grams
Ethyl alcohol, 60 per cent ..... 40.00 cc.

Dissolve the solids in the alcohol by grinding in a mortar, adding 10 cc. of the alcohol first, and then the remainder slowly. This alcoholic solution may be kept several years.

**Mordant for Fontana's Stain.—**

Tannic acid ..... 2.50 grams
Distilled water to make ..... 40.00 cc.
Phenol, 5-per-cent solution ..... 10.00 cc.

**Obermayer's Solution.—**

Concentrated hydrochloric acid, C.P. ..... 1000.00 cc.
Ferric chloride, C.P. ..... 2.00 grams

**Reagents for Determining the Presence of Nitrites, Solution A.—**

Sulfanilic acid, C.P. ..... 8.00 grams
5/N acetic acid ..... 1000.00 cc.

**Reagents for Determining the Presence of Nitrites, Solution B.—**

a-naphthylamine ..... 5.00 grams
5/N acetic acid ..... 1000.00 cc.

Filter through washed absorbent cotton.

**Ruge's Fluid.—**

Glacial acetic acid ..... 1.00 cc.
Formalin ..... 2.00 cc.
Distilled water ..... 100.00 cc.

**Safranin, 10-per-cent Aqueous Solution.—**

Saturated alcoholic solution safranin ..... 10.00 cc.
Distilled water ..... 90.00 cc.

**Schaudinn's Solution.—**

Saturated mercuric-chloride solution in normal saline ..... 65.00 cc.
Alcohol, 95 per cent ..... 33.00 cc.
Glacial acetic acid ..... 2.00 cc.

**Silver-Nitrate Solution for Fontana's Stain.—**

Silver-nitrate crystals ..... 1.00 gram
Distilled water ..... 50.00 cc.

When the crystals are dissolved, add ammonia drop by drop. A brownish precipitate will be formed. Continue to add ammonia cautiously until the brown precipitate disappears and a faint milky appearance remains. If excess ammonia is added, the solution clears and may be readjusted by the cautious addition of more silver nitrate.

(A 10-per-cent silver-nitrate solution may be used for this readjustment.)

**Sodium Nitrite, 0.01-per-cent Aqueous Solution for Determining the Presence of Indol (Salkowski's Method).—**

Sodium nitrite, 0.1-per-cent aqueous solution ..... 1.00 cc.
Distilled water ..... 9.00 cc.

**Wright's Stain.—**

Prepared stain ..... 0.30 gram
Methyl alcohol ..... 100.00 cc.

## TISSUE SECTIONS

**Acid Alcohol (for Decalcifying Tissues).—**

Alcohol, 70 per cent ..... 90.00 cc.
Nitric acid ..... 10.00 cc.

**Acid Alcohol.—**

Alcohol, 70 per cent ..... 99.00 cc.
Hydrochloric acid, C.P. ..... 1.00 cc.

**Alcohol, 80 per cent.—**

Prepare in a 1000-cc. graduate by adding to 800 cc. of 95-per-cent alcohol enough distilled water to make 950 cc.

Any desired percentage of alcohol may be prepared from 95-per-cent alcohol in a similar way. Take as many parts of alcohol as the percentage required and add enough water to make 95 parts.

**Anilin and Xylol.—**

Anilin ..... 50.00 cc.
Xylol ..... 50.00 cc.

Mix and keep in a well-stoppered bottle.

**Carbol Xylol.—**

Carbolic-acid crystals ..... 5.00 grams
Xylol ..... 100.00 cc.

**Delafield's Hematoxylin.—**

Hematoxylin crystals ..... 4.00 grams
Alcohol, 95 per cent ..... 25.00 cc.
Ammonia alum, saturated aqueous solution ..... 400.00 cc.

Dissolve the hematoxylin in the alcohol and add to the ammonia alum in a wide-mouth bottle. Stopper tightly with cotton and allow it to stand in a light place for about two weeks. Filter through paper and add:

Glycerin (tested purity) ..... 100.00 cc.
Alcohol, 95 per cent ..... 100.00 cc.

Allow the mixture to stand in the light until it assumes a deep magenta color. Then filter and keep in a tightly stoppered bottle.

**Eosin, Stock Solution.—**

Eosin, yellowish, water soluble ..... 5.00 grams
Distilled water ..... 100.00 cc.
Thymol ..... 1 crystal

**Eosin, 1-per-cent Solution.—**

Eosin, stock solution ..... 10.00 cc.
Water ..... 65.00 cc.
Alcohol, 95 per cent ..... 25.00 cc.

**Formalin (10 per cent).—**

Distilled water ..... 2700.00 cc.
Formalin, neutralized ..... 300.00 cc.

**Formalin (Neutralized).—**

Commercial formalin, saturated aqueous solution, formaldehyde (40 per cent).
Calcium carbonate ..... q.s.

**Goodpasture's Carbol Anilin Fuchsin.—**

Alcohol, 20 per cent ..... 100.00 cc.
Basic fuchsin ..... 0.50 gram
Anilin ..... 1.00 cc.
Phenol crystals ..... 1.00 gram
Add anilin and phenol only when ready to use.

**Gram's Iodine (Weigert's Modification).—**

Iodine crystals ..... 4.00 grams
Potassium iodide ..... 8.00 grams
Distilled water ..... 400.00 cc.
Grind the iodine and potassium iodide in a mortar and gradually add the water.

**Harris' Hematoxylin.—**

Hematoxylin ..... 1.00 gram
Absolute alcohol ..... 10.00 cc.
Alum (ammonium or potassium) ..... 20.00 grams
Distilled water ..... 200.00 cc.
Mercuric oxide ..... 0.50 gram
Glacial acetic acid ..... 8.00 cc.
Dissolve the alum in the water with moderate heating. Dissolve the hematoxylin in the alcohol and add it to the warm alum solution. Quickly bring the mixture to the boiling point and add the mercuric oxide. As soon as the mixture assumes a dark purple color, cool by

plunging the container in cold water. When cold, add the glacial acetic acid.

**Kaiser's Glycerin Jelly.—**

Gelatin ..... 40.00 grams
Water ..... 210.00 cc.
Glycerin ..... 250.00 cc.
Phenol crystals ..... 5.00 grams

Soak the gelatin in the water for two hours. Add the glycerin and the phenol and heat in a water-bath until the gelatin is dissolved (15 minutes) stirring constantly. Filter through filter paper in the 54°C.-incubator.

**Mallory's Phosphotungstic Acid Hematoxylin.—**

Hematein ammonium ..... 0.10 gram
Water ..... 100.00 cc.
Phosphotungstic-acid crystals ..... 2.00 grams

Dissolve the hematein in a little water by the aid of heat, and after it is cool, add it to the rest of the solution. Ripen, by adding 5 cc. of a 0.25-per-cent aqueous solution of potassium permanganate. If hematoxylin is used, add 10 cc. of the permanganate solution.

**Mayer's Albumin Fixative.—**

Egg white
Glycerin, C.P. ..... equal parts
Sodium salicylate ..... 1 per cent

Mix, shake, and filter through several thicknesses of gauze.

**Methyl-Alcohol Fixative for Negri Bodies.—**

Methyl alcohol, C.P. neutral ..... 100.00 cc.
Picric acid, 10-per-cent solution in neutral methyl alcohol... 1.00 cc.

The alcohol is neutralized by adding 0.5 per cent of sodium carbonate, shaking, and allowing the excess to settle out. Add the picric acid just before use.

**Scharlach R Stain for Fat.—**

Alcohol, 70 per cent ..... 50.00 cc.
Acetone, pure ..... 50.00 cc.
Scharlach R, enough for a saturated solution.

Filter before using.

**Stirling's Gentian Violet.—**

Gentian violet ..... 5.00 grams
Alcohol, 95 per cent ..... 10.00 cc.
Anilin ..... 2.00 cc.
Distilled water ..... 88.00 cc.

Mix the alcohol and anilin and add these to the water. Thoroughly

mix the alcohol, anilin, and water, and then add them to the gentian violet in the mortar. Triturate thoroughly and filter through paper.

**Unna's Methylene Blue.—**

Methylene blue ..... 1.00 gram
Potassium carbonate ..... 1.00 gram
Distilled water ..... 100.00 cc.

Dissolve the methylene blue and the potassium carbonate in the water, and bottle. Allow to ripen two weeks before using. Dilute this stock solution with from 5 to 15 parts of water before use.

**Van Gieson's Stain (Williams' Modification for Negri Bodies) (5).—**

Saturated alcoholic solution basic fuchsin ..... 0.50 cc.
Saturated alcoholic solution methylene blue ..... 10.00 cc.
Distilled water ..... 30.00 cc.

Make up fresh before use or keep on ice. More fuchsin is usually necessary to secure satisfactory results (0.7 to 1.0 cc.).

**Zenker's Fluid.—**

Potassium bichromate ..... 125.00 grams
Mercuric chloride ..... 250.00 grams
Water ..... 5000.00 cc.

Dissolve the mercuric chloride and potassium bichromate in the water with the aid of heat. Use an Erlenmeyer flask or an enameled pan. Add 5-per-cent glacial acetic acid immediately before use but not to the stock solution.

**Zenker Formalin or Helly's Fluid.—**

Zenker's fluid ..... 90.00 cc.
Formalin, neutralized, undiluted ..... 10.00 cc.

## METHODS OF STAINING

**Albert's Method (6).—**

Solution I ..... 2 minutes
Rinse with water and blot dry.
Solution II ..... 1 minute
Rinse with water, blot dry, and examine.

**Casares-Gil's Flagella Stain (7).—**

Preparation of culture: Use a 15- to 18-hour agar-slant culture. Transfer a small loopful of the growth to 2 cc. of sterile distilled water which has been incubated with the culture, and is, therefore, of the same temperature. Since the flagella are easily detached, distribute the organisms by gently shaking the tube and never by mixing with the loop. Incubate this suspension for from thirty minutes to two hours at 37°C. As a control, prepare a suspension of a known motile organism in the same manner.

Preparation of slides: Take special care in cleaning the slides. Allow them to stand in cleaning solution for at least fifteen minutes, and wash thoroughly in running tap water. Then cover them with 95-per-cent alcohol, each 25 cc. of which contains from 10 to 20 drops of ammonium hydroxide, and allow to stand for fifteen minutes. Wipe dry with lens paper, being careful not to touch the surfaces of the slides with the fingers.

With a very small loop, place four drops of the suspension on a slide. Allow the drops to dry in the air and then fix with the flame.

Procedure for staining: Just before using, dilute the mordant with one or two parts of water, and filter. Flood the slides with the filtrate and allow it to act for one minute.

Wash in running water.

Blot.

Methylene blue, 0.1 per cent aqueous, or carbolfuchsin..1 minute

Wash in running water.

Blot dry.

**Eosin Methylene Blue.**—

Use Zenker-fixed tissue or mordant formalin-fixed tissue in Zenker's fluid (minus the acetic acid).

Eosin, 5 per cent aqueous at 55°C. at least 20 minutes, or at room temperature.....8 to 12 hours

Wash thoroughly in tap water.

Unna's alkaline methylene blue diluted 1:5 with distilled water until the sections are dark blue in color.

Wash in tap water.

Decolorize in 95-per-cent alcohol to which several drops of 10-per-cent colophonium in alcohol has been added. Dehydrate, clear, and mount in Canada balsam.

**Fontana's Stain for Treponemata (8, 9).**—

Fixative: Ruge's fluid..... 1 minute
Wash in water.

Mordant: Tannic acid and phenol solution.
Heat until steaming.....30 seconds
Quickly wash in water.
Blot dry.

Stain: Prepare each time used.
Ammoniacal silver-nitrate solution
Heat until steaming.....30 seconds
Wash in water.
Blot dry.

**Goodpasture's Carbol-Anilin-Fuchsin Stain (10).—**

Use Zenker-fixed tissues.

Goodpasture's carbol anilin fuchsin.....10 minutes

Wash in water.

Blot lightly.

Decolorize in 95-per-cent alcohol until pink.

Wash in water.

Loeffler's alkaline methylene blue.....15 to 60 seconds

Dehydrate and remove excess blue in absolute alcohol.

Xylol..... 3 minutes

Mount in balsam.

**Goodpasture's Peroxidase Stain (11).—**

Do not fix in the flame.

Goodpasture's stain..... 1 minute

Add to the slide an equal quantity of distilled water containing 0.5 cc. of hydrogen peroxide per 100 cc., mix, and let stand..... 3 minutes

Wash in distilled water until the film appears bright red.

Dry in air.

**Gram's Method for General Differentiation of Bacteria.—**

For controlling this stain, place at the end of each slide a film preparation of *B. coli* and one of *Staphylococcus aureus* from 24-hour cultures.

Anilin gentian violet, freshly prepared (not over one week old).

Filter before using if precipitate is formed..... 2 minutes

Blot dry.

Gram's iodine..... 1 minute

Blot dry.

Alcohol, 95 to 100 per cent.....until decolorized

Wash in running water.

Safranin (10 per cent aqueous).....30 seconds

Wash in running water.

Dry by blotting or draining.

**Hematoxylin and Eosin.—**

Transfer the slides from water to Harris' hematoxylin.

2 to 5 minutes

Wash in tap water to remove excess stain from cytoplasm.

Lithium carbonate, saturated aqueous solution.....5 minutes

Wash in water to remove all traces of the lithium carbonate.

Eosin (1-per-cent aqueous solution).....2 to 5 minutes

Rinse in tap water.

Dehydrate and clear by transferring successively to alcohol, 90 per cent.....1 minute

Alcohol, 95 per cent.....1 minute
Alcohol, absolute.....2 minutes
Absolute alcohol and xylol (equal parts).....2 minutes
Carbol xylol.....1 minute
Xylol.....1 minute or until clear
Mount in Canada balsam.

**Hiss' Method for Capsules (12).—**

Do not fix in the flame.
Aqueous gentian violet (5 per cent).....heat until steaming
Remove stain by washing with 20-per-cent aqueous solution of copper-sulfate crystals.
Blot dry.

**Levaditi's Method for Treponema Pallidum.—**

Cut blocks of formalin-fixed tissue, 1 to 2 mm. in thickness, and treat as follows:
Rinse in distilled water.
Alcohol, 95 per cent..... 24 hours
Distilled water, until the blocks sink.
Silver nitrate, 3-per-cent aqueous solution. (Wrap the bottle to exclude all light.).....3 to 5 days, at 37°C.
Rinse in three changes of distilled water.
Place in following solution..... 3 days:
Pyrogallic acid..... 3 grams
Formalin, 40 per cent..... 5 cc.
Distilled water.....100 cc.
Wash in distilled water, 3 changes.
Dehydrate and embed in paraffin as usual.
Cut thin sections, dehydrate, clear, and mount without staining.

**Loeffler's Method.—**

Loeffler's alkaline methylene blue.....1 minute
Wash in running water.
Dry by blotting or draining.

**MacCallum's Stain for Bacteria.—**

Fix tissues in Zenker's fluid or Zenker formalin, or mordant formalin-fixed sections in Zenker's fluid.
Goodpasture's carbol anilin fuchsin.....20 minutes
Wash in tap water.
Place the slide in fresh, undiluted formalin (which has not been used for this purpose before) for about 30 seconds or until some of the bright red color has washed away leaving the section definitely red.
Wash in tap water.

Saturated aqueous solution picric acid..... 5 minutes
Wash thoroughly in running tap water.
Differentiate in 95-per-cent alcohol, washing out some of the red and yellow.
Wash in water.
Stirling's gentian violet.....4 minutes
Wash thoroughly in water.
Gram's iodine solution.....3 minutes
Blot dry without washing.
Decolorize in anilin xylol (equal parts) until no color comes away and the section is clear and transparent.
Xylol.
Mount in balsam.

**Phosphotungstic-Acid Hematoxylin.**—

If sections are not Zenker-fixed, mordant them as described under tissues, anatomic examination of.
Then treat the sections as follows:
Potassium permanganate, 0.25-per-cent aqueous solution
10 to 20 minutes
Wash in tap water.
Oxalic acid, 5-per-cent aqueous solution.....5 to 10 minutes
Wash thoroughly in running tap water.
Stain in phosphotungstic-acid hematoxylin.....2 to 12 hours
Alcohol, 95 per cent.....½ to 2 minutes
Alcohol, absolute.....½ to 1 minute
Xylol.
Mount in balsam.

**Stain for Acid-Fast Bacilli in Tissues.**—

As a control, always stain at the same time, with the same reagent, and in the same way, a section of tuberculous tissue in which acid-fast bacilli have been previously found.
Cover the slide with carbolfuchsin and steam.....5 minutes
Wash in water.
Decolorize in acid alcohol.
Wash in water.
Counterstain with methylene blue, saturated aqueous solution.
1 minute
Wash in water.
Blot lightly.
Dehydrate and clear rapidly in graded alcohols and xylol.
Mount in balsam.

**Stain for Fat in Sections.—**

Cut frozen sections.

Dip for an instant in 70-per-cent alcohol, then transfer, unmounted, to alcohol-acetone solution of Scharlach R (taking care to prevent evaporation and the formation of a precipitate) .....5 minutes

Rinse sections quickly in 70-per-cent alcohol.

Transfer to water.....1 to 2 minutes

Stain lightly in Harris' hematoxylin.

Wash in water.

Transfer to lithium carbonate (saturated aqueous solution) for a few moments.

Wash in water.

Mount in glycerin or glycerin jelly.

**Wright's Stain.—**

Do not fix in the flame.

Wright's stain.....1 minute

(The film is fixed during this period by the methyl alcohol in the stain.)

Distilled water.

Add an equal amount of the distilled water to the stain on the slide and mix until a metallic luster appears.....about 5 minutes

Wash off the stain in distilled water. Do not pour off the stain, as this procedure is apt to allow some of the precipitate to remain on the film.

Dry in air.

**Ziehl-Neelsen Method.—**

Carbolfuchsin.....5 minutes steaming

Blot dry or wash.

Acid alcohol (3-per-cent hydrochloric acid).....until decolorized

Wash in running water.

Methylene blue (0.1-per-cent aqueous).....1½ minutes

Wash in running water.

Dry by blotting or draining.

# CHAPTER 2

# THE USE OF EXPERIMENTAL AND TEST ANIMALS

## OBTAINING THE ANIMALS

Order all laboratory animals by requisition. Enter on the requisition the date, number, kind and weight of animals required, name of the worker, and any additional information which may be of assistance in filling the order. Put the requisitions in the correspondence basket in the office, marked "Requisitions for animals" from which they are collected at 8:00 a.m. and 1:00 p.m. Place orders, as far as possible, not later than the day before the animals are required. Telephone orders only in an emergency, when they will be filled with the least possible delay. Make out the requisition at the time of telephoning and put it in the basket before the next collection. When unusually large numbers of animals are needed, give notice as far in advance as possible.

Animals which have survived certain tests such as potency tests of antitoxin, are returned to stock and redistributed. When such animals are suitable, the word "Used" is entered on the requisition.

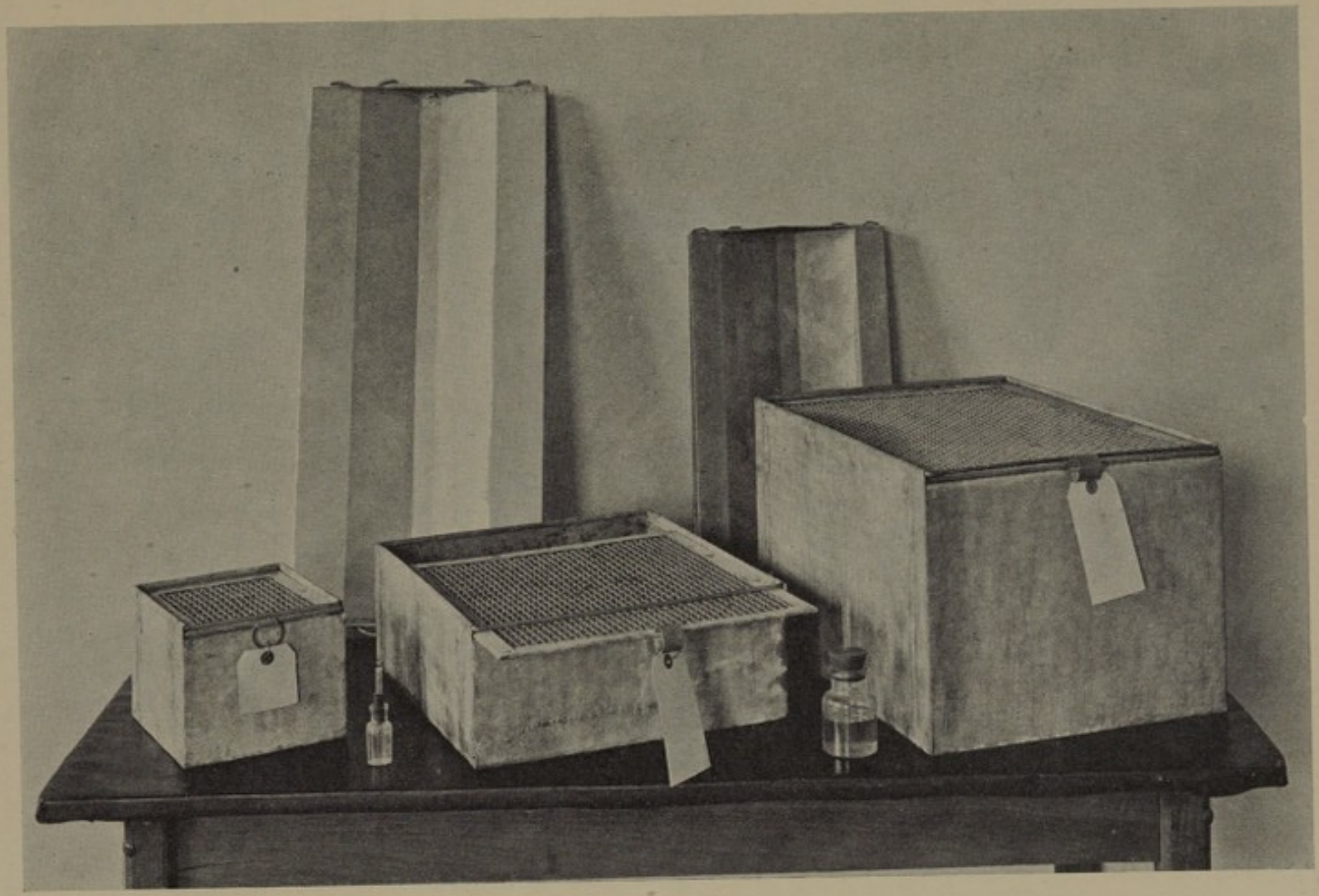
## HOUSING THE ANIMALS

### *Rabbits and Guinea Pigs*

As soon as possible after the animals are received, transfer them from the metal boxes to the cages assigned for experimental and test animals (See: plate IX). When animals are not transferred to cages until after the last regular feeding of the day, see that they have food. Attach a "Normal animal" tag to each cage, giving the number of animals, the number of the laboratory group which is to use them, and the name of the worker.

Cages are assigned to the different groups or workers according to their requirements; additional cages being reserved for the use of groups whose assignments may be temporarily inadequate, or for workers carrying on special investigations. A list of the regular assignments is posted.

When more than the assigned space is required, apply to the person in charge of the quarters for experimental and test animals. Do not place animals in unassigned cages or in temporarily empty cages assigned to other workers, without the approval of the person in charge.



ANIMAL BOXES, WATER BOTTLES, AND AUTOPSY TRAYS

### *Rats and Mice*

Keep rats in metal boxes with sliding wire covers; mice, in smaller metal boxes or in glass jars with wire covers. Tag each box or jar and provide it with a bottle containing fresh water. When the boxes are not placed in the section for test animals until after the last regular feeding of the day, see that the animals have food. The boxes are shown in plate II.

When an unusually large number of boxes is required, notify in advance the person in charge of the animal quarters.

### *Larger Animals*

When quarters for larger animals are required, notify the person in charge.

## OPERATIVE PROCEDURES

The routine operative procedures include the injection, test feeding, and bleeding of animals. Other operative procedures may be employed in special investigations as required. The operating room is used for operative procedures which involve the use of small laboratory animals (mice, rats, guinea pigs, rabbits, cats, and dogs) with the exception that procedures requiring special aseptic precautions or freedom from interruption are carried on in the special operating room, and tests on material that is extremely infectious or is suspected of containing pathogenic spore-bearing bacilli are made in a room reserved for such tests. Certain other exceptions to the rule may be made at the discretion of the bacteriologists in charge of the laboratory departments. In special procedures requiring assistance not otherwise available, members of the staff may call on the worker in charge of the operating room.

During and following all operations, the utmost consideration of the animals must be observed. Rules regarding animals are posted in the operating room, and any infringement of these requirements must be reported immediately to the bacteriologist in charge of the department.

## IDENTIFICATION OF ANIMALS BEFORE USE

Identify rabbits and guinea pigs by fastening a numbered aluminum tag to the ear. (An exception is made when guinea pigs are bled for complement, in which case the tag attached to the cage is sufficient identification.) Cleanse the ear with 70-per-cent alcohol or 1-per-cent

crude cresol, place the tag against the ventral or concave side of the thinnest portion and pass the small sharp staple through the eyelet of the tag and the ear with a quick thrust. Then bend the ends of the staples together to form a clamp on the dorsal or convex side of the ear.

Identify mice and rats by marking different parts of their bodies with stains or picric acid and recording the location of the marks.

Marking the tail with a ring or series of rings has been found to be satisfactory.

Identify the cages by attaching a separate tag for each animal, giving the identification number (and when desirable for reference, the source), the date and purpose of the test (virulence test, tetanus antitoxin, etc.), the number of the laboratory group, and the name of the worker. Also give on the tag a brief description of the animal, if desirable for further identification.

Attach only one tag to the cage if the directions for a given test specify that the data for all the animals are to be entered on a single tag. Replace promptly tags which have become soiled or illegible.

## INJECTION

Animals may be injected by intracutaneous, subcutaneous, intravenous, or intraperitoneal methods. In all methods, the skin of the animal is first disinfected with 70-per-cent alcohol or 1-per-cent crude cresol. The needle should always be inserted with the lumen up.

### *Preparation of the Animal*

When it is necessary to remove the hair from the area to be injected, either of the following methods may be used.

1. Clip a small area as closely as possible, lather it well, and shave it with a safety-razor blade.

2. Clip, as for shaving, and apply a thin paste made by adding water to a mixture of equal parts of barium sulfide and corn starch. Allow the paste to remain for three or four minutes, wash it off thoroughly with lukewarm water, and dry the animal with a clean towel. Be careful to keep the animal warm until thoroughly dry. (Since this procedure may cause some irritation of the skin, it is advisable to remove the hair on the day before the injection or at least several hours in advance.)

### *Preparation of the Syringe*

In making injections, observe carefully all aseptic precautions. Use a glass syringe and a hypodermic needle that have been sterilized by

hot air or in boiling water. For the former method, sterilize the needle, barrel, and plunger separately in test tubes or wrap the barrel and plunger separately in paper. For the latter method, place the needle, barrel, and plunger in a sterilizer and boil for five minutes immediately before using. Be careful to allow time for the needle and syringe to cool before filling.

In filling the syringe, place the lumen of the needle well below the surface of the material to be injected and draw up into the barrel a little more than is necessary for the injection. Then invert the syringe to a perpendicular position and, covering the lumen of the needle with carbolized cotton if the material is infectious, force out any air bubbles that may have entered.

*Rabbits*

For intracutaneous injection, use white or light-colored animals. Use 26-gauge needles, one-quarter to three-eights of an inch long. Have the rabbit held by an assistant or fasten it to a board; it should be held securely, but not so that the skin is stretched taut at the point of injection. Introduce the material between the layers of the skin, taking care that the needle does not go through to the subcutaneous tissue. During the procedure, watch the site of injection for the bleb which forms as the material is being injected, if the technic is correct. Also watch the graduations on the syringe. Note and record any leakage of material (See: fig. 7).

For subcutaneous injection, use needles 24- to 19-gauge, three-quarters to one and one-half inches long. Have the animal held as for intracutaneous injection. Introduce the material under the skin, taking care to avoid puncturing the peritoneal wall if injections are made over the abdomen. To facilitate the procedure, raise the skin slightly just in front of the point of insertion (See: fig. 8).

When introducing solid material that cannot be emulsified, proceed as follows: Shave and disinfect the skin as previously described. Anaesthetize the animal with ether and then with sterile sharp-pointed scissors snip the skin to form a V-shaped opening about 1 cm. in length. Lift up the lower edge of the opening with sterile forceps and force the closed blades of sterile scissors between the skin and the peritoneal wall. Open the blades to form a pocket and then close and withdraw them. With sterile forceps, insert the inoculum into the pocket, disinfect the opening with alcohol and close it with one stitch, using a surgical needle held with needle forceps. When very little material is inoculated and the opening is small, close it with collodion.

For intraperitoneal injection, use needles 24- to 19-gauge, three-eighths to one and one-half inches long. Have the animal held by an assistant. Introduce the needle with the point toward the head, as for subcutaneous injection, anterior to the region of the groin, then slant the point of the needle downward and pierce through the peritoneal wall.

For intravenous injection, use needles 26- to 19-gauge, three-eighths to one and one-half inches long. Place the animal in a special box with a sliding cover (See: fig. 11) or have it held by an assistant. To put the rabbit in position for injection, place it on the table and then grasp it under the abdomen in such a way that the hind legs are held firmly between the thumb and second finger while the index finger rests against

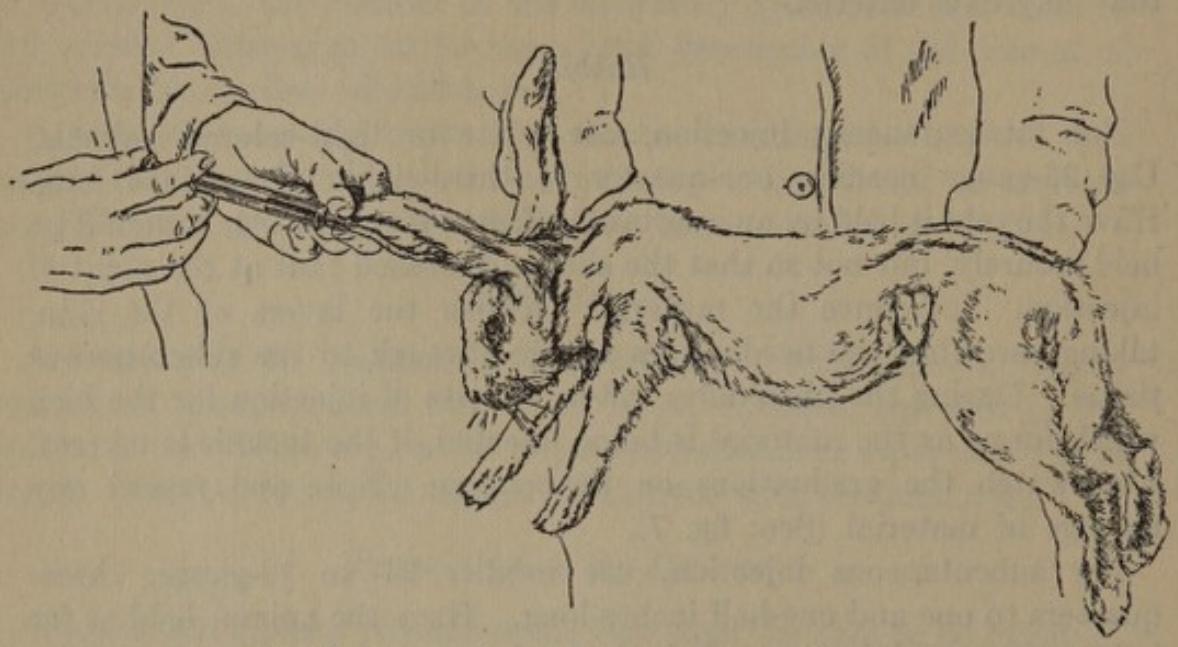


FIG. 6. INTRAVENOUS INJECTION OF A RABBIT

the inner side of the right hind leg. Then grasp the forelegs in a similar manner, lift the animal from the table, and stretch it at full length (See: fig. 6).

Remove the hair from the margin of the ear, rub the area lightly until the veins become prominent, and disinfect. Make sure that all the air is out of the syringe, and then, resting the ear against the first three fingers of the left hand and holding it down with the thumb, introduce the needle, directed toward the head, into the marginal vein as nearly parallel to the vessel as possible. Inject a very small amount of the material. If a bleb forms, showing that the needle is not in the vein, withdraw the needle at once and insert it again nearer the base of the ear. If the needle is in the ear vein, continue with the injection until all the material is injected. Remove the needle, wipe the ear with

70-per-cent alcohol or sterile cotton, and if there is any bleeding, place a small piece of cotton over the point of injection.

When several injections are to be made in the same vein, begin toward the tip of the ear so that space will be left for later injections.

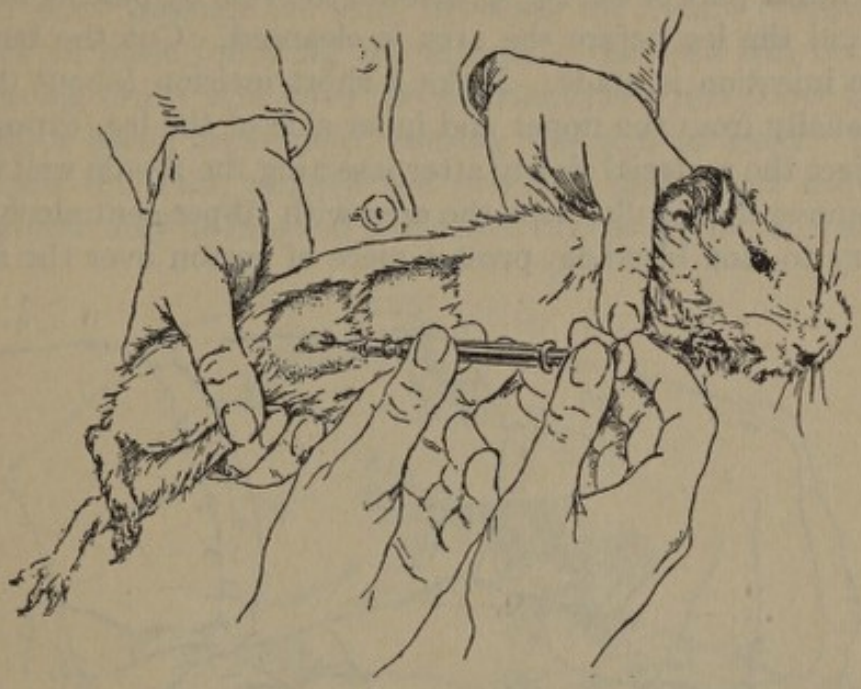


FIG. 7. INTRACUTANEOUS INJECTION OF A GUINEA PIG

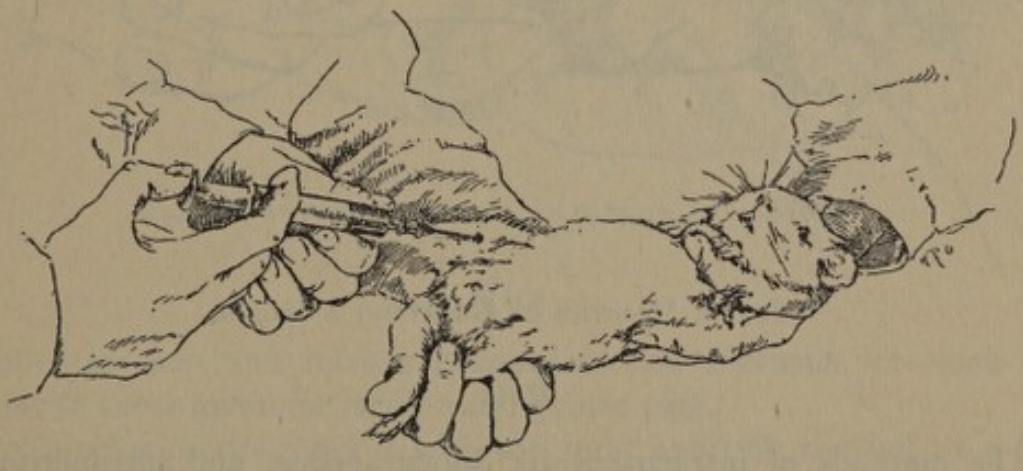


FIG. 8. SUBCUTANEOUS INJECTION IN THE GROIN OF A GUINEA PIG

*Guinea Pigs*

Intracutaneous, subcutaneous, and peritoneal injections are made in the same way on the guinea pig as on the rabbit (See: figs. 7 and 8).

For intravenous injection, use 26-gauge needles, one-quarter to three-eighths of an inch long.

Tie the animal securely, abdomen down, to a small board and shave the right leg. Cleanse the shaved area with 70-per-cent alcohol or 1-per-cent crude cresol, and slip a support, such as a 12-mm. test tube, between the leg and the board. Have an assistant apply pressure across the upper part of the leg, or distend the vein by placing an elastic band around the leg before the area is cleansed. Cut the band just before the injection is made. Make a short incision (about 0.5 to 1 cm.) diagonally from the upper and inner side of the leg, exposing the vein. Inject the material slowly after inserting the needle well into the vein. Remove the needle, wipe the area with 70-per-cent alcohol, and, if necessary to stop bleeding, press a piece of cotton over the incision.

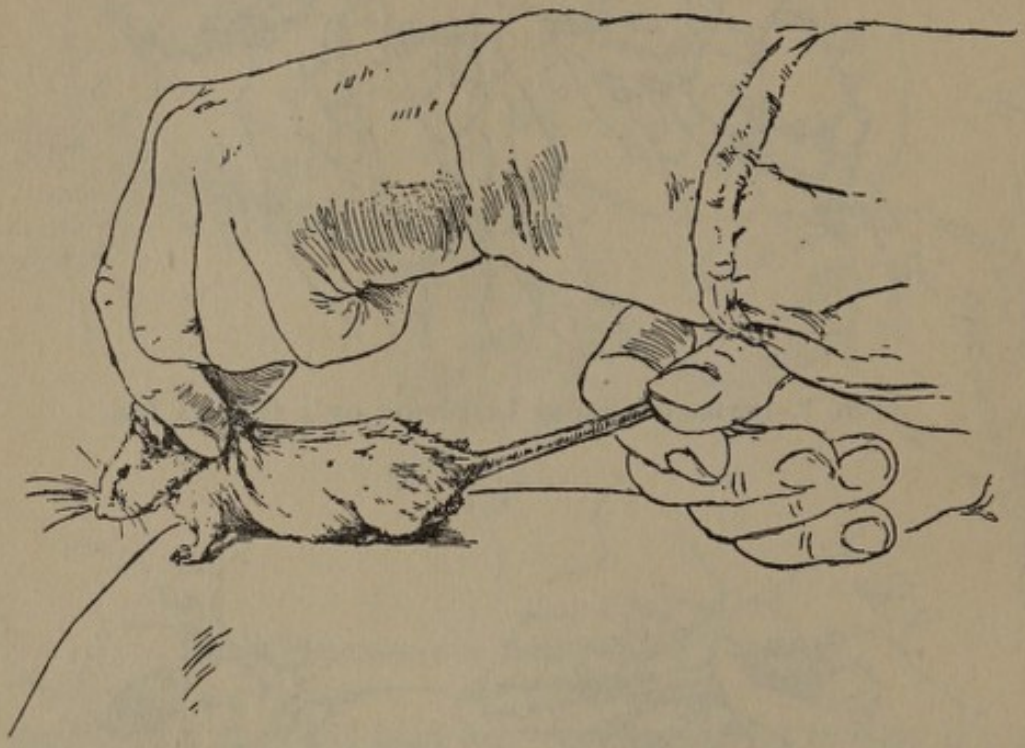


FIG. 9. MANNER OF GRASPING A MOUSE

*Rats*

The methods of intracutaneous, subcutaneous, and intraperitoneal injections of rats are the same as those used with rabbits and guinea pigs.

Have the rat held by an assistant wearing a glove. Grasp the rat by the tail with the ungloved hand and at the back of the neck with the gloved hand, then turn the animal and hold it securely in the position desired for injection.

For intravenous injection, use 26-gauge needles, three-eighths to one-

half an inch long. Fasten the rat by its four legs to a special board. Shave an area over the saphenous vein of one hind leg and disinfect the area with 70-per-cent alcohol. Then, with the finger placed under the leg, roll the skin slightly to one side and with a scalpel make a cut (about 1 cm.) through the skin, exposing the vein. (When the skin falls back in place following the injection, the vein will be covered.) Hold the leg firmly and have the assistant press just above the shaved area with a blunt instrument, causing the vein to swell. Insert the needle into the vein, remove the pressure, and inject the material. (The material may be injected into one of the coccygeal veins of the tail.)

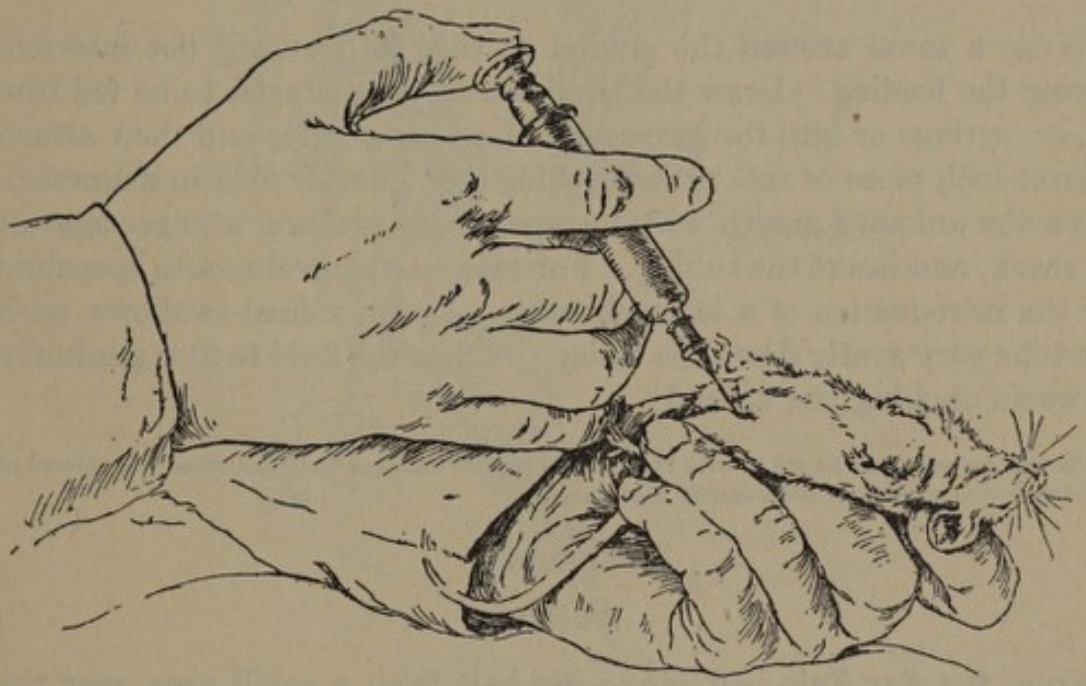


FIG. 10. INTRAPERITONEAL INJECTION OF A MOUSE

*Mice*

Subcutaneous- and intraperitoneal-injection methods for mice are similar to those given for rabbits and guinea pigs.

To inject a mouse, hold it with the right hand by the tail on a flat surface and allow it to stretch out to its greatest length. Then with the thumb and index finger of the left hand quickly and firmly grasp the skin at the back of the neck. Turn the hand palm up and keeping the mouse stretched out, secure the tail and left hind leg against the palm with the second, third, and fourth fingers. Then let go with the right hand (See: figs. 9 and 10).

For intravenous injection, just before injecting, dip the tail of the mouse into warm water (50°C.) for from one to two minutes to produce

distention of the veins. Have an assistant hold the animal by the skin at the back of the neck with the right hand and press the root of the tail with the thumb and index finger of the left, to retard circulation temporarily. Grasp the tail with the left hand and introduce a needle (27 gauge, five-eighths of an inch long, short bevel) into one of the distended lateral veins. Inject slowly, while the assistant releases the pressure at the root of the tail. If several injections are to be made on the same animal, begin toward the end of the tail, making each succeeding injection nearer the body.

### TEST FEEDING

Wrap a towel around the animal so that its feet will not interfere during the feeding. Draw the emulsion of the material to be fed into a 5-cc. syringe or into the larger end of a 5-cc. pipette and then attach a three-inch piece of soft rubber tubing (one-quarter inch in diameter). Open the animal's mouth with forceps, or by pressing a finger against its cheek, and insert the tubing. For cats, use an oval mouth speculum for the introduction of a stomach tube. As the animal swallows, push the tube very gently down the throat. Allow the fluid to flow gradually to avoid choking the animal.

The test material may be mixed with the animal's regular food, but by this method the amount of material consumed cannot be accurately determined.

### BLEEDING

#### *Rabbits*

**From the Ear Vein.**—Remove the hair from a small area over the marginal vein and along the edge and lower surface of the ear to avoid contamination during the bleeding. Stimulate the circulation by rubbing the ear lightly, sterilize the shaved areas with 1-per-cent crude cresol, and make a short incision with a sterile razor blade or scalpel. Collect the blood in a sterile container. If a steady flow of blood cannot be obtained, place the rabbit in a special box equipped with a jacket for warm water. Close the box carefully to avoid injuring the animal and then turn it up on end so that the animal's head is down. When sufficient blood has been obtained, wipe the ear with 70-per-cent alcohol or sterile cotton, and if the blood continues to flow, place a piece of cotton held with an artery clamp over the incision.

*Bleeding Box for Rabbits.*—Water-jacketed, galvanized-iron (22 gauge) box 6 by 6¾ by 17½ inches outside dimensions, 5 by 5 by 16½ inches inside. An adjustable cover of galvanized iron with a curved piece cut from one end to fit

over the animal's neck slides in any one of three grooves, 2¾, 3½, or 4¼ inches from the bottom of the box; an adjustable end piece of galvanized iron slides in grooves 11½, 13½, and 15½ inches from the closed end (See: fig. 11).

**From the Heart.**—When more than 20 cc. of blood are required or the animal is to be "bled out," use for collecting the blood a 200-cc. bottle with a rubber stopper fitted with two pieces of glass tubing, one having a cotton-filled bulb, and the other connected by means of

FIG. 11. BLEEDING A RABBIT FROM THE EAR VEIN (ANIMAL PLACED IN SPECIAL BOX)

heavy-walled rubber tubing with a sharp needle (17- or 19-gauge, 1½ inches long) protected by a cotton-plugged test tube. Since it is essential that the apparatus be air-tight, expand the glass tubing slightly at the connections with the rubber tubing. Sterilize the apparatus in an autoclave. Tie the animal securely on the tray. With sharp curved scissors, clip the hair from an area on the chest about one and one-half inches in diameter. Determine the point of maximum pulsation of the heart by palpation and disinfect the area with iodine. Introduce the needle at the point of maximum pulsation. As the needle

enters the heart, apply suction by mouth through a piece of heavy-walled rubber tubing previously attached to the outlet tube of the bottle. Continue the suction as necessary until a sufficient quantity of blood has been obtained. Then carefully and quickly withdraw the needle. If a small amount of blood (10 to 20 cc.) is required, collect it in a glass syringe, sterilized and rinsed with 0.85-per-cent salt solution immediately before use. If the blood is not forced into the syringe by the heart action, draw up the plunger very gradually. Empty the syringe immediately into a sterile container. Always give an animal water after taking any quantity of blood. Blood should be taken from an animal without anaesthesia only by workers experienced in bleeding from the heart.[1]

Approximately from 20 to 30 cc. of blood can be taken from a 2000-gram rabbit at intervals of two or three weeks.

**From the Carotid Artery.**—Etherize the animal on an operating tray slanted slightly so that the head is low. Clip the hair from the anterior surface of the neck, paint the area with iodine, or wash with 1-per-cent crude cresol and make a medial longitudinal incision through the skin. Lay back the skin on either side and, using fresh forceps and scalpel, cut down very carefully until the carotid artery is exposed and freed from surrounding tissues. With artery clamps, shut off a small portion of the artery where the incision is to be made. Raise the artery carefully with forceps, make a very small V-shaped cut with fine sharp scissors, and, near the point of the incision, insert the tip of a dissecting needle bent at right angles. Then, just below the needle, insert the glass cannula attached to the bottle in which the blood is to be collected. Hold or tie the artery firmly on the point of the cannula and without pulling the artery, release the upper clamp, and allow the blood to flow freely into the bottle until all the blood has been obtained. Remove at once and seal the capillary opening in a flame.

*Guinea Pigs*

**From the Heart.**—Follow the procedure described for bleeding rabbits from the heart.

Approximately from 10 to 15 cc. of blood can be taken from a 700-gram guinea pig at intervals of two or three weeks.

**From the Carotid Artery (for Complement).**—Etherize the animal until it is relaxed, then cut away the skin from the throat exposing the

[1] This method in experienced hands has been found apparently to cause the animal less discomfort than etherization.

trachea and blood vessels. Dissect or cut the carotid artery or cut transversely the veins on either side of the trachea. (The blood flows more rapidly by the former method.) Hold the guinea pig with the head bent backward at right angles and allow the blood to collect in sterile Petri plates.

## OBSERVATION, CARE, AND TREATMENT

### *Routine Inspection*

The worker in charge of the test is responsible for the animal's general condition and for observing it as frequently as may be required.

Following the injection or test feeding, watch the condition of the animal and record any symptoms of illness. When spontaneous infections develop among the test and experimental animals, report the fact at once to the veterinarian in charge.

**Weight Determination.**—When a record of the weight of the animals under observation is required, weigh them at approximately the same hour each day, preferably in the morning before feeding.

**Temperature Determination.**—Use a tested, clinical thermometer with a long slender bulb. Be sure that the mercury has been shaken down. Then grease the thermometer with vaseline, insert it in the rectum up to the end of the mercury reservoir and hold it in place for the required period of time. To prevent breakage, hold the thermometer firmly throughout the procedure.

### *Inspection at Night*

When night inspections are necessary, fasten a green tag on the cage and leave a note for the night watchman, specifying which animals are to be observed. In the case of sections where regular nightly inspections are required, no note is needed. (Unless otherwise directed, the night watchman will make two inspections during the night.)

### *Removal of Dead Animals*

Remove a dead animal from a cage as soon as found. When an immediate autopsy cannot be made, place the animal in a can in the cold room. Should the ear tag be missing, fasten a tag to the leg or, as in the case of rats and mice, wrap the animal in paper and write the necessary information on the wrapper.

On Sundays and holidays, at the time of feeding, or at night when inspections are made, dead animals are removed and placed in the cold room by the caretaker or the night watchman who enters the date and hour found dead and his

initials on the cage tag, or when the animal has no individual tag, on the paper in which the animal is wrapped, together with the number of the cage from which the animal is taken.

### *Discharge of Used Animals*

Immediately on the completion of a test, discharge all surviving animals. Attach to the compartment or box, a tag on which the word "Discharged" is written in red. On this tag enter the date of discharge, the number of animals (or ear-tag number of each animal), the purpose for which they were used, and the number of the group, and initials of the worker by whom discharged. Enter the same information on a requisition form and send directly to the worker in charge of small animals.

### *Special Instructions for Treatment of Cages*

When an animal is known to have died, or is suspected of having died, from an infection transmissible to other animals, attach to the cage a tag marked "Sterilize" and place no other animals in that cage until the tag has been removed.

Since all vacated boxes and jars are sterilized as a routine procedure, they need not be marked.

## POST-MORTEM EXAMINATIONS

Perform all autopsies as soon after death as possible. Select the required instruments, allowing a fresh set of forceps and scissors for each step in the procedure. Sterilize the instruments by boiling in water for at least five minutes.

Tie the dead animal to a metal autopsy tray, or, in the case of a mouse, fasten to a board with push pins, ventral surface exposed, and wet the hair with 1-per-cent crude cresol. Using forceps and blunt-pointed scissors (a sharp scalpel or safety-razor blade may be used), make the primary incision through the skin only, along the extreme length of the trunk, from the symphysis pubis to the root of the neck. Cut through the skin at both ends of this incision, loosen and turn back the skin, exposing the whole ventral surface, and note the condition of the axillary and inguinal glands exposed. Note also the condition of the skin at the site of injection.

Examine the abdominal wall and make cultures, when indicated, from any lesion which may be present, the choice of media being determined by the kind of material injected and the nature of the lesion.

Using fresh sterile instruments, make a mid-line incision in the peri-

toneal wall extending to the diaphragm. If cultures are to be made from the peritoneal fluid, make them immediately. Then cut the peritoneal wall near the diaphragm and at the lower end of the incision, if necessary; lay the folds back, exposing the organs. Examine each organ carefully to note any abnormality and make cultures from lesions if present.

Open the pleural cavity with fresh sterile instruments, making an inverted V-incision from the base to the apex of the thorax, by cutting costal cartilages. (Use blunt-pointed scissors and take care not to pierce the lungs.) Connect the two incisions by cutting along the upper margin of the diaphragm. Turn back the sternum, exposing the pleural cavity and make cultures from the fluid immediately, if required. If a blood culture is to be made, lift the heart and hold it in position with haemostatic forceps. Make a cut in the pericardium and draw it back, exposing the heart. Sear an area on the right ventricle and with a fresh sterile scalpel, or sharp-pointed scissors, make a short incision. Insert a sterile loop, transfer some of the blood to suitable culture medium, and make a preparation for morphologic examination.

To make a blood culture from a mouse, sear very lightly the apex of the heart, and cut off the tip.

If the lungs appear abnormal, make cultures from them in the same manner, selecting the portions which show lesions.

Make direct preparations from the infected areas which are cultured, and stain by Gram's and other methods, as indicated.

If anatomic examination of tissue is required, proceed as directed under "Tissue for Anatomic Examination." Always obtain material for bacteriological examination, before removing the sections of tissue.

As soon as the autopsy has been completed, wrap the animal in heavy paper and place it in the can reserved for dead animals.

Autopsy trays: Trays of galvanized iron, 12 by 26 inches for rabbits, and 10 by 18 inches for guinea pigs, with a central longitudinal depression 1½ inches deep. Two 2-inch cleats are attached at each end (See: plate II).

### *Tissue for Anatomic Examination*

When an anatomic examination of tissue is necessary, notify the pathologist immediately and proceed as soon as possible with the autopsy. If the pathologist cannot be present, place representative sections of the tissue, not over 1 by 1 by 0.5 centimeters in size, in

Zenker's fluid and in 10-per-cent formalin, using five parts of fixative to one part of tissue.

## CARE OF THE OPERATING ROOM AND EQUIPMENT

### GENERAL INSTRUCTIONS

After use, clean and disinfect operating trays, bleeding boxes, table tops, etc., with 1-per-cent crude cresol. If infectious material has, or is suspected of having, come in contact with the table, box, or tray, allow the disinfectant to remain for a few minutes, or thoroughly flame the area. Place all waste paper, cotton, etc., in the cans provided for the purpose, taking care that the covers are replaced securely on the cans.

Return all bottles, trays, etc., to their proper places. Do not remove from the room any apparatus or supplies which are part of the operating-room equipment. Do not leave in the room any supplies or other materials which are not part of the operating-room equipment.

When removing hair from animals or performing other work which causes an accumulation of waste, see that it is not scattered about the room but is placed immediately in the proper receptacle.

#### *Care of Instruments*

Immediately after use, boil the instruments for five minutes in water containing 5-per-cent sodium carbonate (about a tablespoonful to a liter of water). Before they have had time to cool, dry them thoroughly by rubbing briskly with a soft towel or cloth, and wipe with a cloth saturated with mineral oil. Always give scalpels a few strokes on a soft stone (carborundum). When the edges become dull, sharpen them on a soft stone and then on a hard stone. Never flame scalpels, scissors, etc. For searing, use a vegetable knife.

#### *Care of Syringes*

Immediately after use, rinse the syringe and needle by drawing up water from and expelling it into the sterilizer. Detach the needle with forceps and place it and the syringe and plunger in the sterilizer. Boil for five minutes. (If material of noninfectious nature has been injected, the syringe may be cleaned by rinsing with water and then allowing it to stand for a short time in cleaning solution, if necessary, to remove any viscous material.)

### *Care of Needles*

Rinse as described above and sterilize by boiling. Clean with a wire if necessary, and then in succession draw up and expel alcohol, ether, and albolene. Finally replace the wire. (If the wires are lost or bent, others should be substituted.) Sharpen needles, when necessary, on a soft stone and rub with a soft or specially prepared cloth. (With proper care, a needle will remain satisfactory for use for a long time.)

## PROCEDURE IN TESTS REQUIRING SPECIAL PRECAUTIONS

### HOUSING AND INSPECTION

When special precautions against the spreading of infection are required (as in the case of animals inoculated with material which contains, or is suspected of containing, *B. mallei*, *B. pestis*, *B. melitensis*, *B. tularense*, *Sp. cholerae*, *B. anthracis*, *B. botulinus*. *B. tetani*, *B. welchii*, *B. sporogenes*, *B. oedematis maligni*, and the rabies virus), keep the animals in special isolation cages, metal boxes, or glass jars in the room reserved for the purpose. If the infection can be spread by insects (*B. pestis*, *B. tularense*), set the cages or jars in shallow pans containing kerosene oil. Label the boxes and jars as previously described, and, in addition, tie on each a large red tag, which will indicate to the worker taking care of the special quarters that no dead animals are to be removed. (In case the animal is found dead on a Sunday or a holiday, however, the hour is recorded on the tag.) When the animals have been inoculated to determine the presence of *B. anthracis*, *B. pestis*, *B. mallei*, *B. tularense*, *B. melitensis*, or rabies, mark the red tags distinctly, "Do not touch or feed," which will indicate that food and bedding are to be left outside the boxes or jars so that the worker in charge of the tests may take entire care of the animals.

When it is necessary to handle the animals after inoculation with *B. tularense*, *B. melitensis*, or *B. pestis*, use rubber gloves, washing them at frequent intervals during the operation, and sterilizing them in an autoclave or by boiling, when the work is completed.

### SPECIAL INSTRUCTIONS

When performing autopsies on special test animals, observe the following precautions:

1. Perform the autopsies in the room set aside for all the work with these organisms. Do not use the operating room.
2. Use metal autopsy trays, reserved for this work, and wet the

animal's hair with 5-per-cent cresol compound. When the autopsies are completed, place the tray in a covered can and put it directly into an autoclave, then loosen the cover. If additional autopsy trays are needed, use pieces of wooden board, but in this case take special precautions to prevent the spreading of contaminated fluid from the animal to the work table.

3. Place all instruments and syringes immediately after use in a deep container with a tight-fitting cover. When the examination is made for *B. pestis*, *B. mallei*, *B. tularense*, *B. melitensis*, and *Sp. cholerae*, boil in water containing 5-per-cent sodium carbonate (about a tablespoonful to a liter of water) for fifteen minutes. When the examination is made for *B. anthracis*, *B. botulinus*, and *B. tetani*, place the instruments in a pail containing 5-per-cent cresol compound and personally place the pail in the autoclave.

When it is necessary to use the instruments again before it has been possible to sterilize them in the autoclave, put them in a large covered container and boil in 5-per-cent cresol compound for thirty minutes.

4. After the autopsy, wrap the animal in several thicknesses of heavy paper and personally place it in the furnace. If a piece of board is used, wrap it with the dead animal.

5. Personally place the boxes or jars occupied by these animals directly in the autoclave.

Always consult the bacteriologist in charge of the department concerning the disposition of special test animals, which have not died during the period of observation. (Such animals are usually not returned to stock but are chloroformed, autopsied, and burned, as previously described.)

# METHODS USED IN THE DEPARTMENT FOR THE PREPARATION OF MEDIA AND GLASSWARE

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## INTRODUCTION

The routine work of the media department comprises the preparation of media, the care and cleaning of glassware, the processes connected with sterilization, and the preparation of diagnostic and other outfits that are distributed from the laboratory.

As the work of preparing and sterilizing glassware and media is considered fundamental, practically all new members of the staff are assigned to this department for training when they first enter the organization. Those workers who show capacity for further development are given opportunity to learn other branches of laboratory work.

Within recent years, the department has been equipped and organized to supply glassware and media not only for the routine work, but also to meet the increasing demands of the research workers. Formerly, new media required for special investigations were prepared in this department, or in their own laboratories, by the research workers themselves. Now, however, all such special work is done by the regular media staff.

When questions in connection with the behavior of routine media arise, members of the group assigned to the preparation of media cooperate with members of other groups in studying them. Members of the staff are also encouraged to undertake independent investigations, but as this department is organized primarily for the service of the other laboratories, a study may have to be put aside for months at a time, in order that the demands for increased service may be met. The department is constantly taking over routine work, formerly performed elsewhere in the laboratory, and frequently supplies workers to fill temporary vacancies in other departments.

As is required by the volume and the variety of the work done in this department, much of the equipment provided is especially adapted for work in a large laboratory and includes: a platform scale of 180-kg. capacity, a motor-driven meat grinder, motor-driven wheels for sharpening returned blood-letting needles, a motor-driven "World Labeler" for labeling mailing cases for diagnostic outfits, alberene steam-heated water-baths, steam-heated vats for boiling glassware, a 40-liter steam kettle for heating media, a dry-air "American" sterilizer heated by steam, under high pressure, circulating in the jacket, a second large "American" sterilizer used for autoclave sterilization of large articles,

such as animal cages, etc., a bank of ten horizontal autoclaves of three different sizes attached to a 30-pound steam line and equipped with positive and negative air pressure.

The standard methods of the media and glassware department have been formulated with a view to being of special assistance to the more or less inexperienced workers entering the laboratory, in obtaining a grasp of the different phases of the work. The detailed directions are intended to be exactly followed by the beginner. No attempt has been made to include discussions of theory or of possible alternative procedures. For such information the workers are referred to standard textbooks on bacteriology, chemistry, and physics, and to other sources. The present collection of methods is in the nature of a guide, and consists mainly of instructions for carrying out procedures in this particular laboratory with the equipment and apparatus in use here.

# SECTION I

## STERILIZATION

Routine sterilization is effected: by exposure for various periods to dry heat, to steam under pressure, to steam at 100°C. (intermittent sterilization); by contact with chemicals, such as crude cresol; and by passage through sterile filter candles.

### SPECIAL APPARATUS

In addition to gas ovens and an inspissator, which require no description, the equipment includes the following special apparatus.

### "AMERICAN" STERILIZERS

There are two of these sterilizers, operated on a steam line of 130-pounds pressure. They are rectangular and approximately 90 by 135 by 105 centimeters inside dimensions. An easily movable carriage with adjustable shelves can be drawn out from the chamber on a track, to facilitate loading. The sterilizers are so constructed that they can be used for sterilization by dry heat or by steam. The jacket is built especially to withstand high pressures. When used for steam-pressure sterilization, the method of operation is similar to that used with autoclaves. The doors of these sterilizers are not self-locking, but are sealed with a gasket and when closed sufficiently tight, they will maintain satisfactorily either moderate pressure or vacuum. Care should be taken, however, that they are not closed too tightly, especially during dry-heat sterilization. If this occurs, the gasket is flattened and cracked, permitting leakage of steam. When operated as dry-heat sterilizers, steam is admitted to the jacket only.

### AUTOCLAVES

The autoclave equipment consists of a bank of ten horizontal sterilizers (Bramhall Deane, special construction), operated on a steam line of 30-pounds pressure.

These autoclaves, as well as the "American" sterilizers, are attached to the positive and the negative air-pressure lines. Special, closely fitting, outer doors, sealed by gaskets and equipped with vacuum-release valves, are closed tight before the vacuum-line valves are

opened. Special care should be taken that the ground surfaces where the inner doors lock into the frames do not become scratched or roughened, as this would permit leakage of steam.

*Registering Apparatus*

The two "American" sterilizers and the four large autoclaves are equipped with "Tycos" recording thermometers which give in permanent form the time and temperature record of each run.

All the autoclaves are equipped with capillary mercury thermometers with index dials, the bulbs located at the point of lowest temperature in the chamber, below the false bottom in front. Pressures, both in the autoclaves and the "American" sterilizers, are registered on gauges connected with the inner chambers and with the jackets. The gauges that are connected with the chambers register both positive and negative pressures.

GENERAL DIRECTIONS FOR LOADING STERILIZERS

Do not place heavy articles on top of lighter ones. In sterilization by steam, protect cotton plugs by caps or layers of heavy paper. Do not pack articles tight in baskets nor place them too close together on the shelves of the sterilizers.

Tight packing causes breakage due to expansion, and in the autoclaves, interferes with free circulation of steam.

**Glassware.**—In general, sterilize glassware in the hot-air sterilizers, except suction flasks, bleeding jars, and heavy tincture bottles. Place bleeding jars and bottles on their sides with heavy paper between them and also between the glass and the sides of the sterilizers, as heavy glassware is very easily broken by sudden changes of temperature.

**Boxes and Pails with Covers.**—Place boxes and pails in the sterilizers with their covers set loosely in place. Close the covers tight as soon as the hot-air sterilizer or autoclave is opened. When boxes with hinged covers are left open, set them in the sterilizer in such a way that the opening is toward the inside, so that when the door is opened, air will not rush into the box.

Large Berkefeld and Mandler filters are sterilized in copper boxes in the autoclave. Small bottles and vials for antitoxins and other products, are sterilized in enamelware pails in the gas ovens.

Sterilize animal boxes and contaminated material collected each day from the various laboratories, in the "American" sterilizer.

**Media.**—The general directions given for packing material in

sterilizers applies with special force to media, especially to tubed media in baskets.

To avoid breakage, place tincture bottles containing solutions, such as infusion and salt, in pails of water which have a protecting pad of cotton in the bottom.

## STERILIZATION BY DRY HEAT

For sterilization by dry heat, gas ovens are used, or high-pressure steam-heated sterilizers, "American" sterilizers.

### *Gas Ovens*

In the ovens the required temperature is secured by heating with gas burners, and regulation is accomplished by adjusting the flames. These ovens are equipped with ordinary mercury thermometers. Considerable care is necessary to prevent wide variations from the temperature required. Experience, checked by means of maximum thermometers, has shown that the temperature varies in different sections of the oven, and that, in general, the heat is more intense on the bottom shelf near the burners. Any glassware to be sterilized by dry heat may be sterilized in the gas ovens. Cans or containers of glassware are placed on the lower shelf where the heat is more intense. Baskets of material wrapped in wax paper are placed on the top shelf, to avoid charring.

**Procedure.**—1. Light the gas and load the oven.

2. When the thermometer registers the required temperature, note the time and attach a tag to the door, indicating the time the gas is to be turned off.

The temperatures and the sterilization periods for different materials to be sterilized in the gas ovens vary.

### *"American" Sterilizers*

The "American" sterilizer can be used for dry-heat sterilization at high temperatures (150 to 162°C.) by admitting steam, under high pressure, to the outer jacket.

**Procedure.**—1. Heat the jacket slowly at first, then more quickly.

This is done to avoid rapid temperature changes in the walls, which would tend to cause leaks.

2. Load the car, run it into the chamber, and close the door.

3. Allow steam to enter the jacket until the maximum pressure is reached.

An hour is usually required to reach the maximum pressure (125 pounds) after the door is closed. An initial pressure of 110 pounds is necessary to bring the temperature of the chamber to 150°C., at which point the sterilization period is started. A pressure of from 110 to 125 pounds of steam is sufficient to maintain a temperature of from 150 to 162°C. The sterilization period is one hour at this temperature.

4. At the end of the sterilization period, shut off the steam supply and open the door slightly. Allow the material to cool somewhat before opening the door wide.

A slip of paper on which the word "sterile" is written with cobalt-chloride solution is placed in each basket, pipette container, and can, which is sterilized by dry heat. The invisible writing darkens between 140 and 150°C., above which the color becomes deeper.

## STERILIZATION BY STEAM UNDER PRESSURE

Sterilization by means of steam under pressure is usually accomplished in the autoclaves. In some instances the "American" sterilizer is used. In operation it is necessary to check carefully the thermometer readings and the recorded steam pressures.

### *Autoclaves*

**Procedure.**—1. Load the autoclave.

2. Close both pressure and vacuum doors and all valves.

3. Admit steam to the jacket and the chamber until 15- or 16-pounds pressure is registered on the two gauges. Open slightly the exhaust valves of the jacket and the chamber, and leave them open during the period of sterilization. When the temperature reaches 121°C., start to time the sterilization period. Keep the temperature and pressure constant by adjusting the exhaust valve of the jacket.

4. When the sterilization period is ended, close the chamber-exhaust and the steam-inlet valves and open the jacket-exhaust valve.

5. When condensation is not objectionable, reduce the temperature quickly by replacing the steam with air, maintaining the pressure.

6. Do not open the inner door until the temperature has fallen to 98°C. or below, if the load includes media.

### *"American" Sterilizer*

**Procedure.**—1. Heat the jacket slowly.

2. Load the car, run it into the chamber, and close the door.

3. After the sterilizer is warm, allow the steam to enter the jacket until a pressure of approximately 18 pounds is recorded on the jacket gauge.

4. Admit steam to the chamber until a pressure of from 15 to 18 pounds is reached, giving a temperature of 121°C. as indicated on the

dial of the recording thermometer. Maintain this temperature and pressure for thirty minutes.

5. Shut off the steam supply.

The door should never be opened until the chamber gauge registers zero. However, the door should then be opened or the vacuum-release valve should be opened slightly, to prevent the creation of a vacuum and the pulling of condensation water into the chamber.

## STERILIZATION BY A MIXTURE OF STEAM AND AIR UNDER PRESSURE IN THE AUTOCLAVE

This method is used only for the coagulation and sterilization of Loeffler's serum medium.

**Procedure.**—1. Load the autoclave.

2. Close the doors and the exhaust valves of both jacket and chamber.

3. Admit steam to both jacket and chamber until a pressure of 15 pounds is reached in the chamber. Shut off steam from chamber. Open exhaust valve of jacket slightly until a temperature of 100°C. is reached. Time the sterilization period from this point and maintain at this temperature, or above, for fifty minutes, by adjusting flow of steam through the jacket, never allowing the pressure to exceed 15 pounds.

4. When the sterilization period is completed, close the steam valve, open the jacket-exhaust valve wide, and open the chamber-exhaust valve slightly.

5. Do not open the door of the chamber until the temperature reaches 98°C.

## INTERMITTENT STERILIZATION

Sterilization at 100°C. is carried out in the autoclaves at 1-pound pressure or less. It is usually carried on for from twenty to forty minutes on three successive days. The time is varied according to the bulk of the material. This method is used only for culture media.

**Procedure.**—1. Load the autoclave and close the inner door tightly.

2. Admit steam to both jacket and chamber. Open both exhaust valves, and pass steam through both jacket and chamber until the air has been displaced and a temperature of 100°C. is reached.

3. Maintain this temperature by regulating the flow of steam by means of the inlet valve. Take care to use no more steam than necessary. Keep the pressure at 1 pound or less to prevent the temperature from rising above 100°C.

4. Time the sterilization period when the thermometer reaches 100°C.

5. When the run is completed, close the chamber-exhaust valve and the steam-inlet valves.

6. Allow the temperature to fall below 98°C. before opening the door.

## STERILIZATION BY INSPISSATION

The process of coagulating and sterilizing Petroff's egg medium without dye is carried out at temperatures between 75 and 85°C., on three successive days.

**Procedure.**—1. Fill the reservoir of the inspissator about three-fourths full of water and light the gas.

2. Line the floor and sides of the chamber with a heavy layer of nonabsorbent cotton.

3. Slant the tubes of medium on pieces of glass tubing placed at convenient intervals.

4. Place two small containers of water near opposite corners of the chamber.

The evaporation of this water during the lengthy sterilizing process helps to retain the water of condensation on the slants of the coagulated medium.

5. Insert a thermometer in the water jacket and place two thermometers inside the chamber in convenient positions for reading. Replace the covers.

6. Watch the temperature closely and regulate it by changing the height of the gas flame.

## STERILIZATION BY THE USE OF CHEMICALS

Crude cresol is used to sterilize rubber stoppers. They are boiled in a 1-per-cent solution and are dried over a flame immediately before use. Crude cresol is also used to sterilize plating rods and pipettes contaminated with nonspore-forming pathogenic microörganisms. These rods and pipettes are left in the solution for several hours.

## STERILIZATION BY FILTRATION

Sterilization by filtration through a candle of diatomaceous earth (Berkefeld or Mandler filter) is employed for liquids which are injured by the application of heat.

## SECTION II

## PREPARATION OF GLASSWARE

All glassware, with the exception of special articles, such as certified pipettes and the glassware used in the chemical laboratories, is cleaned and prepared for use by the glassware group. The work includes certain definite processes—preliminary sterilization and cleaning; boiling and washing with soapsuds; rinsing; treatment with cleaning solution, if necessary; drying and plugging, preparatory to final sterilization. General directions for this work are given.

### *Apparatus*

**Vats.**—There are three vats of alberene stone, lined with tinned copper, approximately 72 cm. square by 50 cm. deep. Each is equipped with cover, water inlets, standpipe, drain, and steam-heating coils.

**Vat Baskets.**—Heavy wire baskets (30 by 30 by 37.5 cm.) with drop handles, are used for holding glassware. They are so constructed that four baskets will fit easily into a vat.

**Mixing Valve.**—Warm water for rinsing purposes is automatically maintained at a suitable temperature (27 to 32°C.) by means of a Leonard mixing valve.

**Rinsing Apparatus for Large Bottles and Flasks.**—For rinsing heavy bottles and large flasks, an apparatus is used which consists of a rubber hose attached to a faucet terminating in a tip of block tin which has perforations at the end and on the sides to disperse the stream of water. A ring stand, with a ring, supports the bottles, and a clamp holds the rinsing apparatus. The bottles are rotated by hand until the streams of water have thoroughly rinsed the interior.

**Rinsing Apparatus for Pipettes.**—This is a simple apparatus consisting of a glass siphon, about 14 mm. in diameter, hooked over the nose of a glass cylinder (450 by 75 millimeters). The current of water through the siphon is automatically made and broken, thus alternately emptying the cylinder and allowing it to fill from a small stream of water from the top. By using Y-tubing, several cylinders may be fed from one tap.

**Baskets.**—For drying and sterilizing, glassware is placed in heavy wire baskets of convenient sizes (25.5 by 18 by 18 centimeters, 30.5 by 30.5 by 20 centimeters, 35.5 by 25.5 by 18 centimeters).

## PRELIMINARY TREATMENT OF USED GLASSWARE

In general, glassware is collected from the various rooms and sterilized by workers of the sterilization group on the afternoon before the glassware is to be cleaned. However, glassware contaminated with exceptionally pathogenic microörganisms is placed directly in the sterilizer by workers accustomed to handling these organisms.

**Sterilization.**—Practically all contaminated glassware is sterilized in the autoclave for thirty minutes at 121°C. before being placed at the sinks to be cleaned. Used porous Petri-plate tops are sterilized by dry heat at from 150 to 162°C. for one hour.

Contaminated glassware which contains serum is partially filled with or immersed in water to prevent coagulation of the serum, and etching of the glass.

## CLEANING

Chromic-acid cleaning solution and alkali-free soap flakes are the most commonly used cleaning agents. For convenience in certain work, there is kept on hand a supply of semifluid soap prepared by making a 10-per-cent solution of soap flakes in boiling water.

**Preliminary Cleaning.**—Remove the solid material remaining in the glassware by means of a flat piece of steel with a bent end. Rinse the glassware in running tap water.

**Boiling in Vats.**—Fill the vats with sufficient water to cover the baskets in which the glassware is to be boiled. Add 500 grams of neutral soap flakes or three cakes of soap the first day, and 200 grams of flakes or one cake of soap, each succeeding day. In the large wire baskets used in the vats, place the tubes, bottles, or sputum jars on their sides, the open ends all pointing in the same direction, so that they may readily fill with water. If it is necessary to place tubes, bottles, and jars in the same basket, place the heavy glassware in the bottom, to prevent breakage. Immerse the baskets in the vat, tipping slightly, to fill the glassware with water. Lower the cover of the vat, turn on the steam, and leave it turned on until the water boils. Keep the water at the boiling point for an hour.

Ordinarily, the water in the vats needs to be changed only once or twice a week; they are always cleaned thoroughly at least once a week.

Small test tubes, for use in serological work, are placed in coarse muslin bags and are boiled in the vats. The soap solution should be cold when the tubes are immersed. These tubes are never boiled with other glassware.

**Boiling in Pails.**—Place the glassware in the pails and cover it with cold water. Add about 60 grams of soap flakes (or one-third of a

cake of soap, shaved). Boil the water over a free flame until the soap is completely dissolved, or as long as it is necessary for the particular glassware being cleaned.

**Washing.**—After it is boiled, wash the glassware in hot soapsuds. Use a brush or cloth to remove all foreign material. When a large amount of glassware is to be cleaned, use one of the lead-lined alberene sinks for this purpose. Add about 250 grams of soap flakes, or two cakes of soap dissolved in boiling water to a sink full of hot water. Remove one basket of glassware at a time from the vats, tip the basket on the edge of the vat in such a manner that the glassware is emptied of water, and then lower the basket into the hot soapsuds in the sink.

Used glassware which is fragile is not boiled before washing in soapsuds; new glassware, unless otherwise specified, is not boiled but is washed in hot soapsuds, then thoroughly rinsed and dried.

**Rinsing.**—Always rinse each article in a running stream of lukewarm or cold tap water, unless otherwise specified. Fill and completely empty every piece of glassware, six such rinsings being sufficient in most cases. First rinse heavy bottles and large flasks well on the outside and then invert them over the rinsing apparatus. Give a final rinsing with distilled water to any article which should be especially brilliant.

### TREATMENT WITH CLEANING SOLUTION

When necessary, use a sulfuric-acid-bichromate solution to remove organic matter not removed by previous washing. Merely rinse flasks and bottles with the solution, but fill used sputum jars, Coplin jars, graduates, pipettes, and glass slides with, or submerge them in, the solution, and allow them to stand for one or more hours.

Very thorough rinsing is necessary after this cleaning solution has been used.

### FINAL TREATMENT OF GLASSWARE

**Drying.**—Dry all glassware at temperatures below 150°C.

**Inspecting and Sorting.**—Inspect all glassware carefully and return to the sinks to be washed any which is not clean. Place tubes of the same size together, open end down, in baskets to await plugging. Plug bottles and flasks at once to avoid repeated handling.

**Plugging with Cotton.**—Place a loosened roll of nonabsorbent cotton, paper side down, on the table. Tear off a piece of cotton, the exact size of which can be determined only by trial and practice. Separate the cotton into two layers, cross the fibers and reinforce the center by adding a small piece of cotton. With the end of a steel plugging rod

placed in the center of the crossed cotton, push the cotton into the tube or neck of the vessel to be plugged.

The finished plug should be of even thickness from one end to the other. It should be about 25 to 35 millimeters in length inside the tube, depending on the size of the tube, or nearly the length of the neck of the bottle or flask (Erlenmeyer). Enough of the cotton should project to form a firm head by which the plug may be grasped.

**Plugging with Cheesecloth and Cotton.**—Place a square of cheesecloth, large enough to cover the finished plug, over the opening to be protected.

Make the plug in the usual manner and push both the cheesecloth and the cotton into place at the same time.

Cheesecloth-covered plugs are sometimes used to prevent the cotton from sticking to the necks of bottles that are steam sterilized, and also in glassware that must be relatively free from cotton fibers.

**Plugging and Corking.**—Unroll a short length of cotton and carefully separate into thin layers, three or four, depending on the thickness of the cotton. Use these thin layers for making plugs, reinforcing the centers with sufficient cotton to make butts as firm as those of the ordinary cotton plugs. Push the plug into the tube with a plugging rod, spreading the thin upper margin of the plug to receive the cork. Put the cork into the tube so that the thin layer of cotton forming the margin of the plug extends upward along the length of the cork. Sterilize by dry heat.

The thin margin of cotton serves, when the cork is paraffined, to bind the plug to the cork, thus forming one continuous stopper which can be easily removed and replaced. It is practically always necessary to trim the cotton somewhat before paraffining the corks.

Tubes stoppered with both cotton plugs and corks are used for preventing evaporation of certain media which are to be kept over a long period of time.

**Corking.**—Untreated corks: Use untreated corks just as they are received from the stockroom. Simply push them into place, previous to sterilization.

Treated corks: Place the sorted corks in a cheesecloth bag and boil for five minutes in 1-per-cent crude cresol. Without removing them from the bag, rinse thoroughly and boil for five minutes in tap water. Dry at a temperature below 100°C. After the corks are thoroughly dry, remove from the bags directly into tightly covered cans, in which they are stored until needed. When ordered, distribute the corks in pails in the following manner: Place them in layers with circular sheets of heavy paper between the layers. On top of the circular sheet, over the upper layer of corks, place a 30-cm. square of the same kind of paper folded in quarters to be unfolded and placed over the pail as a protection from dust while the corks are being used.

**Capping.**—With heavy paper: Place heavy paper over plugs or corks when specified and tie about the neck of the bottle or flask with a cord, using a slip bowknot.

With tinfoil: Place a piece of tinfoil large enough to come well down over the neck, smoothly over the cork, fold down, and crease tightly about the neck.

With tinfoil and muslin: In addition to a layer of tinfoil, place a circle of muslin over the stopper on top of the foil, and tie firmly about the neck.

With muslin: Place a circle of muslin over the stopper and tie firmly about the neck.

With soft filter paper: Place a loose-fitting cap of soft filter paper over the stopper.

### STERILIZATION

Most glassware is sterilized by dry heat, but heavy glass articles and apparatus made up of glass and rubber, such as dispensing apparatus, are sterilized by steam under pressure. In the detailed working directions, typewritten copies of which are kept in the department, the methods of sterilization to be used for each type of glassware are given.

## PREPARATION OF SPECIAL OUTFITS

### APPARATUS FOR BLEEDING HORSES

**For Defibrinated Blood.**—Fit a 480-cc. wide-mouth bottle with a 2-hole rubber stopper. Insert two glass tubes 1 cm. in diameter; one, a straight tube about 12 cm. long, the other, a right-angle tube, with both arms about 8 cm. long.

The straight tube serves as an air release, and the other, attached to the needle by a rubber hose, as an inlet tube.

Plug the straight tube tightly with cotton. Attach to one end of the right-angle tube a piece of rubber hose 225 to 250 mm. long. Insert into the other end a short length of glass tubing, pushing it up from 15 to 25 mm. (to keep the hose from collapsing). Slip the end of a 13-gauge needle $2\frac{1}{2}$ inches long, into the hose and wire in place. Leave an excess of oil on the needle to prevent rusting. Cover the bottom of the bottle with glass beads (short lengths of rod) and tie the stopper firmly in place. Slip a pinchcock over the rubber hose, insert the needle into a test-tube guard and plug with cotton. Tie the test-tube guard to the neck of the bottle, and attach a manila tag. Wrap in heavy paper and sterilize by steam at 121°C. for twenty minutes.

**For Serum.**—This outfit, which is known as a bleeding jar (See: fig. 12), consists of a cylindrical battery jar, a stamped metal cover, which fits well over the rim of the jar and a heavy metal weight. The weight has on its lower surface teeth or pegs (for the purpose of gripping the clot and preventing the weight from sliding and breaking the jar) and, on its upper surface, a small stud with a hole drilled horizontally through it. This stud passes through an opening in the center

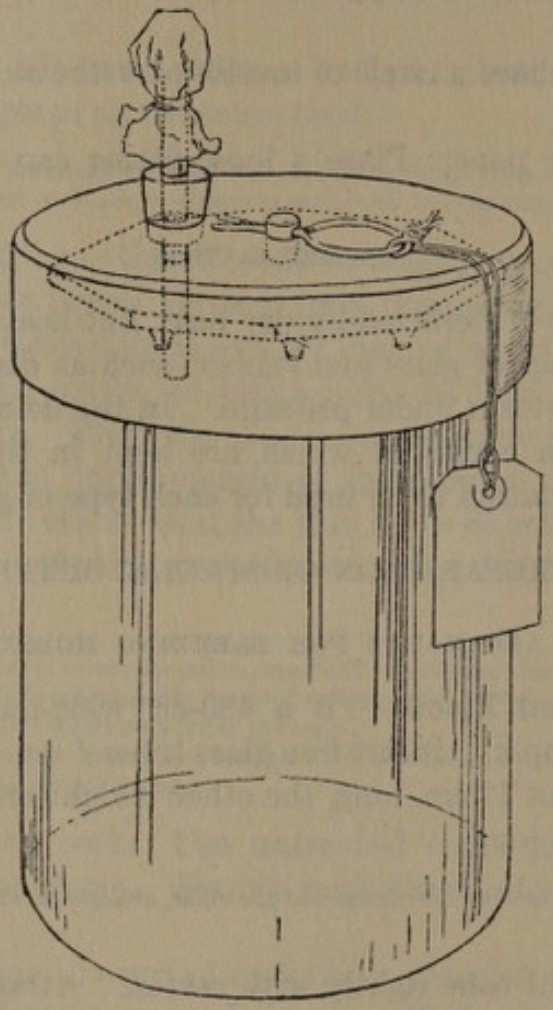

FIG. 12. BLEEDING JAR

of the cover. The weight is supported directly beneath the cover by means of a brass cotter pin which passes through the hole in the stud. A glass tube through which the blood is allowed to enter passes through holes in the weight and the cover, and is held in place by means of a rubber stopper which fits tightly in the hole in the cover. The space between the glass jar and the metal cover is filled by a tightly fitting pad of "silence" cloth or canton flannel.

**Procedure.**—Fasten weight in cover by means of the cotter pin and

wind a small piece of cotton around the stud under the pin to protect the opening. Attach to the pin a cord and tag on which is written "Do not cut off or pull this tag." Use a cord long enough to allow the tag to hang half way down the side of the jar, and place it so that one pull will remove the pin and allow the weight to fall. Insert the stopper with glass tube, making sure it fits tightly. Place the pad smoothly around the top of the jar and put on the cover, which should fit snugly and be pressed down over the pad. Cover the top of the jar with heavy paper, allowing it to reach 3 or 4 cm. below the rim of the cover and tie firmly in place (not shown in fig. 12).

Cut a hole in the paper so that the inserted tube may project. Protect the end of the tube with cotton, and cover with paper tied in place. Wrap in heavy paper and sterilize by steam at 121°C. for thirty minutes.

# SECTION III

# PREPARATION OF MEDIA

## CHAPTER 1

## PREPARATION

Although a large number of different culture media are prepared for various kinds of organisms, certain ingredients and procedures are more or less common to the preparation of large groups of media. An attempt has, therefore, been made to formulate a section dealing, as far as possible, with materials and procedures in general, leaving matters of a special nature to be described in the individual formulae.

As for ingredients, a search of the market is constantly being made for dependable products, the laboratory animals furnish blood and serum, while the hospitals of the district are depended upon for ascitic fluid.

In the general discussion of the procedures, it has been found convenient to divide media into two main groups. The first group consists of media which, after all the materials are combined, may be subjected to a sufficiently high temperature to effect sterilization. In the second group are placed media prepared by combining sterile components, observing the precautions necessary to maintain the sterility of the finished product; such media are usually prepared in a draft-free room and generally contain an enrichment substance, some desired property of which is unfavorably affected by exposure to a temperature sufficiently high for sterilization.

All media are ready for use when delivered, except certain media to which sugar solutions are added at the time of inoculation.

### HEAT-STERILIZED MEDIA

#### MATERIALS

**Water.**—Distilled water is used in the preparation of all culture media and reagents.

**Meat Extract.**—Liebig's meat extract is used.

**Peptone.**—Digestive Ferments Company's Bacto-peptone is used for general purposes. Other peptones are used as specified in the formulae.

**Sugars.**—C.P. sugars are used in all media to which sugar is added as a nutrient. Only biologically tested sugars are used to obtain information regarding biologic reactions.

**Agar.**—A. H. Thomas' No. 40 white agar is used.

**Gelatin.**—Wilson-laboratory gelatin is used.

**Meat.**—The cut known as top round of beef, fat- and bone-free, is purchased. Fascia and any remaining fat are trimmed off in the laboratory; the meat is then cut into small pieces and ground. Veal is bought in thick pieces also free from bone and fat. It is trimmed and ground in the same way as the beef. Beef heart and beef liver are used for vitamin media.

**Chemicals.**—C.P. salt and other C.P. chemicals are used.

## WEIGHING

The different individual formulae specify the amount of each ingredient to be used in the preparation of the various media. All media and all solutions prepared in large quantities, are made by weight with no reference to volume; only solutions prepared in small quantities are made up to volume. The department is supplied with two balances and two scales, all of different capacities and varying in sensitiveness from a torsion balance with a rider beam graduated in 5 mgm., to a platform scale, the slide beam of which is not graduated below 20 grams.

New workers are carefully instructed in the use of the scales and balances. All weighings of the ingredients of a medium performed by one worker are checked by a second worker. The first worker puts the weights on the scales and enters on his record card each separate weight used, while a second worker removes and checks these weights.

## HEATING TO DISSOLVE INGREDIENTS

All the common ingredients used in media making, with the exception of agar, are readily soluble upon slight heating. The nature and the quantity of the ingredients determine, to a large extent, the manner of heating. In the preparation of all media, loss of weight, due to evaporation, is made up with distilled water after each heating.

### *Apparatus*

The apparatus used for heating media includes:

Free flame.

Autoclaves.

Steam kettle.—The steam-jacketed kettle is of approximately 40-liter capacity and is equipped with a faucet to permit liquids to be drawn off easily, and

with a strainer to prevent the clogging of the outlet. The kettle is attached to the 30-pound pressure steam line and is equipped with a reducing valve and a bypass.

Steam-heated water-bath.—The steam-heated water-bath is a part of the standard equipment of the laboratory. It is an alberene water-bath heated by steam coils. The flat alberene top (60 by 70 centimeters), containing openings of various sizes, as well as the bath itself, is used for dissolving materials and for keeping them hot.

**Procedure.**—For small quantities of broth, dissolve ingredients, with frequent stirring, over free flame. Dissolve gelatin solutions in the steam-heated water-bath. Dissolve agar in water or in infusion by heating in the autoclave for thirty minutes at 121°C.

### TITRATION AND ADJUSTMENT

As soon as all the ingredients of a heat-sterilized medium are dissolved and combined, the reaction of the medium is adjusted to a point suitable for the growth of the organisms for which the medium is being prepared. As change of reaction of a medium frequently occurs upon sterilization, allowance for this change is made, as far as possible, in the formulae. Great care should be taken not to add excess alkali in the adjustment of a medium, as the addition of acid to bring the medium to the proper reaction is undesirable.

It seems advisable to state in the formulae both the pH value and the approximate phenolphthalein value that have been found equivalent for the particular medium in question.

#### *Phenolphthalein Method*

**Apparatus Required.**—

1. 50-cc. burette, graduated in tenths of a centimeter and connected by means of a side arm with a bottle of N/20 NaOH.
2. 1-cc. pipette (need not be graduated).
3. 5-cc. pipette (need not be graduated).
4. 50-cc. cylinder, graduated, and other graduated cylinders of convenient sizes.
5. 2 white porcelain evaporating dishes.
6. Glass stirring rod.
7. Bunsen burner and tripod.

**Solutions Required.**—

1. N/20 NaOH for titrating most media.
2. N/1 NaOH for adjusting most media.
3. 0.5-per-cent solution of phenolphthalein in 50-per-cent alcohol.

All these solutions, except the phenolphthalein, are prepared by a chemist. Other solutions are used in the titration and adjustment of certain media. These are specified in the formulae.

**Procedure.**—Into each of two evaporating dishes measure 5 cc. of the medium and 45 cc. of distilled water which has been freshly boiled and cooled to between 40 and 45°C. If a raw-meat infusion is to be adjusted, boil it until a coagulum is formed and the solution is clear, and cool before titrating. Fill the burette to a convenient level with N/20 NaOH and record the burette reading. To one dish of diluted medium add 1 cc. of phenolphthalein solution. Allow the alkali to drop slowly from the burette into this dish, stirring constantly with a glass rod and noting any change of color as compared with the other dish of medium used as a control. After the end-point, a delicate but distinct pink color, is reached, read the burette again. Subtract the first reading from the second, the difference being the amount of N/20 alkali required to neutralize 5 cc. of the medium, or the amount of N/1 alkali required to neutralize 100 cc. Divide the number of cubic centimeters of medium to be adjusted by 100 and multiply the quotient by the difference in the burette readings, the product being the amount of N/1 NaOH to be added, to neutralize the entire batch of medium.

If the medium is not to be neutralized, but is to be adjusted to a certain number of points acid to phenolphthalein, subtract this number of points from the difference in the burette readings and multiply by this difference instead of by the difference in the burette readings. If the medium is to be adjusted to a certain number of points alkaline to phenolphthalein, add this number of points to the difference in the burette readings and multiply by this sum. After the computed amount of N/1 NaOH has been added to the medium, titrate again.

Two or more adjustments of a medium are sometimes necessary before the desired reaction is obtained.

Express phenolphthalein reactions in terms of percentage of acidity or alkalinity, that is, express the reaction of a medium acid to phenolphthalein, as the number of points acid to the neutral point of that indicator with the plus sign before it. Express the reaction of a medium alkaline to phenolphthalein as the number of points alkaline to the neutral point of that indicator with a minus sign before it. Use N/20 HCl to titrate a medium alkaline to phenolphthalein. Proceed as for titrating an acid medium with N/20 NaOH.

When titrating with acid it is simpler to continue adding N/20 HCl until the pink color just disappears and then to subtract 0.1 cc. from the burette reading to compensate for going beyond the end-point.

### *Colorimetric Method of Adjusting Media to a Definite Hydrogen-Ion Concentration*

Titration with phenolphthalein gives practically correct results when adjusting a medium to the neutral point of that indicator, as the end-

point indicates a hydrogen-ion concentration of approximately from pH 7.8 to 8.0. As the phenolphthalein-titration method is not suitable, however, for adjusting media to other hydrogen-ion concentrations, the colorimetric method is used.

This method is based upon the fact that stable, standard solutions can be prepared covering the whole range of pH values, and that, when proper indicators are added to these solutions, a graduated series of colors is obtained. These color standards represent definite hydrogen-ion concentrations to which media can be adjusted. For the preparation of standard solutions and other procedures relating to them, see hydrogen ions, determination of.

**Apparatus Required.—**

1. Comparator block with holes large enough to hold 150-by-16-millimeter tubes.
2. 3 Pyrex tubes (150 by 16 millimeters).
3. 50-cc. burette (like that used for phenolphthalein titration).
4. 3 1-cc. pipettes (graduated).
5. 5-cc. pipette (need not be graduated).
6. 10-cc. pipette (need not be graduated).

**Solutions Required.—**

1. N/20 NaOH for titrating.
2. N/1 NaOH for adjusting.
3. Pyrex tubes (150 by 16 millimeters) of standard solutions, with suitable indicators, covering the range from pH 6.0 to pH 9.4, in graduations of 0.2 pH. The Clark and Lubs solutions and indicators are used.

The indicators and standard solutions are furnished by the chemist in charge of this work. The color standards, each consisting of 15 cc. of a 1:3-dilution of a standard solution in distilled water plus 0.5 cc. of an indicator, are made up once every two weeks by a member of the media group under the direction of the chemist referred to. The most frequently used series is that covering the range from pH 6.8 to 8.4, phenol red being used as an indicator. Brom thymol blue is used as an indicator for the range between pH 6.0 and 7.6, and thymol blue from pH 8.0 to 9.6. The color standards are kept in the cold room, but are warmed to room temperature before being used.

**Titration and Adjustment.**—Place the standard tube of the reaction to which the medium is to be adjusted in the right-hand hole of the back row of the comparator block. In the adjoining hole of the same row, place a tube of distilled water. In the holes in front of these, place tubes that have been thoroughly cleaned, and rinsed with distilled water. Into each of these put 5 cc. of the medium to be titrated,

and 10 cc. of distilled water which has recently been boiled and cooled to between 40 and 45°C.

A final dilution of medium is thus obtained which corresponds to the dilution of standard solution in the standard tube.

To the tube of diluted medium directly in front of the tube of distilled water, add 0.5 cc. of the indicator used in the standard tube to be matched.

Fill the N/20 NaOH-burette to a convenient level and record the reading. Add the alkali slowly to the tube containing medium plus indicator, shaking after each addition, until the color shades of the two sets of fluids, viewed through the horizontal openings of the comparator block, match.

Determine the calculation in the same way as when making the adjustment to the neutral point of phenolphthalein, since 5 cc. of medium and the same strength of reagents are used in both cases. That is, read the percentage of N/1 solution required directly from the burette. After adding the calculated amount of alkali to the medium, determine the reaction and, if necessary, again titrate and adjust.

**Determination of Reaction when No Adjustment is to be Made.**—Sometimes a medium is prepared, the reaction of which is not adjusted; the determination of the reaction, however, is usually made. Likewise, the reactions of all media are determined after sterilization, and occasionally, also after bacterial growth has taken place.

**Procedure.**—Prepare the tubes of diluted medium and place them in the block exactly as for titration and adjustment. Then place the various standards in turn in the opening behind the medium-control tube until the colors of the two pairs match. If a perfect match is not found, determine between which two color standards the value lies. Record the value as plus to one or minus to the other, but if halfway between, take the intermediate figure.

In adjusting turbid media to the true neutral point, it is sometimes necessary to use litmus paper as an indicator; when the medium is sufficiently clear however, it is preferable to adjust it by the pH method, to from pH 6.8 to 7.0.

## CLARIFICATION

After all the ingredients of a heat-sterilized medium have been dissolved, and the medium has been adjusted, it must be rendered more or less clear. This is usually accomplished by further heating, followed by filtration. When a specially clear broth medium is desired, it is heated for a few minutes in the autoclave at a temperature

slightly higher than the sterilization temperature to be used. When a specially brilliant agar medium is desired, it is cooled to between 50 and 55°C., a solution of egg albumen is added, and the medium is then heated as is any other agar. When such special procedures are to be followed, they are specified in the individual formulae.

### *Heating*

**Procedure.**—Heat large quantities of infusions and broths, for varying periods, in the steam kettle. Apply steam under pressure of from 4 to 5 pounds to heat contents to the boiling point; then reduce the pressure to 1 pound. Stir the contents occasionally with a wooden paddle while heating. Heat small quantities of infusions and broths over the free flame; heat the broths to boiling, stirring frequently. Boil for several minutes. Heat agars in the autoclave at 100°C. for one hour. Heat gelatins in the steam-heated water-bath at 95°C. for twenty minutes.

### *Filtration*

In general, infusions and broths are filtered through soft filter paper (sometimes after a preliminary straining through cheesecloth); agars, through cheesecloth and cotton; gelatins, through paper pulp. Vitamin media are filtered through glass wool.

**Cheesecloth.**—Line with a double thickness of cheesecloth a glass funnel or preferably, if available, the wire basket specially designed for the purpose with hooks to hold it in place inside the rim of a stock pot. Pour the material to be strained into the cheesecloth. Strain by twisting the cloth and pressing the bag thus formed against the side of the funnel or wire basket.

**Filter Paper.**—Fold a filter paper of suitable size through the center, dividing it into halves and quarters. Then crease it into fan-like folds, dividing it into small sections. Place a smaller filter paper, similarly folded, with its apex well down in the apex of the funnel. Then place the larger paper inside the smaller. Rinse with cold, distilled water.

**Cheesecloth and Cotton.**—Cover the mouth of a 960-cc. wide-mouth bottle with two layers of cheesecloth. Over this, place three layers of nonabsorbent cotton, the fibers crossed at right angles. Cover with another layer of cheesecloth and tie firmly with twine around the neck of the bottle. Trim away the cotton and cheesecloth which extend below the twine, leaving only enough to prevent the twine from slipping or loosening.

Invert the bottles in the melted agar, cooled to from 50 to 55°C., and heat them in the autoclave at 100°C. for one hour. When the heat has been turned off for about ten minutes, straighten the bottles and allow them to stand in the sterilizer for about an hour or until the medium has all filtered up.

**Paper Pulp in Buchner Funnel.**—For filtering by this method, see biologic products, filtration of.

**Glass Wool.**—Use rubber gloves in handling this material. Place a mat of the glass wool in a funnel and rinse well with distilled water before using. Pass the filtrate several times through the coarse coagulum that is retained by the filter until the medium is rendered as clear as possible.

## DISPENSING

Heat-sterilized media are dispensed in various types of bottles, flasks, and tubes, depending upon the purpose for which they are to be used. In general, a medium is dispensed immediately after filtration from the containers into which it has been filtered. However, all the filtered portions are pooled when additional substances, such as carbohydrates or indicators, are to be added.

Twenty cubic centimeters of each heat-sterilized medium are placed in a test tube and sterilized with the medium. When intermittent sterilization is used, three such reaction tubes are filled.

In all dispensing, care should be taken not to wet the necks of the containers. In placing tubes and bottles of media in baskets, tight packing should be avoided. Agar media should be dispensed rapidly to prevent solidification; while the process of filling is going on, the bulk of the material should be kept hot. The steam-heated water-bath is convenient for this purpose.

**Apparatus Required.**—

1. Graduated cylinders of convenient sizes.
2. Funnels 20 cm. in diameter.
3. Funnels 15 cm. in diameter, equipped with a short rubber connection, a pinchcock, and a delivery tip for dispensing media into tubes.
4. Pipettes, graduated.
5. Burette with side arm connected by a siphon with an elevated container for medium.
6. Tubes, bottles, flasks.
7. Tube for taking sample of medium for determining reaction after sterilization (reaction tube).

A Pyrex-glass test tube (175 by 22 millimeters) is used when the medium is to be dispensed in Pyrex-glass containers. A common glass tube (165 by 22 millimeters) is used when the medium is to be dispensed in containers of common glass.

When large, measured amounts of material are to be dispensed, use a graduated cylinder. Pour the medium into the cylinder, using a dipper and funnel if necessary. From the cylinder, pour correctly measured quantities into the containers. Replace the cotton plugs.

If the medium is to be stored, place caps of heavy paper over the plugs and tie down firmly over the necks, using a single bowknot. If the medium is to be delivered as soon as completed, use a flat-folded, envelope-like cap which will fit well down over the neck and stay in place without tying.

When small, measured amounts of small quantities of material are to be dispensed, select a graduated pipette of convenient size and pipette the material carefully into the containers.

When small, measured amounts of large quantities of material are to be dispensed, use a burette.

When small amounts of small or large quantities of material are to be dispensed in only approximately equal volumes, use a funnel equipped with rubber connection and delivery tip. Use a measured quantity as a gauge.

## LABELING

Attach tags to all large bottles and flasks of media, and to all baskets containing tubes, small bottles, or small flasks. Label small bottles and flasks after sterilization. On each tag enter the following data:

Kind of medium (the percentage of agar and carbohydrate when employed, must be plainly indicated; i.e., 2-per-cent beef-infusion agar, 1-per-cent dextrose).
Date made.
Name of worker dispensing material.
Peptone used, if a special brand.
Number of tubes, if in a basket.

Attach a perforated, printed tag to one container or basket of each lot of medium. On this tag, record data concerning the sterilization procedure, i.e., the name, date, method and period of sterilization of the medium, and the name of the worker.

The worker in charge of the sterilization of media fills in additional data regarding the actual process, and the tags are separated from the stubs and filed for reference.

## STERILIZATION

Media in bulk, sterilized by steam under pressure, are usually run for thirty minutes at 121°C.; media in tubes and small containers, for twenty minutes; routine carbohydrate solutions and media for use in

fermentation tests, for twelve minutes at 121°C. Special media are always sterilized as the individual formulae direct.

### DISPOSITION OF SOLID MEDIA AFTER STERILIZATION

Allow media in flasks and bottles to solidify in an upright position, with the exception of agar in Blake bottles for mass growth of organisms. Place these bottles flat on their sides.

Allow media in tubes for plates or deep-tube cultures, and semi-solid medium in tubes, to harden in an upright position.

Place tubes of hot medium for slant cultures on "slant boards" constructed for this purpose, or on glass tubing of suitable diameter. For slants where only the slant surface is to be inoculated, distribute most of the medium in the slant and only a small portion in the butt, being careful that the material does not come in contact with the plugs. When the butt, as well as the slant, is to be inoculated, control the distribution of the material in the butt as well as in the slant. Leave the tubes in the slanted position until the medium has solidified.

Cool gelatin rapidly by placing in the cold room as soon as it is taken from the autoclave.

### INCUBATION

Two temperatures of incubation are commonly employed, 20°C. (room temperature) and 37°C., the period varying from eighteen hours to two weeks.

Media sterilized by heat are rarely contaminated, but the purposes for which certain heat-sterilized media are used make it desirable to take every precaution to be sure that they are sterile. Media for use in sterility tests are incubated at 37°C. for from forty-eight to ninety-six hours, and then left at room temperature for an additional forty-eight hours before being placed in the cold room.

### STORAGE

All heat-sterilized media are stored in the cold room at a temperature of approximately 6°C. The paper caps tied over the plugs of all large bottles, before sterilization, are left in place to protect the plugs from dust and to prevent mold spores from falling on the cotton.

Paraffin is used to diminish evaporation in certain tubed media. The cotton plug is trimmed even with the top of the tube and, if the medium is solid, the tube is dipped in melted paraffin; if the medium is liquid, the paraffin is put on with a brush. Media for which there is

only an occasional demand but which have to be kept in stock are dispensed in tubes which are plugged with cotton and stoppered with corks, covered with a heavy layer of paraffin. Sterile rubber stoppers, in place of cotton plugs, are sometimes used to protect material from evaporation.

### *Biologic, Physical, and Chemical Tests*

Some media are subjected to certain tests before being distributed for use. These tests are, for the most part, of a biologic nature, though the determination of the melting point is performed on each batch of gelatin medium prepared, and chemical tests (reduction of copper solutions) are occasionally made for detecting the presence of a simple sugar as an impurity in a more complex carbohydrate. The tests to be made are indicated in the individual formulae.

## ENRICHMENT MEDIA (MEDIA PREPARED BY COMBINING STERILE COMPONENTS)

Certain materials, known as enrichment substances, are used in the preparation of media for the growth of some microörganisms, either because of the difficulty of cultivating those organisms on unenriched, heat-sterilized media or because certain biologic reactions dependent upon these substances are to be studied. These constituents are added to a heat-sterilized component known as the base.

The ingredients most frequently used for enriching media are: defibrinated horse and rabbit blood, horse and rabbit sera, ascitic fluid, carbohydrate solutions, and blood extract. They are either sterile when obtained or, if contaminated, are rendered sterile, usually by filtration through a filter candle.

The preparation and sterilization of some of these substances for use in combined media entail considerable work, a large part of which is performed in a draft-free room. The technic used in dispensing them is similar to that used in dispensing the enrichment media themselves, in that the same type of apparatus is employed and that two trained workers are usually required.

### ENRICHMENT MATERIALS

**Blood.**—Bottles for collecting defibrinated blood are prepared and sterilized by workers of the media group.

As soon as the blood is received, a rubber stopper of suitable size is boiled for ten minutes in 1-per-cent crude cresol and is substituted for the rubber stopper of the bleeding apparatus.

For taking small quantities of blood from the heart of a rabbit, a

sterile syringe is used. The blood is discharged from the syringe into a sterile 120-cc. French square bottle containing beads. It is shaken for five minutes to defibrinate.

Blood from both the horse and the rabbit is generally used the same day it is drawn. Contaminations are rarely encountered. Blood may be stored a short time in the cold room, but after about two weeks, hemolysis renders it unsatisfactory for use in the preparation of certain media.

**Sera.**—Both horse and rabbit sera are obtained from blood drawn with aseptic precautions.

Horse serum which is received from the farm is placed in the cold room until needed. It is then filtered through a sterile filter candle into 4-liter bottles by the worker in charge of pressure filtration, assisted by a member of the media group. It is redispensed immediately into smaller, sterile, cotton-plugged, paper-capped bottles. (For apparatus required for dispensing, see figs. 13 and 14.) The quantities dispensed are regulated by the volumes used in the enrichment media containing serum. After the serum has been dispensed, the cotton plugs of the bottles are replaced with sterile rubber stoppers. A piece of cotton wrung out of 5-per-cent phenol solution is wrapped about the junction of the stopper and the bottle. The stoppers are tied firmly in place and the paper caps replaced over them and tied down securely about the necks of the bottles. Rabbit serum is simply pipetted off the blood clot and centrifugalized. It is practically always sterile and is never filtered.

Sera may be stored indefinitely in the cold room. On long standing, however, there is danger of protein precipitates forming and clouding the fluid. This fact has to be taken into account when a specially clear enrichment medium is desired and a large proportion of serum is used, as in serum semisolid agar. Serum in which a precipitate has formed can, however, be used for some media.

**Ascitic Fluid.**—This fluid is obtained from various pathologic conditions and when received by this laboratory is practically never sterile. It is filtered, dispensed, and stored in the same manner as serum.

**Filtered Carbohydrate Solutions.**—These materials are filtered only in small amounts (100 cc. or less), by suction through a small sterile candle into 240-cc. French square bottles. The work is performed by two workers of the media group in a draft-free room.

**Blood Extract.**—When it is necessary to filter this material, the same procedure is followed as for carbohydrate solutions.

## STERILITY TESTS

Wherever experience has demonstrated the advisability of the procedure, sterility tests are made on enrichment substances to make sure that they are sterile before being used in the preparation of media. Extensive tests are necessary with serum and ascitic fluid, as slow-growing contaminants and filter-passers have occasionally been encountered. Less extensive tests have been found necessary with blood, filtered carbohydrate solutions, and blood extract. All sterility tests are made in a draft-free room by members of the media group. The tests are incubated at 37°C. and inspected daily for a period of two weeks. When contaminations occur, slide preparations and, occasionally, subcultures are made.

Sterility tests on enrichment materials are made at three different times: immediately after filtering the enrichment substance; preliminary to using it; and at the time of using it.

After filtration, during the process of dispensing serum or ascitic fluid from a 4-liter bottle into smaller containers, eight sterility tests are taken, four in 80-cc. amounts of Hitchens' medium in French square bottles and four in 30-cc. amounts of sterility broth in Smith fermentation tubes. A few cubic centimeters of enrichment material are run at intervals during the dispensing process, alternately into Hitchens' medium and into sterility broth. The last Smith tube of sterility broth is saved for testing the last of the material from the dispensing apparatus.

Filtered carbohydrate solutions and blood extract are tested immediately after filtration by inoculating 3 cc. of material into an aerobic and an anaerobic tube of sterility broth and into a tube of Hitchens' medium, putting approximately 1 cc. into each tube.

Preliminary to use, each separate container into which serum or ascitic fluid has been dispensed is tested at least two weeks before the serum or fluid and the base are combined. About 5 cc. of material is used for the test and is divided between a Smith tube of sterility broth and an 80-cc. amount of Hitchens' medium. Several bottles of tested material are kept on hand so that requisitions for enrichment media may be filled promptly.

At the time of using, tests are taken on all enrichment substances when they are added to a heat-sterilized base. A few drops of the material are saved in the pipette or other dispensing apparatus and are added to an aerobic and an anaerobic tube of sterility broth.

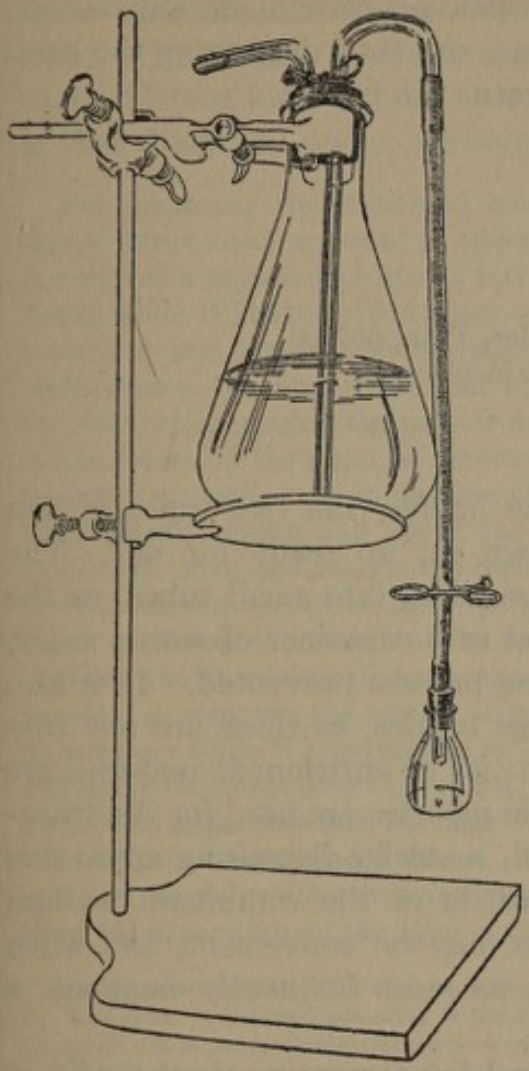

FIG. 13

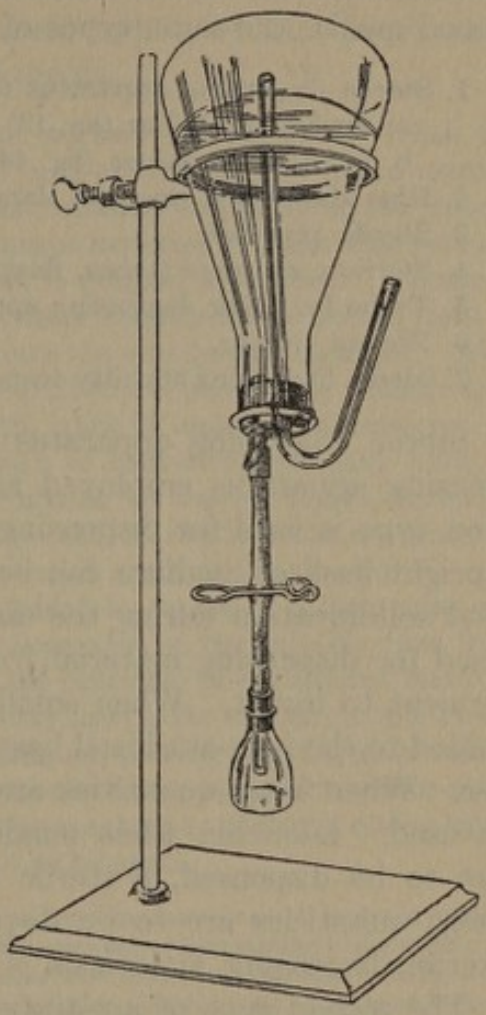

FIG. 14

FIG. 13. DISPENSING APPARATUS FOR UPRIGHT CONTAINER

This apparatus which is used for dispensing material from upright containers consists of a siphon fitted tightly into a two-hole rubber stopper. Through this stopper is passed a long, bent, glass tube, U- or V-shaped, with one long and one short arm, and a short right-angle tube, air vent, with the outer end closed with a cotton plug. The long arm of the U- or V-shaped tube is of such length as just to escape touching the bottom of the container of material to be dispensed. To the short arm is attached a length of rubber tubing with pinchcock and filling tip protected by a glass bell. The glass bell is wired firmly to a rubber stopper, through which the filling tip passes. All connections must be as tight as possible to prevent loosening during the dispensing.

FIG. 14. DISPENSING APPARATUS FOR INVERTED CONTAINER

In this apparatus the glass tube to which the rubber tube and filling tip are attached, is short and straight, extending less than 1 cm. above the stopper, inside the flask. The air vent is an acutely angled tube with the long arm extending to the bottom of the flask.

### APPARATUS REQUIRED

For the processes connected with dispensing enrichment substances, adding them to the heat-sterilized bases, and then dispensing the combined media, the same types of apparatus are used.

1. Sterile dispensing apparatus for
   a. upright container (fig. 13).
   b. inverted container (fig. 14).
2. Ring stand with ring and clamp.
3. Sterile pipettes.
4. Sterile containers (tubes, flasks, bottles, Petri plates).
5. Twine for tying dispensing apparatus in place.
6. Bunsen burner.
7. Media for taking sterility tests.

Sterile dispensing apparatus: In the figures, the two types of dispensing apparatus employed are shown set up ready for use. The first type is used for dispensing agar medium into small tubes, as the upright flask of medium can be placed in a container of warm water, and solidification during the dispensing process prevented. It is also used for dispensing material from large bottles, as these are not convenient to invert. When small quantities of enrichment material are added to the heat-sterilized base, sterile pipettes are used for the transfer. When large quantities are added, a sterile dispensing apparatus is used. Likewise, when small quantities of the combined medium are to be dispensed, a sterile pipette may be convenient, and when large quantities are to be dispensed, as most frequently happens, a sterile dispensing apparatus is used.

The second type of apparatus is used for dispensing most media in flasks as it is more saving of material. It may even be used by rapid workers in place of the first type for dispensing agar media into small tubes. In fact, the second type is preferred wherever it is possible to use it.

These two types of apparatus are prepared for sterilization before being used, by tying a paper over the bell and slipping the glass tubing which is to be inserted in the container into a large envelope fastened together with paper clips. This envelope is long enough to protect the stopper also. The whole apparatus is wrapped in heavy paper, fastened with paper clips, and sterilized in the autoclave.

### COMBINING THE ENRICHMENT SUBSTANCES AND THE HEAT-STERILIZED BASE

Supplies of the various heat-sterilized bases and enrichment substances are kept in the media cold rooms in readiness for use. The bases

are heat sterilized, nutrient broths and agars, both meat extract and meat infusion, and also miscellaneous broths and agars not containing meat extractives. There is a separate formula for the preparation of each different kind of base and the general technic of their preparation is that described under heat-sterilized media.

For preparing the combined media, two workers are required. When the sterile enrichment material is added to the heat-sterilized base with a pipette, A removes a pipette of suitable size from its container and picks up the Bunsen flame, while B holds the container of enrichment material in a slanting position toward A and removes the stopper, holding it pointed downward. A immediately flames the open neck and holds the flame slightly above and in front of the neck while passing the pipette directly into the enrichment material. After A has drawn up the required amount of material in the pipette, he withdraws it from the container, again flaming the mouth, while B replaces the stopper. B then quickly slants and opens the container of heat-sterilized base while A flames the neck and inserts the pipette, permitting all the enrichment material, except a few drops to be used for a sterility test, to run into the container of base. A flames the mouth of the container and B replaces the stopper.

When the sterile enrichment material is added by means of a dispensing apparatus, A removes the sterile, dispensing apparatus of the suitable type from its wrapper while B removes the stopper of the container of enrichment material and holds the flame over the mouth. A quickly inserts the rubber stopper of the dispensing apparatus into the neck of the container, passing the long glass tubing straight down through the flame. The stopper is then pushed firmly into place and tied securely with twine and the apparatus is set up as shown in either figure 13 or 14, depending on the type of apparatus to be used.

If a dispensing apparatus for an upright container is used, the apparatus is set up as shown in figure 13. The height is adjusted according to the height of the container of heat-sterilized base so that the filling bell will fit conveniently over the neck of the container when the stopper shall have been removed.

If a dispensing apparatus for an inverted container is used, the apparatus is set up as shown in figure 14. Before the container is inverted, the pinchcock is closed and during the process of inverting, the worker blows into the air vent to prevent the material from running into it.

B slants the container of base so that the neck is directly under the bell of the filling apparatus. He then removes the stopper while A flames the bell and the neck of the container and brings the container to an upright position under the bell. B starts the flow of enrichment material by applying air pressure to the air vent, if the container is upright, leaving the pinchcock open. The material is allowed to run out until the container is nearly empty, only a few drops being saved for a sterility test, which is later taken directly from the filling apparatus.

### DISPENSING OF THE COMBINED MEDIUM

As soon as the enrichment material has been added to the base, if the medium is to be distributed with a dispensing apparatus, as is the common practice, A removes from its wrapper a sterile, dispensing apparatus of the suitable type, while B lifts the bell of the enrichment-fluid apparatus off the neck of the container of combined medium and

immediately applies the flame over its mouth. A quickly inserts the dispensing apparatus, as previously described for enrichment materials. A then runs the few drops of enrichment material from the first dispensing apparatus directly into the tubes of sterility broth. This apparatus is then taken down and washed immediately. The apparatus for dispensing the combined medium is set up at a convenient level.

If the medium is to be dispensed in test tubes, it is filled directly into these tubes from the dispensing apparatus. If the medium is to be dispensed in Petri plates, unplugged, sterile 150-by-19-millimeter tubes, sterilized in cans, are used. The material is filled by one worker into these tubes while an assistant opens the plates just far enough so that the first worker can pour the material into them.

### INCUBATION AND STORAGE

All combined media are incubated before they are used. The period varies from twenty-four hours at 37°C. for blood media, to ninety-six hours for media containing ascitic fluid and serum. All media are left at room temperature for at least twenty-four hours longer.

The media are examined for contaminations before delivery or storage. If evidence of growth is noted, microscopic examinations are made. All contaminated media are sterilized and discarded. However, when the procedures for testing enrichment substances are strictly followed, a batch of contaminated enrichment medium is of rare occurrence.

# CHAPTER 2

## FORMULAE FOR THE PREPARATION OF MEDIA

The arrangement of the formulae is that which has been found most convenient for the workers using them. The division is into seven more or less natural groups based upon similarity of ingredients and of method of preparation.

The first group is composed of meat infusions as they form the basis for many different kinds of media both solid and liquid. Broths and agars form the second and third groups respectively. They are subdivided into meat-extract and meat-infusion media. Within these subdivisions broths are arranged alphabetically, while it has been found more advantageous to arrange agars in order of increasing percentages of that ingredient. Gelatins form a small fourth group.

The fifth division consists of media with some other base than meat extract or meat infusion, grouped, for want of a better classification, under miscellaneous media. These are arranged alphabetically within the group.

The sixth division is a large one consisting of solutions and miscellaneous materials. These are subdivided into solutions which are to be added to media, and other solutions and materials. The former is subdivided further into filtered, heat-sterilized and unsterilized solutions; the latter, into heat-sterilized and unsterilized solutions and materials.

The seventh is a distinct and important group consisting of all media prepared from already sterile components by combining them, with precautions to maintain sterility. These media are designated as enrichment media. The formulae under this group are arranged alphabetically.

A small subgroup under this large one comprises media, such as Loeffler's blood-serum medium and Petroff's egg medium prepared with the precautions used in preparing media from sterile components, but heated to coagulate and sterilize subsequent to preparation though at temperatures too low to kill resistant spores.

As far as possible, references have been given for the various formulae though the wording is practically never the same as in the original, as all formulae have been more or less adapted to the equipment and procedures of this laboratory.

### MEAT INFUSIONS

*Double-strength infusions, beef or veal*

Meat, ground.....1 kg.
Distilled water.....1 kg.

**Procedure.**—Weigh meat and add the required quantity of water. Stir thoroughly. Infuse overnight in the cold room. Strain through cheesecloth, pressing by twisting the cloth. Save meat for residue infusion. Weigh meat juice and, if its weight is less than that of the amount of water added to the meat, press the meat again until the amount of juice recovered equals the amount of water added. (Re-pressing of the meat is rarely necessary.) Cook in steam

kettle (small amounts should be cooked over the free flame), stirring occasionally, and boil until the infusion is clear and the coagulum brown. Strain through cheesecloth and filter through paper. Weigh and, if necessary, add water to give 1 kg. of infusion for each kilogram of meat. Adjust to pH 7.2 (+0.3 to +0.4). Dispense in 2-liter amounts in 2.5-liter bottles and sterilize by steam at 121°C. for thirty minutes.

This is a routine stock infusion and is convenient to use in preparing agars, as the agar can be dissolved in water, thus saving one heating of the infusion. When less than the full amount of sterile infusion is taken from a bottle, it is withdrawn in a draft-free room by means of a sterile dispensing apparatus (See: dispensing apparatus for upright container fig. 13) into a graduated container. The date and the amount of infusion withdrawn from the bottle are entered on the tag.

*Double-strength infusion, sugar free*

Meat, ground.....1 kg.
Distilled water.....1 kg.

Procedure.—Proceed as for double-strength infusion up to the point of adjustment. Do not adjust the reaction at this point. Warm the meat juice to between 35 and 37°C. and add 10 cc. per liter of an eighteen- to twenty-four-hour broth culture of *B. coli*. Incubate at 37°C. overnight. Heat in autoclave at 100°C. until the infusion is clear and the coagulum is brown. Strain through cheesecloth and filter through paper. Make up any loss in weight. Fill three Smith fermentation tubes and dispense the remainder in convenient amounts in Erlenmeyer flasks. Sterilize by steam at 100°C. for thirty minutes. Tip out any bubbles present in the arms of the Smith tubes. Cool and inoculate with a vigorous young culture of *B. coli*. Incubate at 37°C. overnight.

If no gas appears in the anaerobic arms of the tubes, the infusion is considered practically sugar free and is sterilized two more days if it is to be put in stock. However, if gas appears in the tubes, the entire batch of material is pooled and again inoculated with *B. coli* and reincubated. It is again tested in Smith tubes. This process of inoculation, incubation, and testing is continued until a sugar-free product is obtained.

This infusion is kept in stock as a basis for agar media containing various carbohydrates, for the differential diagnosis of organisms difficult to cultivate, especially the meningococcus.

*Meat-residue infusions, beef or veal, single strength*

Meat, ground (residue from double-strength infusion).....1 kg.
Distilled water.....1 kg.

Procedure.—Weigh meat residue and add the required quantity of water. Stir thoroughly. Infuse overnight in the cold room. Heat to from 45 to 55°C. in the steam kettle or in the water-bath, and hold at this temperature for thirty minutes, stirring occasionally. Bring to boiling temperature and cook until meat and coagulum are brown and the infusion is clear. Strain through cheesecloth and filter through paper. Weigh and, if necessary, add water to give 1 kg. of infusion for each kilogram of meat residue infused. Adjust to pH 7.2 (+0.3 to +0.4). Dispense and sterilize in the same way as double-strength infusions.

These infusions are used chiefly as single-strength infusions in the preparation of stock infusion broths.

*Single-strength infusions, beef or veal*

Meat, ground.....500 grams
Distilled water..... 1 kg.

**Procedure.**—This procedure is the same as for double-strength infusion except that the meat is given an additional pressing in the meat press and the meat residue is discarded.

This infusion is not carried as a routine stock infusion, though it is occasionally prepared. It is used for the same purposes as meat-residue infusion.

*Vitamin infusion (beef liver) (13)*

Beef liver, ground.....1 kg.
Distilled water.....1 kg.

**Procedure.**—Weigh liver and add the required quantity of water. Heat in a covered container in the autoclave at 100°C. for twenty minutes. Open autoclave and stir infusion with a glass rod. Heat again at 100°C. for ninety minutes. Strain through a wire sieve to remove coagulated mass. Filter through glass wool. Dispense in Erlenmeyer flasks and sterilize by steam at 121°C. for thirty minutes.

## BROTHS (HEAT STERILIZED)

### MEAT-EXTRACT BROTHS

*Beef-extract broth (14)*

Distilled water.....1 kg.
Beef extract.....0.3 per cent or 3 grams per kg.
Peptone.....0.5 per cent or 5 grams per kg.

**Procedure.**—To the required quantity of water, add the other ingredients. Heat over flame to dissolve, stirring frequently. Adjust to pH 7.0 (+0.2 to +0.5). Boil for about five minutes. Make up lost weight, filter through paper until clear, and dispense. Sterilize by steam at 121°C. for twenty minutes.

*Beef-extract broth with lactose or dextrose (15)*

Distilled water.....1 kg.
Beef extract.....0.3 per cent or 3 grams per kg.
Peptone.....0.5 per cent or 5 grams per kg.
Lactose or dextrose.....0.5 per cent or 5 grams per kg.

**Procedure.**—To the required quantity of water add the extract and peptone. Heat over flame to dissolve, stirring frequently. Adjust to pH 7.0 (+0.2 to +0.5). Boil for about five minutes. Make up lost weight. Add the sugar, dissolve, and filter through paper until clear. Dispense in Durham fermentation tubes with outer tube 150 by 19 millimeters and inner tube 75 by 11 millimeters in size. Fill a small amount of broth (about 1.5 cm. depth) into the large tube, fill the small tube completely, and invert in the large tube. Sterilize by steam at 121°C. for twelve minutes.

Lactose broth is made double strength and dispensed in a larger size Durham tube, with outer tube 175 by 25 millimeters and inner tube 100 by 12 millimeters in size. These tubes are filled and sterilized in the same manner as the tubes of single-strength broth except that the broth in the outer tube is filled to about 2 cm. in depth.

### MEAT-INFUSION BROTHS

#### *Beef-infusion broth*

| | |
|---|---|
| Beef infusion, single | 1 kg. |
| Peptone | 1 per cent or 10 grams per kg. |
| Sodium chloride | 0.5 per cent or 5 grams per kg. |

Procedure.—Adjust the required amount of stock meat-residue (or single-strength) infusion to pH 7.6 (+0.1 to +0.3) and add the other ingredients. Autoclave at from 123 to 125°C. for five minutes. Make up lost weight and filter through paper until clear. Dispense in 2-liter amounts in 3-liter flasks and sterilize by steam at 121°C. for thirty minutes. Place in stock. When needed, filter through paper to remove whatever precipitate has formed, dispense as ordered and sterilize by steam at 121°C. for twenty minutes.

This method of preparing infusion broth has been found to furnish a product practically free of precipitate.

#### *Beef-infusion broth with 0.1 per cent dextrose: Sterility broth (also used in culturing blood)*

| | |
|---|---|
| Beef, ground | 500 grams |
| Distilled water | 1 kg. |
| Peptone | 1 per cent or 10 grams per kg. |
| Sodium chloride | 0.5 per cent or 5 grams per kg. |
| Dextrose | 0.1 per cent or 1 gram per kg. |

Procedure.—Weigh meat and add the required quantity of water. Stir thoroughly. Infuse overnight in the cold room. Strain through cheesecloth and press off juice from meat with meat press. Weigh.

The amount of meat juice recovered always exceeds the amount of water added to the meat, but the making up of subsequent losses due to evaporation is based on the amount of water originally added to the meat.

Heat gradually in the steam kettle (small amounts are made over the free flame), stirring occasionally, until the infusion is clear and almost colorless and the coagulum is brown. Strain through cheesecloth and filter through paper. Make up weight to give 1 kg. of infusion for each 500 grams of meat. Adjust reaction to from pH 8.0 to pH 8.2 (±0.0 to −0.2). Add peptone and salt, and autoclave at from 123 to 125°C. for from five to ten minutes, depending on the bulk of the material. Make up weight to that of infusion plus the other ingredients. Add dextrose, dissolve, and filter through paper until clear. Dispense and sterilize by steam at 121°C. for twenty minutes. Incubate at 37°C. for seventy-two hours.

Sterility broth is dispensed in 120-cc. French square bottles, 50 cc. in each, and in 165-by-22-millimeter test tubes, 21 cc. in each. One-half of the tubes should contain sufficient paraffin, mineral-oil mixture (about 1.25 cc.) to form a firm layer over the broth when cold. This makes an efficient seal for maintaining anaerobic conditions.

**Keidel Blood-Culture Tubes.**—Keidel culture tubes are prepared from this broth with the addition of 0.5 per cent sodium citrate. These tubes are large ampules, about 85 by 25 millimeters with a 50-millimeter stem. The tubes are inverted in pails containing enough freshly made broth to provide approximately 20 cc. for each tube. The pails are placed in the autoclave and a 22-inch vacuum is pulled. (The temperature of the broth must not be high enough to cause boiling at this reduced pressure.) The vacuum is compensated by admitting compressed air, which forces the broth into the tubes. The filled tubes, in groups of three, held together with rubber bands, are placed upright in a container of water, boiled vigorously over the free flame for from three to four minutes to allow the water vapor formed from the broth to displace the air in the tubes. They are sealed while boiling by playing a micro-gas flame on the tips of the stems. The tubes are then tested for imperfections in sealing by immersing them directly from the boiling water into tepid, soapy water to which sufficient methylene blue has been added to give a deep-blue color.

After the tubes have been thoroughly dried, they are fitted with a piece of rubber tubing, 3 millimeters in diameter, and a 22-gauge blood-letting needle with glass window. The needle is protected by a small, lipped test tube 112 by 12.5 millimeters which is held in place over the needle by means of a narrow strip of nonabsorbent cotton, wound around the juncture of the rubber tubing with the ampule.

The complete outfits are sterilized by steam at 121°C. for twenty minutes and incubated for four days at 37°C.

Each lot of broth used in these tubes is tested for its suitability for supporting the growth of delicate organisms, by inoculation with the pneumococcus.

*Cooked-meat medium for anaerobes (16, 17)*

| | |
|---|---|
| Beef, ground | 1 kg. |
| Distilled water | 1 kg. |

**Procedure.**—Weigh meat and add the required quantity of water. Stir thoroughly. Infuse overnight in the cold room or, if convenient, prepare the medium at once. Heat in water-bath to 95°C., stirring frequently. Place over free flame and bring to boiling point, stirring constantly. Cool in ice bath and skim off fat.

Filter about 15 cc. of the medium through filter paper, for use in titrating. Adjust the bulk of the medium to pH 8.0 ($\pm 0.0$ to $-0.1$). Filter another small amount of the medium and check the reaction. Strain through a sieve until the meat is comparatively dry. Place meat in a funnel with a short wide stem (outside diameter slightly under 15 mm.) and tube the meat in 150-by-15-millimeter tubes by pushing it through the stem of the funnel by means of a glass rod. Fill tubes to a depth of between 25 and 30 mm. with the meat, then add the liquid to the same depth above the meat, by means of an ordinary tubing funnel. Sterilize by steam at 121°C. for thirty minutes.

*Botulinus-toxin broth: van Ermengem's double-strength veal-infusion broth (18)*

| | |
|---|---|
| Veal, ground | 1 kg. |
| Distilled water | 1 kg. |
| Peptone (Eimer and Amend) | 1 per cent or 10 grams per kg. |
| Sodium chloride | 0.5 per cent or 5 grams per kg. |
| Dextrose | 2 per cent or 20 grams per kg. |

Procedure.—Weigh the meat and add the required quantity of water. Stir thoroughly and infuse overnight in the cold room. Strain through cheesecloth and press off juice from meat with the meat press. Weigh. Boil until the infusion is clear and almost colorless and the coagulum is brown. Strain through cheesecloth and add the peptone and salt. Make up weight to that of the infusion (1 kg. for each kilogram of meat) plus the other ingredients. Adjust to pH 8.4 (−0.6 to −0.4). Boil for five minutes. Make up lost weight and filter through paper. Dispense, filling tubes and flasks as full as possible, after making allowance for the addition of 2 per cent dextrose in 20-per-cent solution. Sterilize by steam at 100°C. for from twenty to forty minutes (depending on the bulk of the material), on three successive days.

Dextrose is added to the flasks and tubes of medium at the time of inoculation. A sterile stock 20-per-cent dextrose solution is used and a sufficient amount is added to the medium to give a final concentration of 2 per cent dextrose.

*Botulinus broth, van Ermengem's modified (for isolation purposes) (18, 19)*

| | |
|---|---|
| Veal, ground | 1 kg. |
| Distilled water | 1 kg. |
| Peptone (Eimer and Amend) | 1 per cent or 10 grams per kg. |
| Sodium chloride | 0.5 per cent or 5 grams per kg. |
| Potassium phosphate, dibasic | 0.5 per cent or 5 grams per kg. |
| Dextrose | 1 per cent or 10 grams per kg. |

Procedure.—The procedure is the same as with the preceding, except that the medium is sterilized by steam at 121°C. for from twenty to thirty minutes. The dextrose is added in the same manner as in the preceding but only half the quantity is used.

*Diphtheria-toxin broth: Sugar-free veal-infusion broth with 0.2 per cent dextrose (20)*

| | |
|---|---|
| Veal, ground | 500 grams |
| Distilled water | 1 kg. |
| Peptone ("Difco" Proteose or Parke Davis) | 2 per cent or 20 grams per kg. |
| Sodium chloride | 0.5 per cent or 5 grams per kg. |
| Dextrose | 0.2 per cent or 2 grams per kg. |

Procedure.—Weigh the meat and add the required quantity of water. Stir thoroughly. Infuse overnight in the cold room. For each kilogram of meat infused, inoculate a 10-cc. amount of infusion broth with *B. coli*. Incubate at 37°C. overnight. The next morning strain the juice from the meat through cheesecloth and press the meat, using the meat press. Adjust the reaction of the

meat juice to +1.0 to phenolphthalein. Warm to between 35 and 37°C. and add the *B. coli* culture. Stir well and incubate at 37°C. overnight. Again adjust the reaction to +1.0 to phenolphthalein and heat in steam at 100°C. until the infusion is clear and almost colorless and the coagulum is brown. Strain through cheesecloth and add the peptone and salt. Make up weight to that of the weight of the water added to the meat, plus the other ingredients. Adjust the reaction to pH 7.6+ (+0.2 to +0.5). Boil for five minutes, stirring well. Again make up lost weight. When the broth is to be dispensed in small amounts, add 0.2 per cent dextrose and filter through paper. When the medium is to be dispensed in large flasks, do not add dextrose but filter and dispense 700-cc. amounts in sterile 2-liter Erlenmeyer flasks. Prepare enough of a 20-per-cent dextrose solution to supply 7 cc. for each flask of medium. Tube in 7-cc. amounts in sterile 150-by-15-millimeter Pyrex tubes. Sterilize the medium and the sugar solution by steam at 100°C. for thirty minutes, on three successive days. When no large flasks of medium are present, reduce the time to twenty minutes.

The 20-per-cent dextrose solution is added to the 700-cc. amounts of medium at the time of inoculation with *B. diphtheriae*. Seven cubic centimeters of a 20-per-cent solution of dextrose added to 700 cc. of the medium gives a final concentration of approximately 0.2 per cent dextrose, the same percentage as is used in the small amounts of medium.

*Dysentery-toxin broth (21)*

| | |
|---|---|
| Beef, ground | 500 grams |
| Distilled water | 1 kg. |
| Peptone (Chapoteaut) | 1 per cent or 10 grams per kg. |
| Sodium chloride | 0.5 per cent or 5 grams per kg. |

Procedure.—Weigh the meat and add the required quantity of water. Stir thoroughly and infuse overnight in the cold room. Heat to 45°C. and hold between 45 and 50°C. for thirty minutes, stirring occasionally. Bring to the boiling point and boil for ten minutes, stirring frequently. Strain through cheesecloth and filter through paper. Add the peptone and salt, and make up weight to that of the infusion (1 kg. for each 500 grams of meat) plus the other ingredients. Adjust the reaction to pH 8.3 (−0.3 to −0.2). Boil for five minutes. Check the reaction and, if it is more acid than pH 8.3, readjust to that point. If it is necessary to readjust, boil an additional three minutes. Make up lost weight and filter through paper. Dispense as ordered and sterilize by steam at 100°C. on three successive days, thirty minutes each day. Incubate for 48 hours at 37°C.

The final reaction should be as close to pH 8.0 as possible. N/1 NaOH is added to this medium, with aseptic precautions, after sterilization, whenever the increase in acidity is greater than anticipated. It is readjusted to pH 8.0.

*Pneumococcus broth*

| | |
|---|---|
| Beef, ground | 500 grams |
| Distilled water | 1 kg. |
| Peptone | 1 per cent or 10 grams per kg. |
| Sodium chloride | 0.5 per cent or 5 grams per kg. |

**Procedure.**—Weigh meat and add the water, reserving about 10 per cent or 100 grams per kilogram to dissolve the other ingredients. Stir thoroughly and infuse overnight in the cold room. Strain through cheesecloth and press off juice from meat with the meat press. Dissolve salt and peptone in the water reserved for the purpose, by heating in the water-bath, and add to meat juice. Weigh. Adjust the reaction to ± 0.0 to phenolphthalein. Boil until the broth is clear and the coagulum brown. Check the reaction and, if it is more acid than pH 8.0 adjust to this point, and boil for 3 minutes. Filter through paper and make up weight to that of the infusion (1 kg. for each 500 grams of meat plus the other ingredients). Fill 3 Smith fermentation tubes and five 150-by-15-millimeter test tubes. Dispense the bulk of the material in 2300-cc. amounts in 3-liter flasks. Sterilize by steam at 100°C. for forty minutes on three successive days. As a precipitate forms on sterilization, filter through a sterile filter and redispense as ordered, using aseptic precautions. Heat this refiltered and dispensed material in steam at 100°C. for ten minutes to insure sterility. Incubate at 37°C. for forty-eight hours.

The apparatus used for removing the precipitate which forms on sterilization consists of a siphon, a covered filter funnel and a dispensing apparatus for an upright container having a 3-hole rubber stopper. The broth passes from its container through the siphon into the filter funnel.

From the stem of the funnel a piece of rubber hose leads to a glass tubing passing through one of the three holes in the rubber stopper of the dispensing apparatus into a sterile 3-liter flask.

The broth is dispensed from this flask. In this way, the filtration is accomplished without exposing the broth to air.

The finished broth is tested for gas production, with *B. coli* by inoculating the Smith tubes with that microörganism, and for its ability to support the growth of the pneumococcus by inoculating the 150-by-15-millimeter tubes with that microörganism. Material which contains so little muscle sugar that no trace of gas is noted in the Smith tubes, usually fails to support a vigorous growth of the pneumococcus.

To broth, poor in muscle sugar, dextrose in sterile 20-per-cent solution is added with aseptic precautions just before filtration through the sterile filter funnel. Sometimes as little as 0.02 per cent and sometimes as much as 0.08 per cent dextrose is added to a broth, to effect a satisfactory growth of the pneumococcus. The refiltered and redispensed material is heated in steam at 100°C. for ten minutes to insure its sterility. Only broth which yields a good growth without the addition of dextrose is used for protection tests.

### *Streptococcus-toxin broth*

| | | |
|---|---|---|
| Beef, ground | 500 grams | |
| Distilled water | 1 kg. | |
| Peptone ("Difco" Proteose) | 2 per cent or 20 | grams per kg. |
| Sodium chloride | 0.5 per cent or 5 | grams per kg. |
| Dextrose | 0.02 per cent or 0.2 | gram per kg. |

**Procedure.**—Weigh the meat and add the required quantity of water. Stir thoroughly. Infuse overnight in the cold room. Strain through cheesecloth and press off juice from meat with the meat press. Bring to boiling point, stirring occasionally, and boil until the infusion is clear and almost colorless and the coagulum is brown. Strain through cheesecloth and filter through paper. Add peptone and salt to the first part of the filtrate to come through, stirring to dissolve. When all the infusion has been filtered, make up weight to give 1 kg. of infusion for each 500 grams of meat, plus the other ingredients. Adjust reaction to pH 8.2 (−0.1 to −0.3). Autoclave at from 123 to 125°C. for from five to ten minutes, depending on the bulk of the material. Make up weight. Check

the reaction and if it is more acid than pH 8.2 readjust to that point. If it is necessary to readjust, boil for three minutes. Filter through paper until clear. Dispense in 1200-cc. amounts in 2-liter Erlenmeyer flasks. Sterilize by steam at 121°C. for twenty minutes.

Dextrose is added to the flasks of medium with aseptic precautions. A sterile stock 20-per-cent solution is used and a sufficient amount is added to the medium to give a final concentration of 0.02 per cent dextrose. After the addition of the dextrose this medium is frequently redispensed with aseptic precautions into smaller containers.

The redispensed material is heated in steam at 100°C. for ten minutes to insure its sterility.

*Tetanus-toxin broth: Veal-infusion broth with 1 per cent dextrose (22)*

| | |
|---|---|
| Veal, ground | 500 grams |
| Distilled water | 1 kg. |
| Peptone (Berna) | 1 per cent or 10 grams per kg. |
| Sodium chloride | 0.5 per cent or 5 grams per kg. |
| Dextrose | 1 per cent or 10 grams per kg. |

**Procedure.**—Weigh the meat and add the water, reserving about 10 per cent or 100 grams per kilogram to dissolve the peptone and salt. Stir thoroughly and infuse overnight in the cold room. Skim off any fat which has floated to the surface. Heat to 45°C. and hold between 45 and 50°C. for one hour, stirring occasionally. Bring to the boiling point, and boil for thirty minutes, stirring frequently. Strain through cheesecloth. Cool infusion in an ice bath and remove fat. Dissolve the peptone and salt in the water reserved for the purpose by heating in the water-bath. Make up weight to that of the infusion (1 kg. for each 500 grams of meat) plus the other ingredients. Adjust the reaction to pH 6.8 (+0.9 to +1.3). Boil for three minutes. Make up lost weight and add the dextrose. Filter through absorbent cotton. Dispense, filling tubes and flasks as full as possible without danger of wetting the plugs during sterilization. Sterilize by steam at 100°C. for thirty or forty minutes (depending on bulk) on three successive days.

*Tuberculin broth*

| | |
|---|---|
| Beef, ground | 500 grams |
| Distilled water | 1 kg. |
| Peptone (Fairchild) | 1 per cent or 10 grams per kg. |
| Sodium chloride | 0.5 per cent or 5 grams per kg. |
| Glycerin C. P | 5 per cent or 50 grams per kg. |

**Procedure.**—Weigh meat and add the water, reserving about 10 per cent or 100 grams per kilogram to dissolve the other ingredients. Stir thoroughly and infuse overnight in the cold room. Heat to 45°C. and hold between 45 and 50°C. for thirty minutes, stirring occasionally. Dissolve the peptone and salt in the water reserved for the purpose by heating in the water-bath. Bring the infusion to the boiling point and boil for five minutes. Strain through cheesecloth to remove meat and coagulum. Cool infusion in an ice bath and remove fat. Add glycerin and solution of peptone and salt. Make up weight to that of the infusion (1 kg. for each 500 grams of meat) plus the other ingredients. Adjust reaction to pH 7.6 (+0.3 to +0.4). Autoclave for five minutes at 121°C. Make up lost weight. If necessary, readjust to pH 7.6 and boil over the free flame for

five minutes. Make up lost weight and filter through paper. Dispense in 960-cc. Blake bottles, 130 cc. in each and sterilize in steam at 100°C. for thirty minutes on three successive days. Incubate for six hours between sterilization periods and incubate the completed material for at least ninety-six hours.

## AGARS (HEAT STERILIZED)

### MEAT-EXTRACT AGARS

*Beef-extract agar 1.5 per cent*

| | |
|---|---|
| Distilled water | 1 kg. |
| Agar | 1.5 per cent or 15 grams per kg. |
| Sodium chloride | 0.5 per cent or 5 grams per kg. |
| Beef extract | 0.3 per cent or 3 grams per kg. |
| Peptone | 1 per cent or 10 grams per kg. |

**Procedure.**—Add the water to the agar, reserving about 10 per cent or 100 grams per kilogram to dissolve the other ingredients. Dissolve the agar by heating in the autoclave for thirty minutes at 121°C. Dissolve the peptone, salt and extract by heating in the water-bath. Combine the two parts and make up weight to that of all ingredients. Adjust to from pH 7.0 to 7.2 (+0.2 to +0.3). Cool to about 50°C. Heat in steam at 100°C. filtering through cheesecloth and cotton. Dispense and sterilize by steam at 121°C. for twenty or thirty minutes, depending on the bulk of the material.

*Beef-extract agar, 1.5 per cent (standard for the bacteriological examination of water and milk) (23)*

| | |
|---|---|
| Distilled water | 1 kg. |
| Agar | 1.5 per cent or 15 grams per kg. |
| Beef extract | 0.3 per cent or 3 grams per kg. |
| Peptone | 0.5 per cent or 5 grams per kg. |

**Procedure.**—Add the water to the agar, reserving about 10 per cent or 100 grams per kilogram to dissolve the other ingredients. Dissolve the agar by heating in the autoclave at 121°C. for thirty minutes. Dissolve the peptone and extract by heating in the water-bath. Combine the two parts and make up weight to that of all ingredients. Adjust to pH 6.6 (+0.3 to +0.4). Cool to about 50°C. Heat in steam at 100°C. filtering through cheesecloth and cotton. Dispense and sterilize by steam at 121°C. for twenty minutes.

*Beef-extract agar, 1.5 per cent, with indicator (base for brilliant-green medium) (24)*

| | |
|---|---|
| Beef-extract agar 1.5 per cent | 1 kg. |
| Andrade's indicator | 3 per cent or 30 cc. per kg. |

**Procedure.**—The procedure is the same as for beef-extract agar, 1.5 per cent, except that the reaction is not adjusted.

Weigh the unadjusted, filtered agar and add 3 per cent Andrade's indicator. Determine the reaction and if it lies between pH 7.2 and 7.6 do not adjust. Otherwise adjust to pH 7.6. (With the special brands of ingredients used adjustment of reaction is practically never necessary.) Dispense five 100-cc. amounts in

240-cc. bottles. Dispense the remainder in 400-cc. amounts in 480-cc. Blake bottles. Sterilize by steam at 121°C. for thirty minutes. As soon as the material is removed from the autoclave, add to each of the five 100-cc. amounts of agar 5 cc. of a sterile solution containing 2 per cent dextrose and 20 per cent lactose. To the first bottle add 0.1 cc., to the second 0.2 cc., to the third 0.3 cc., to the fourth 0.4 cc., and to the fifth 0.5 cc. of a 0.1-per-cent aqueous solution of brilliant green dye. (These amounts may vary with different lots of brilliant-green dye.) From each bottle pour six plates, marking the amount of brilliant-green solution and the date of preparation of agar on the cover of each plate. Send these plates to the diagnostic laboratories for standardization.

When brilliant-green, agar plates are ordered by the diagnostic laboratories, add to the required number of 400-cc. amounts of the agar melted in steam at 100°C., 20 cc. of a sterile solution containing 2 per cent dextrose and 20 per cent lactose. Then add the required amounts of 0.1-per-cent aqueous solution of brilliant green as indicated on the record card showing the results of standardization. Pour into porous-top plates and mark "Gs" and "Gw" for green strong and green weak, respectively, according to the amount of dye added.

*Beef-extract agar, 1.5 per cent with indicator and 1 per cent lactose*

| | |
|---|---|
| Beef-extract agar 1.5 per cent | 1 kg. |
| Andrade's indicator | 3 per cent or 30 cc. per kg. |
| Lactose | 1 per cent or 10 grams per kg. |

**Procedure.**—Proceed as for beef-extract agar, 1.5 per cent, with indicator. After the reaction has been determined (and the adjustment made if necessary) add the lactose and dissolve. Dispense sufficient medium in 125-by-13-millimeter test tubes to give a deep butt and a moderate slant. Sterilize by steam at 121°C. for twelve minutes. As soon as completed send eight tubes of each lot to the laboratories for sanitary and analytical chemistry, to be tested.

*Beef-extract agar 1.5 per cent, with indicator and sugars. (Krumwiede's triple-sugar, Russell's modified double-sugar medium) (25, 26)*

| | |
|---|---|
| Beef-extract agar 1.5 per cent | 1 kg. |
| Andrade's indicator | 3 per cent or 30 cc. per kg. |
| Lactose | 1 per cent or 10 grams per kg. |
| Saccharose | 1 per cent or 10 grams per kg. |
| Dextrose | 0.05 per cent or 0.5 gram per kg. |

**Procedure.**—The procedure is the same as for beef-extract agar, 1.5 per cent, with indicator and lactose except that two more sugars, saccharose and dextrose, are added. Eight tubes of each lot of this medium are sent to the diagnostic laboratories to be tested.

*Beef-extract agar 2 per cent (base for eosin-methylene-blue medium) (27)*

| | |
|---|---|
| Distilled water | 1 kg. |
| Agar | 2 per cent or 20 grams per kg. |
| Sodium chloride | 0.5 per cent or 5 grams per kg. |
| Beef extract | 0.3 per cent or 3 grams per kg. |
| Peptone | 1 per cent or 10 grams per kg. |

**Procedure.**—The procedure is the same as for 1.5-per-cent beef-extract agar, reaction pH 7.0 to 7.2 (+0.2 to +0.4).

Dispense beef-extract agar which is to be used as a base for eosin-methylene-blue plates, in 750-cc. amounts in 960-cc. Blake bottles. When ordered by the diagnostic laboratories, add to 750 cc. of the agar melted in steam at 100°C., 37.5 cc. of a solution containing 10 per cent saccharose and 10 per cent lactose, 10 cc. of a 3-per-cent aqueous solution of eosin and 15 cc. of a 0.5-per-cent aqueous solution of methylene blue. Mix well, pour into porous-top plates, and mark "Eb."

*Beef-extract agar 2.5 per cent (base for Robinson and Rettger's modification of Endo's medium) (28, 29)*

| | |
|---|---|
| Distilled water | 1 kg. |
| Agar | 2.5 per cent or 25 grams per kg. |
| Beef extract | 0.5 per cent or 5 grams per kg. |
| Peptone | 1 per cent or 10 grams per kg. |

**Procedure.**—Add the water to the agar, reserving about 10 per cent or 100 grams per kilogram to dissolve the other ingredients. Dissolve the agar by heating in the autoclave for thirty minutes at 121°C. Dissolve the peptone and extract by heating in the water-bath. Combine the two parts and make up weight to that of all ingredients, using 1-per-cent $Na_2CO_3$ for titrating and 10-per-cent $Na_2CO_3$ for adjusting. Adjust to pH 7.0 (+0.3 to +0.4) and add 10 cc. of 10-per-cent $Na_2CO_3$ per kilogram in excess or adjust to pH 8.5 and do not add excess carbonate. Cool to 50°C. Heat in steam at 100°C. filtering through cheesecloth and cotton. Dispense in 960-cc. Blake bottles, 750 cc. in each. Sterilize by steam at 121°C. for thirty minutes. When ordered by the diagnostic laboratories add to 750 cc. of the agar melted in steam at 100°C., 37.5 cc. of a 20-per-cent solution of lactose, 1.9 cc. of saturated alcoholic basic fuchsin and 7.5 cc. of 10-per-cent sodium bisulfite. Pour into porous-top plates and mark "E."

The basic medium prepared in this way will have a reaction of pH 7.6 to 8.0 after the decolorized fuchsin (Endo's) indicator has been added, if sodium bisulfite is used in the preparation of the indicator. If the slightly alkaline sodium sulfite is used, the base should be adjusted to a less alkaline point, pH 7.6 to 7.8 and, of course, no excess carbonate should be added.

*Beef-extract agar 3 per cent (base for Kendall and Walker's modification of Endo's medium) (28, 30)*

| | |
|---|---|
| Distilled water | 1 kg. |
| Agar | 3 per cent or 30 grams per kg. |
| Beef extract | 0.5 per cent or 5 grams per kg. |
| Peptone | 1 per cent or 10 grams per kg. |

**Procedure.**—Add the water to the agar, reserving about 10 per cent or 100 grams per kilogram to dissolve the other ingredients. Dissolve the agar by heating in the autoclave for thirty minutes at 121°C. Dissolve the peptone and extract by heating in the water-bath. Combine the two parts and make up weight to that of all ingredients. Titrate with N/20 NaOH and adjust with N/1 $Na_2CO_3$ to from pH 7.4 to 7.6 (+0.1 to + 0.2). Heat in steam at 100°C. filtering through cheesecloth and cotton. Dispense in 960-cc. Blake bottles, 600 cc. in each. Sterilize by steam at 121°C. for thirty minutes.

## MEAT-INFUSION AGARS

### *Hitchens' semifluid agar 0.12 per cent (31)*

Sterility broth..... 1 kg.
Agar, 3 per cent, aqueous.....40 cc. per kg.

**Procedure.**—Prepare sterility broth (beef-infusion broth, 0.1 per cent dextrose) as usual. Prepare the required amount of 3-per-cent aqueous agar, dissolving the agar by heating in the autoclave at 121°C. for thirty minutes. Filter the broth until it is clear. Bring to boiling point. Add the melted aqueous agar. Line the bottom of a Buchner funnel with two layers of filter paper cut to fit. Over these, place a thick pad of nonabsorbent cotton. Using a small amount of negative pressure, and keeping the medium hot, filter it into a 4-liter suction flask. Dispense and sterilize by steam at 121°C. for twenty minutes.

This semifluid agar, or agar broth, readily maintains anaerobic conditions, and when dispensed in moderately tall columns supports vigorous growth of many anaerobes as well as aerobes. The agar concentration is based on a final 0.1 per cent oven-dried agar, or 0.12 per cent market agar. For use in sterility tests, this medium is dispensed in 165-by-22-millimeter test tubes, 22 cc. in each; in 50-cc. round bottles, 45 cc. in each; in 120-cc. French square bottles, 90 cc. in each; and in 240-cc. bottles, 170 cc. in each, to allow for final volumes, after sterilization, of at least 20 cc., 40 cc., 80 cc., and 160 cc. respectively.

### *Beef-infusion agar, 0.5 per cent (base for ascitic-fluid, semisolid medium)*

Beef infusion, double strength.....500 grams
Distilled water.....500 grams
Agar.....0.5 per cent or 5 grams per kg.
Sodium chloride.....0.5 per cent or 5 grams per kg.
Peptone.....1 per cent or 10 grams per kg.
Egg albumen..... 1 gram per kg.

**Procedure.**—Prepare a solution of egg albumen by adding a small quantity of water to the dry albumen (about 2 cc. per gram) and allowing it to stand overnight to dissolve. Add the water to the agar and dissolve by heating in the autoclave for thirty minutes at 121°C. Meanwhile, dissolve the peptone and salt in the infusion. Combine the previously warmed peptone-salt solution with the dissolved agar and make up weight to that of all ingredients. Adjust to from pH 7.4 to 7.6 (+0.2 to +0.3). Cool to 50°C., and add the dissolved albumen. Heat in steam at 100°C. filtering through cheesecloth and cotton. Dispense (usually in 400-cc. amounts in 2-liter Erlenmeyer flasks) and sterilize by steam at 121°C. for thirty minutes.

### *Beef-infusion agar, 0.5 per cent with 1 per cent dextrose (dextrose, beef-infusion, semisolid medium)*

Beef-infusion agar 0.5 per cent..... 1 kg.
Dextrose.....1 per cent or 10 grams per kg.
Egg albumen.....1 gram per kg.

**Procedure.**—Prepare 0.5-per-cent beef-infusion agar and adjust to pH 7.8 (±0.0). After filtering, weigh and add the dextrose. Dispense and sterilize by steam at 121°C. for twenty minutes.

*Beef-infusion agar, 1.5 per cent (base for tellurite, dextrose, serum agar and 0.2 per cent dextrose-serum agar)*

Beef infusion, double strength.....500 grams
Distilled water.....500 grams
Agar..... 1.5 per cent or 15 grams per kg.
Sodium chloride..... 0.5 per cent or 5 grams per kg.
Peptone..... 1 per cent or 10 grams per kg.
Egg albumen..... 1 gram per kg.

**Procedure.**—Proceed as for 0.5-per-cent beef-infusion agar. Adjust to from pH 7.4 to 7.6 (+0.2 to +0.4).

*Beef-infusion agar, 1.75 per cent (for slants; also used plain for vaccine production)*

Beef infusion, double strength.....500 grams
Distilled water.....500 grams
Agar..... 1.75 per cent or 17.5 grams per kg.
Sodium chloride..... 0.5 per cent or 5 grams per kg.
Peptone..... 1 per cent or 10 grams per kg.

**Procedure.**—The procedure is the same as for 0.5-per-cent beef-infuson agar except that no egg albumen is used. It is adjusted to from pH 7.4 to 7.6 (+0.2 to +0.4).

*Beef-infusion agar, 1.75 per cent, with 0.2 per cent dextrose (for plating and deep-tube culture)*

Beef-infusion agar, 1.75 per cent..... ..1 kg.
Dextrose.....0.2 per cent or 2 grams per kg.
Egg albumen.....1 gram per kg.

**Procedure.**—Prepare 1.75-per-cent beef-infusion agar and adjust to pH 7.8 (±0.0 to +0.1). Add solution of egg albumen. After filtering, weigh, and add the dextrose. Dispense, and sterilize by steam at 121°C. for twenty minutes.

*Beef-infusion agar 2 per cent (base for dextrose, serum-agar slants and blood-agar plates and slants; also used plain in vaccine production)*

Beef infusion, double strength.....500 grams
Distilled water.....500 grams
Agar..... 2 per cent or 20 grams per kg.
Sodium chloride..... 0.5 per cent or 5 grams per kg.
Peptone..... 1 per cent or 10 grams per kg.

**Procedure.**—The procedure is the same as for 0.5-per-cent beef-infusion agar, except that no egg albumen is used. It is adjusted to pH 7.6 (+0.2 to +0.4).

*Beef-infusion agar, 2 per cent, sugar-free, with indicator (base for carbohydrate, Andrade, agar slants with or without ascitic fluid)*

Beef infusion, sugar free double strength.....500 grams
Distilled water.....500 grams

| | |
|---|---|
| Agar | 2 per cent or 20 grams per kg. |
| Sodium chloride | 0.5 per cent or 5 grams per kg. |
| Peptone | 1 per cent or 10 grams per kg. |
| Andrade's indicator | 3 per cent or 30 cc. per kg. |

Procedure.—The procedure is the same as for 2-per-cent beef-infusion agar save that sugar-free infusion is used and no adjustment is made before filtration.

To the unadjusted, filtered agar, add 3 per cent Andrade's indicator. If the reaction lies between pH 7.4 and 7.8 do not adjust, otherwise adjust to pH 7.6. Dispense in 200-cc. amounts in 500-cc. Erlenmeyer flasks.

*Beef-infusion agar, 2.25 per cent (base for serum, semisolid agar)*

| | |
|---|---|
| Beef infusion, double strength | 500 grams |
| Distilled water | 500 grams |
| Agar | 2.25 per cent or 22.5 grams per kg. |
| Sodium chloride | 0.5 per cent or 5 grams per kg. |
| Peptone | 1 per cent or 10 grams per kg. |
| Egg albumen | 1 gram per kg. |

Procedure.—The preliminary procedure is the same as for 0.5-per-cent beef-infusion agar.

Adjust to from pH 7.4 to 7.6. Dispense in 2-liter flasks, 150 cc. in each, and sterilize by steam at 121°C. for thirty minutes.

*Beef-infusion agar, 2.5 per cent, alkaline (for Sp. cholerae)*

| | |
|---|---|
| Beef infusion, double strength | 500 grams |
| Distilled water | 500 grams |
| Agar | 2.5 per cent or 25 grams per kg. |
| Sodium chloride | 0.5 per cent or 5 grams per kg. |
| Peptone (Witte) | 1 per cent or 10 grams per kg. |

Procedure.—This medium is adjusted with N/1 $Na_2CO_3$ to from pH 9.0 to 9.4. Otherwise, except for the omission of egg albumen, the procedure is the same as for 0.5-per-cent beef-infusion agar. Dispense in 300-cc. amounts in 500-cc. Erlenmeyer flasks. Sterilize by steam at 121°C. for twenty minutes. Redispense in slants when needed, with aseptic precautions.

*Beef-infusion agar, 2.5 per cent, with 5 per cent glycerin (for B. pertussis, etc.; also base for coagulated-blood agar for B. pertussis)*

| | |
|---|---|
| Beef infusion, double strength | 500 grams |
| Distilled water | 500 grams |
| Agar | 2.5 per cent or 25 grams per kg. |
| Sodium chloride | 0.5 per cent or 5 grams per kg. |
| Peptone | 1 per cent or 10 grams per kg. |
| Glycerin, C. P | 5 per cent or 50 grams per kg. |

Procedure.—The procedure is the same as for 0.5-per-cent beef-infusion agar save that no egg albumen is added.

Adjust to pH 7.4 (+0.1 to +0.3). After filtering, weigh, and add the glycerin. Dispense, and sterilize by steam at 121°C. for thirty minutes.

If ordered for growing *B. tuberculosis*, sterilize by steam at 100°C. for twenty minutes on three successive days. Incubate for 6-hour periods between sterilizations. Incubate the completed material for forty-eight hours.

*Testicular-infusion agar (base for gonococcus medium) (32, 33)*

| | |
|---|---|
| Beef testicle | 500 grams |
| Distilled water | 1 kg. |
| Agar | 2.5 per cent or 25 grams per kg. |
| Peptone | 2 per cent or 20 grams per kg. |
| $NaH_2PO_4$ | 0.3 per cent or 3 grams per kg. |
| Dextrose | 0.2 per cent or 2 grams per kg. |

Preparation of concentrated broth: Wash testicles well in running water and remove the tunic and connective tissue as completely as possible. Grind, and weigh, and add one-half the total quantity of water (i.e., 1 kg. per kilogram of tissue). Infuse overnight in the cold room. Heat to 50°C. in the water-bath, and hold at this temperature for one-half hour. Bring the water-bath to the boiling point and heat the medium, stirring constantly, until the proteins coagulate and tend to settle out. Strain through cheesecloth or a fine wire sieve. Add water to bring up to the volume of water originally used. To each 500 grams of infusion add 20 grams peptone, and 3 grams monobasic sodium phosphate, and dissolve.

Preparation of aqueous agar: To the other half of the water add agar, 25 grams for each 500 grams of water. Dissolve by heating in the autoclave at 121°C. for thirty minutes.

Procedure.—To 500 grams of concentrated broth, add 500 grams of the 5-per-cent agar. Adjust to pH 7.8 (±0.0 to +0.1). Add and dissolve the dextrose. Dispense, and sterilize by steam at 121°C. for thirty minutes.

*Veal-infusion agar, 0.5 per cent (veal-infusion, semisolid agar) (34)*

This medium is the same as beef-infusion, semisolid agar, save that veal is substituted for the beef. The reaction is adjusted to pH 7.8 (±0.0).

*Veal-infusion agar, 2.5 per cent with 5 per cent glycerin (base for coagulated-blood agar for B. influenzae)*

| | |
|---|---|
| Veal-infusion agar 2.5 per cent | 1 kg. |
| Glycerin, C. P | 5 per cent or 50 grams per kg. |

Procedure.—Prepare 2.5-per-cent veal-infusion agar, using the same procedure as with the 0.5-per-cent beef-infusion agar except that egg albumen is omitted. Adjust to pH 8.2 (−0.1 to −0.3). After filtering, weigh, and add the glycerin. Dispense, and sterilize by steam at 121°C. for thirty minutes.

*Vitamin agar, 0.5 per cent (Huntoon's "hormone" semisolid agar for the gonococcus) (35)*

| | |
|---|---|
| Beef heart, chopped | 500 grams |
| Distilled water | 1 kg. |
| Agar | 0.5 per cent or 5 grams per kg. |
| Peptone | 1 per cent or 10 grams per kg. |

| | |
|---|---|
| Sodium chloride | 0.5 per cent or 5 grams per kg. |
| Whole egg | 1 per kg. |

Procedure.—Add the water to the chopped heart and egg and mix well. Heat on steam-bath (or in double boiler) to 60°C. and maintain at this temperature for five minutes, stirring constantly. Add peptone, salt, and the agar (previously flaked and soaked in water). Raise the temperature until the medium becomes brown (68°C.), stirring constantly. Adjust to +1.0 to phenolphthalein using N/1 $Na_2CO_3$. Place in a flask or a tall container, and heat in steam at 100°C. for one hour. Loosen the clot from the sides of the container, and repeat the heating for another hour. Clear, by straining through a fine wire sieve and filtering through glass wool. (No cotton, paper or other absorbent material may be used.) Weigh filtrate, and adjust to pH 6.8, using N/1 $Na_2CO_3$. Heat in steam at 100°C. or in steam-bath for fifteen minutes. Dispense, and sterilize by steam at 100°C. for thirty minutes on three successive days.

For early generations of gonococcus strains, this medium should be enriched with ascitic fluid (10 to 15 per cent). A similar medium, containing from 1.5 per cent to 1.8 per cent agar may be used as a very favorable solid medium for many organisms, especially when enriched with blood, ascitic fluid, etc. A very soft, vitamin agar (1.16 per cent) plus half as much blood as agar is a most satisfactory plating medium for the cultivation of the Ducrey bacillus.

*Vitamin agar 2 per cent for B. abortus (beef-liver infusion) (13)*

| | |
|---|---|
| Beef-liver infusion, double strength | 500 grams |
| Distilled water | 500 grams |
| Agar | 2 per cent or 20 grams per kg. |
| Peptone | 1 per cent or 10 grams per kg. |
| Sodium chloride | 0.5 per cent or 5 grams per kg. |
| Egg albumen | 1 per cent or 10 grams per kg. |

Procedure.—Add the water to the agar, and dissolve by heating in the autoclave for thirty minutes at 121°C. Dissolve the peptone and salt in the infusion. Combine the agar and previously warmed peptone-salt solution and make up weight to that of all ingredients. Adjust to pH 7.0. Cool to 50°C. and add the dissolved egg albumen. Heat at 100°C. for ninety minutes. Strain through wire sieve and glass wool, previously washed with distilled water. Readjust to pH 7.0. Dispense, and sterilize by steam at 121°C. for thirty minutes.

## GELATINS (HEAT STERILIZED)

*Beef-extract gelatin*

| | |
|---|---|
| Distilled water | 1 kg. |
| Gelatin | 10 per cent or 100 grams per kg. |
| Beef extract | 0.3 per cent or 3 grams per kg. |
| Peptone | 0.5 per cent or 5 grams per kg. |

Procedure.—Add the other ingredients to the water. Heat gradually in a water-bath until all of the gelatin is dissolved. Titrate, and adjust to pH 7.0 (+0.4 to +0.6). Let stand, and cool to 45°C. without stirring. Hold between 95 and 100°C. for twenty minutes, with occasional stirring. Make up lost weight.

Filter through hot paper pulp in a Buchner funnel. Dispense in 150-by-19-millimeter test tubes, from 12 to 14 cc. in each. Sterilize by steam at 121°C. for fifteen minutes. Upon removal from the autoclave, place immediately in the cold room.

The day following the preparation of the medium the melting point is determined. If this lies below 25°C. the medium is discarded. This happens very rarely.

**To Determine the Melting Point of Gelatin.**—Place three tubes in a beaker of cold water, padded with a layer of absorbent cotton. Place a thermometer in the water.

Break the contents of one of the tubes of gelatin by running a second thermometer through the gelatin nearly to the bottom of the tube and withdrawing it again. Push the thermometer into the second tube half way down the column of gelatin and leave it there. Leave the third tube of medium intact.

Place a low flame under the beaker, and watch the temperature very carefully, stirring the water gently with the intact tube of gelatin. Whenever the thermometer in the water registers more than two degrees higher than the thermometer in the tube of gelatin, remove the flame until the temperatures equalize. Be especially careful after the temperature of the water has reached 20°C.

Record the temperatures at which the following phenomena occur: (1) The broken-up portions of gelatin in the first tube begin to coalesce; (2) the thermometer in the second tube begins to sink; (3) the contents of the third tube start to move when the tube is tipped. (If the process is carried out carefully, the recorded temperatures for the three tubes will be found to correspond within a fraction of a degree.)

*Beef-infusion gelatin (for stock cultures) (36)*

| | |
|---|---|
| Beef, chopped | 500 grams |
| Distilled water | 1 kg. |
| Peptone | 1 per cent or 10 grams per kg. |
| Sodium chloride | 0.5 per cent or 5 grams per kg. |
| Gelatin | 9 per cent or 90 grams per kg. |

**Procedure.**—Add the water to the meat, and infuse overnight in the cold room. Heat in a water-bath to from 50 to 55°C. and hold for one hour. Strain through cheesecloth, weigh, and, if necessary, add water to give 1 kg. of infusion for each 500 grams of meat. Titrate and adjust to +1.0 to phenolphthalein. Add the peptone, salt, and gelatin, and dissolve by heating in a water-bath. Make up lost weight, and filter through a thin layer of absorbent cotton wet with hot, distilled water, in a Buchner funnel. Weigh and adjust to pH 7.8 (+0.1 to +0.3). Hold between 95 and 100°C. in water-bath for twenty minutes, with occasional stirring. Filter through hot, paper pulp in a Buchner funnel. Dispense, and sterilize by steam for fifteen minutes at 115°C.

## MISCELLANEOUS MEDIA (HEAT STERILIZED)

*Bordet-Gengou medium (base for blood agar for B. pertussis) (37)*

| | |
|---|---|
| Distilled water | 1250 grams |
| Glycerin C. P. | 20 grams |

| | |
|---|---|
| Potatoes, sliced | 250 grams |
| Sodium chloride | 5 grams |
| Agar | 30 grams |

**Procedure.**—Add 500 grams of water and the glycerin to the potatoes, and heat in the autoclave for thirty minutes at 121°C. Decant the liquid. Add the balance of the water, the salt, and the agar to 250 grams of this liquid. Dissolve the agar by heating in the autoclave at 121°C. for thirty minutes. Heat in steam at 100°C. Dispense, and sterilize by steam at 121°C. for thirty minutes.

*Dunham's solution, modified (38)*

| | | |
|---|---|---|
| Distilled water | 1 kg. | |
| Peptone (Parke-Davis) | 1 | per cent or 10 grams per kg. |
| Potassium nitrate C. P | 0.01 | per cent or 0.1 gram per kg. |

**Procedure.**—Dissolve the peptone in the water, warming if necessary. Adjust to pH 7.0. Add the nitrate, and filter through paper. Dispense, and sterilize by steam at 121°C. for twelve minutes.

Parke-Davis peptone, or some other brand known to be satisfactory for indol production, must be used.

*Milk medium*

Milk, certified or grade A, unpasteurized.....1 qt.

**Procedure.**—Pour the milk into a separatory funnel, and place in the cold room overnight to allow the cream to rise. Draw off the fat-free milk. Dispense, and sterilize by steam at 100°C. for twenty minutes on three successive days.

*Milk with litmus*

| | |
|---|---|
| Milk, certified or grade A, unpasteurized | 1 qt. |
| Alcoholic-litmus solution | 2.5 per cent by vol. or 2.5 cc. per 100 cc. |

**Procedure.**—Separate the milk from the cream as above. Add the required volume of litmus solution. If necessary, adjust the reaction to neutral to the indicator. Dispense, and sterilize by steam at 100°C. for twenty minutes on three successive days.

*Peptone water with indicator (substrate for carbohydrate media) (39)*

| | | |
|---|---|---|
| Distilled water | 1 kg. | |
| Peptone | 1 | per cent or 10 grams per kg. |
| Sodium chloride | 0.5 | per cent or 5 grams per kg. |
| Andrade's indicator | 1 | per cent or 10 cc. per kg. |

**Procedure.**—Add the peptone and salt to the water, and dissolve, warming if necessary. Filter through paper. Add the indicator and adjust to from pH 7.2 to 7.4. Dispense, and sterilize by steam at 121°C. for twenty minutes.

*Potato medium*

Large, sound potatoes.....about 8 or 10, to make 50 slants

Procedure.—Wash and pare the potatoes. Cut into pieces shaped to fit the size tube desired, and to give a good slant surface. Wash the pieces in running water overnight. Insert into tubes, cover plugs with tinfoil and sterilize by steam at 121°C. for twelve minutes.

As these slants are kept in stock for several months, it is customary to have the tubes sterilized in covered, glass fruit jars containing about a 2-cc. depth of mercuric chloride (1:1000). After sterilization, the jars are sealed. Practically no slants have to be discarded because of drying or becoming contaminated with molds.

*Sabouraud's agar for molds and yeasts (40)*

Distilled water.....1 kg.
Peptone.....1.5 per cent or 15 grams per kg.
Agar.....1.5 per cent or 15 grams per kg.
Dextrose.....2 per cent or 20 grams per kg.
Glycerin C. P.....0.5 per cent by vol. or 5 cc. per kg.

Procedure.—Add the peptone and the agar to the water, and dissolve by heating in the autoclave at 121°C. for thirty minutes. Heat by steam at 100°C., filtering through cheesecloth and cotton. To the measured filtrate, add the required amount of dextrose and glycerin. Dispense as ordered, and sterilize by steam at 121°C. for twenty minutes.

*Serum water with indicator (substrate for carbohydrate media) (39, 41)*

Distilled water.....80 per cent or 800 grams per kg.
Horse serum.....20 per cent or 200 grams per kg.
Andrade's indicator.....10 cc. to each kg. of diluted serum

Procedure.—Mix the serum and water, and heat in steam at 100°C. for twenty minutes. Add the indicator, and cool to room temperature. Titrate with N/20 HCl, using phenol red as the indicator. Adjust to pH 6.8 with N/1 HCl, adding the acid drop by drop. Dispense in convenient amounts in 300-cc. or 500-cc. Erlenmeyer flasks, and sterilize by steam at 121°C. for twenty minutes.

It is important that the N/1 HCl be added slowly to the cooled medium as too rapid addition of acid to hot serum water causes a flocculent precipitate to form.

## SOLUTIONS AND MISCELLANEOUS MATERIALS

### SOLUTIONS TO BE ADDED TO MEDIA (FILTERED)

*Blood extract (42)*

Sheep or horse blood, defibrinated.....1 part
Sodium-chloride solution, 0.85 per cent.....4 parts

Procedure.—Mix ingredients thoroughly in an Erlenmeyer flask. Place in a water-bath, and heat to 80°C., holding at this temperature for fifteen minutes.

Agitate frequently during this period. Filter through paper and through a sterile filter candle into a sterile container.

To avoid filtration, the procedure may be conducted with aseptic precautions. Pipette sterile blood and sterile salt solution into sterile centrifuge cups. Heat as above, shaking frequently. (Place thermometer in a tube of water and put in bath as a temperature control.) Centrifugalize for about one-half hour, and pipette supernatant blood extract into sterile, corked 20-cc. vials.

### *Carbohydrate solutions (filtered)*

For careful study of fermentation reactions, filtered carbohydrate solutions are used, save in the case of carbohydrates which are relatively stable at high temperatures. The most commonly used filtered solutions are lactose, saccharose, maltose, and dextrin.

Procedure.—Dissolve the carbohydrate in a portion of the distilled water and make up to volume. Sterilize by passage through a sterile candle into a 240-cc. French square bottle. Stopper with a sterile rubber stopper. Wrap stopper with carbolized cotton, and cap with tinfoil and heavy paper.

### *Tellurite solution 1 per cent (43, 64)*

Potassium tellurite ($K_2TeO_3$) C. P..... 1 gram
Distilled water to make.....100 cc.

Procedure.—Weigh 1 gram of potassium tellurite on torsion balance. Triturate in a small, dry mortar. Measure out 100 cc. of distilled water. Add 10 cc. of the water to the tellurite and macerate well; then add 20 cc. more of water, and stir. Allow the undissolved portion to settle and decant supernatant fluid into a 100-cc. graduate. Add more of the water to the residue in the mortar, macerate, add more water, stir, let settle, and pour off supernatant fluid into the 100-cc. graduate. Repeat. Rinse the mortar with the remainder of the water and add this water to the material in the graduate to make up to 100 cc. Filter through a small, sterile filter candle, and dispense in 11-cc. amounts in sterile, corked, 20-cc. vials.

The potassium tellurite thus far used has been only from 58 to 59 per cent $K_2TeO_3$ and has contained a small amount of insoluble material. There is a slight loss of $K_2TeO_3$ on filtration, which, for practical purposes, is negligible.

## SOLUTIONS TO BE ADDED TO MEDIA (HEAT STERILIZED)

### *Carbohydrate solutions*

Lactose and saccharose are heat sterilized in aqueous solution for use in the dye agars.

Dextrose is aways heat sterilized but, as it breaks down considerably when sterilized in media, and practically not at all when sterilized in aqueous solution. sterile aqueous solutions, dispensed in convenient amounts, are kept in stock for various purposes.

*Dextrose*
Dextrose.....20 per cent or 200 grams per 1000 cc.

*Dextrose-lactose*

Dextrose..... 2 per cent or 20 grams per 1000 cc.
Lactose.....20 per cent or 200 grams per 1000 cc.

*Lactose*

Lactose.....20 per cent or 200 grams per 1000 cc.

*Saccharose-lactose*

Saccharose.....10 per cent or 100 grams per 1000 cc.
Lactose.....10 per cent or 100 grams per 1000 cc.

Procedure.—Dissolve the carbohydrates in a small portion of the distilled water. Make up to volume. Filter through paper. Dispense dextrose-lactose in 20-cc. amounts, and lactose and saccharose-lactose in 37.5-cc. amounts in 50-cc. bottles for dye media. Dispense dextrose in 11-cc. amounts in 20-cc. vials, and in 50-cc. amounts in 120-cc. French square bottles. Sterilize by steam at 121°C. for fifteen minutes.

### *Mineral oil*

Procedure.—Sterilize mineral oil which is to be added to media, by dry heat for at least one hour at from 165 to 175°C.

### *Paraffin mineral-oil mixture*

Paraffin.....100 grams
Mineral oil.....200 grams

Procedure.—Dissolve the ingredients in the water-bath. Pipette about 1.25 cc. into unplugged 165-by-22-millimeter tubes. (The tubes are warmed to allow the mixture to run to the bottom, and are then cooled, plugged, and sterilized by dry heat at from 150 to 162°C. for one hour.)

### *Vaseline*

Procedure.—The same procedure is followed as with mineral oil.

## SOLUTIONS TO BE ADDED TO MEDIA (UNSTERILIZED)

### *Andrade's indicator (decolorized acid fuchsin) (44, 45)*

Acid fuchsin
Distilled water.....1000 cc.
N/1 NaOH..... 160 cc.

It is necessary to test out each new lot of fuchsin received. This is done by making up a small amount of the indicator, using the same amount of fuchsin as was used from the preceding lot for the preparation of the indicator. This new indicator is incorporated into triple-sugar medium. This medium is tested with suitable organisms by the diagnostic department.

If the uninoculated medium of suitable reaction is decidedly pink in color when cold, less fuchsin should be used. As much as 5 grams and as little as 1.5 grams have been found suitable. At present 2.5 grams of Coleman and Bell's acid fuchsin is being used.

**Procedure.**—Dissolve the fuchsin in the water, and add the alkali. Mix thoroughly, and allow to stand for from one to two hours. Filter through paper.

*Litmus solution (46)*

Litmus, granular..... 80 grams
Alcohol, 40 per cent.....300 cc.
(126 cc. 95 per cent diluted to 300 cc.)

**Procedure.**—Grind the litmus in a mortar with 150 cc. of the alcohol. Transfer to a flask, and boil gently for one minute on a steam-bath. Decant the fluid, add the remainder of the alcohol, and again boil for one minute. Decant and add the fluid to the first extract. Allow to settle overnight, decant, and make up to 300 cc. with 40-per-cent alcohol. Add N/1 HCl, drop by drop, shaking from time to time, until the solution becomes purple (this usually requires from 1 to 2 cc. of acid per 100 cc. of extract). (Solution of the correct reaction should be blue in tap water (alkaline) and mauve in boiled, distilled water.)

*Sodium-carbonate solution, normal*

$Na_2CO_3$, 10 per cent.....265 cc.
Distilled water to make.....500 cc.

**Procedure.**—Pour 265 cc. of 10-per-cent $Na_2CO_3$ (furnished by the chemical laboratories) into a 500-cc. dilution flask. Make up to volume with distilled water. Shake thoroughly.

MISCELLANEOUS MATERIAL (HEAT STERILIZED)

*Bile (47)*

Fresh ox bile.

**Procedure.**—Ox bile is secured from the slaughter house, from recently killed animals.

Heat in steam at 100°C. for twenty minutes to coagulate and precipitate substances affected by heat. Filter through paper while hot. Dispense, and sterilize by steam at 100°C. for twenty minutes on three successive days.

*Borate sodium-chloride solution*

Borate solution..... 1 liter
Sodium-chloride solution, 0.85 per cent..... 9 liters

Preparation of borate solution:

Boric acid—$H_3BO_3$..... 6.698 grams
N/1 NaOH.....53 cc.
N/1 HCl.....46 cc.

Weigh the boric acid accurately in a small beaker. Transfer to a 1000-cc. volumetric flask, and add the N/1 NaOH, rinsing all the particles of boric acid out of the beaker with the NaOH. Then add N/1 HCl. Bring the volume up to 1000 cc. with distilled water. Shake until boric acid is in solution.

**Procedure.**—To each part of the borate solution, add 9 parts of 0.85-per-cent salt solution. Dispense in approximately equal amounts in six 2-liter Erlenmeyer flasks, and sterilize by steam at 121°C. for thirty minutes.

*Distilled water*

**Procedure.**—Dispense distilled water, and sterilize by steam at 121°C. for twenty or thirty minutes.

*Glycerin, C.P.*

**Procedure.**—Dispense C.P. glycerin as ordered, and sterilize by steam at 121°C. for thirty minutes.

*Glycerin sodium-chloride solution (48)*

Sodium chloride, 0.6-per-cent solution...70 per cent or 700 cc. per liter
Glycerin T.P. (reagent).....30 per cent or 300 cc. per liter

Determine the amount of sodium-chloride solution necessary for the preparation of the required amount of the glycerin-salt solution.

Preparation of sodium-chloride solution:

Sodium chloride.....0.6 per cent or 6 grams per liter
Distilled water to make up to volume.

Dissolve the salt in part of the water and make up to volume.

**Procedure.**—To each seven parts of the 0.6-per-cent salt solution, add three parts of the reagent glycerin. Filter through paper.

Dispense in 76-by-21-millimeter tubes, about 10 cc. in each. Stopper loosely with rubber stoppers and place in baskets, lined with heavy paper. Cover the tops of the tubes with heavy paper, and tie in place, to prevent the stoppers from being driven out during sterilization. Sterilize by steam at 121°C. for twenty minutes. Immediately after removing from the autoclave, push the stoppers securely into the tubes. Invert the tubes in baskets and leave overnight. Examine, and discard leaky tubes. Pool the solution from these tubes and add it to the next lot of material prepared.

*Paper pulp*

Scrap filter paper (soft).....180 grams
Distilled water to make approximately..... 6 liters

**Procedure.**—Add about two liters of hot, distilled water to the paper and let stand a few minutes. Pour off water. Rinse the paper with warm, distilled water until the water comes off clear. Work the pulp with the hands until it is broken up into fine particles. Make up the volume to about six liters. Aerate vigorously in an 8-liter bottle, using positive pressure. Dispense in 2½-liter bottles, and sterilize at 121°C. for thirty minutes.

*Sodium-chloride solution, 0.85 per cent*

Sodium chloride, C.P.....0.85 per cent or 8.5 grams per kg.
Distilled water to make.....1 kg.

**Procedure.**—Add the required quantity of water to the salt. Dissolve and filter through paper. Dispense, and sterilize by steam at 121°C. for twenty or thirty minutes, depending on the bulk.

It is important that the salt solution used as a diluent for reagents in the complement-fixation test should not be hypotonic. A test for chlorides is made by a chemist of the laboratories for sanitary and analytical chemistry as a check on each lot of this salt solution. If this test shows that the percentage of NaCl is above or below 0.85 per cent, the concentration is corrected by the addition of water or salt. It is unnecessary to compensate for the slight increase in concentration which occurs on sterilization. (Concentration after sterilization is usually about 0.88 per cent.)

*Sodium-citrate solution ($2\ Na_3C_6H_5O_7 \cdot 11\ H_2O$) 17 per cent*

Sodium citrate, C.P.....17 per cent or 170 grams per kg.
Distilled water to make..... 1 kg.

**Procedure.**—Add the required quantity of water to the citrate. Dissolve by heating. Cool about 200 cc. of the material to 20°C. and determine the specific gravity by means of an hydrometer (approximately 1.110 + at 20°C.). If any correction is necessary, have it made by the head of the group. Filter citrate solution through paper. Dispense in 3800-cc. amounts in 4-liter bottles, and sterilize by steam at 121°C. for thirty minutes.

Upon receiving an order from the worker in charge of bleeding horses for antitoxin which is to be concentrated, the sodium-citrate solution is redispensed by means of a dispensing apparatus in 222- and 777-cc. amounts in sterile 4- and 8-liter bottles, respectively. The cotton plugs of these bottles are replaced with rubber stoppers, freshly boiled for 10 minutes in 1-per-cent crude cresol. The stoppers are covered with carbolized cotton and capped with tinfoil and heavy paper. Each 3800-cc. amount of citrate solution is tested in the following manner to make sure that, through some blunder, sodium chloride was not used instead of sodium citrate in preparing the solution: A few drops of the solution are run from the dispensing apparatus into a test tube, and to this are added a few drops of silver nitrate. A white precipitate forms. Concentrated nitric acid is then added to dissolve the precipitate. If the precipitate does not dissolve completely in the nitric acid, the presence of chloride is indicated.

*Sodium-citrate, sodium-chloride solution*

Sodium citrate.....2 per cent or 20 grams per kg.
Sodium chloride.....0.5 per cent or 5 grams per kg.
Distilled water to make.....1 kg.

**Procedure.**—The procedure is the same as for 0.85-per-cent salt solution.

MISCELLANEOUS MATERIAL (UNSTERILIZED)

*Cleaning solution—chromic acid*

Potassium dichromate, saturated solution.....500 cc.
Crude sulfuric acid, concentrated.....800 cc.

**Procedure.**—Place the dichromate solution in a large evaporating dish and set the dish in cold water. Add the acid very slowly, stirring constantly with a

glass rod. (When the acid is added gradually and the mixture is kept cool, a large amount of chromic-acid crystals is formed.)

The solution may be used repeatedly until the crystals have disappeared and the solution assumes a greenish tinge.

*Cobalt-chloride solution (sympathetic ink)*

Cobalt chloride ($COCl_2$) ..... 2 per cent or 2 grams per 100 cc.
Hydrochloric acid, 30 per cent, C.P. 10 per cent or 10 cc. per 100 cc.
Distilled water to make .....100 cc.

**Procedure.**—Dissolve, and test the efficiency of the mixture.

*Cresol-compound solution 2 per cent*

Cresol compound.....2 per cent or 20 cc. per liter
Tap water to make.....1 liter

**Procedure.**—Mix the ingredients thoroughly, and dispense as ordered.

*Crude-cresol solution 1 per cent*

Crude cresylic acid (97–99 per cent straw color).....80 cc.
Warm (40 to 50°C.) tap water to make..... 8 liters

**Procedure.**—Mix thoroughly in an 8-liter bottle, and filter through cotton. (This material is always kept on hand in large quantities for use throughout the laboratory.)

*Dressing wax (for alberene equipment)*

Paraffin.....450 grams
Gasoline .....340 cc.
Kerosene.....340 cc.

**Procedure.**—Melt paraffin in water-bath, cool to about 60°C. and add the kerosene slowly, stirring constantly. Then add the gasoline and mix well. Keep tightly corked and do not bring near a gas flame.

*Phenol solution 5 per cent*

Phenol, loose crystals, C.P..... 5 per cent or 50 grams per kg.
Distilled water.....95 per cent or 950 grams per kg.

**Procedure.**—Add the phenol to the required quantity of water previously heated to approximately 70°C. Dissolve and filter through paper.

Only pure phenol (crystals) is satisfactory for use in biologic products. The phenol is kept in tightly stoppered, amber bottles in the cold room.

*Phenolphthalein solution, alcoholic, 0.5 per cent*

Phenolphthalein..... 1 gram
Alcohol 95 per cent.....105.3 cc.
Distilled water..... 94.7 cc.

**Procedure.**—Dissolve phenolphthalein in alcohol and add water.

MEDIA PREPARED FROM STERILE COMPONENTS (ENRICHMENT MEDIA)

*Alkaline egg medium for Sp. cholerae (49, 50)*

Special 3-per-cent agar base.....87 cc.
Alkaline egg solution.....25 cc.

Preparation of agar base:

| | | |
|---|---|---|
| Distilled water | 1 kg. | |
| Agar | 3 | per cent or 30 grams per kg. |
| Sodium chloride | 0.5 | per cent or 5 grams per kg. |
| Beef extract | 0.3 | per cent or 3 grams per kg. |
| Peptone (Witte) | 1 | per cent or 10 grams per kg. |
| Dextrose | 0.5 | per cent or 5 grams per kg. |

Add the water to the agar, reserving a sufficient volume to dissolve the other ingredients. Dissolve the agar by heating in the autoclave for thirty minutes at 121°C. Add the dissolved peptone, extract, and salt. Make up weight to that of all ingredients. Heat in steam at 100°C., filter. Add dextrose, dispense 90 cc. in 120-cc. bottles, and sterilize by steam at 121°C. for twenty minutes.

Preparation of alkaline egg solution:

One or more eggs.
Distilled water, an equal volume.
7.6-per-cent sodium carbonate, monohydrate ($Na_2CO_3 \cdot H_2O$), a volume equal to the volume of the combined egg and water.

Break the eggs and measure. Place in a flask containing glass beads; add an equal volume of water and mix thoroughly by shaking. Add to the mixture an equal volume of 7.6-per-cent sodium carbonate. Mix well and filter through glass wool. Dispense in convenient quantities (30 cc. in 50-cc. Erlenmeyer flasks or in 180-by-25-millimeter test tubes). Sterilize in steam at 100°C. for thirty minutes. (The excessive alkalinity makes it unnecessary to heat on more than one day to render sterile.)

**Procedure.**—Melt the agar, and add the alkaline egg solution. Mix well and dispense in Petri dishes. Invert the opened plates in the incubator, and dry for several hours. Replace cover, incubate at 37°C. for twenty-four hours.

This is a modification of Krumwiede's medium, lying between it and the Goldberger modification in alkalinity.

*Ascitic-fluid, semisolid agar*

Beef-infusion agar, 0.5 per cent.....1 part or 400 cc.
Ascitic fluid.....1 part or 400 cc.
Mineral oil

**Procedure.**—Warm the ascitic fluid to 50°C. Cool the previously melted agar to 50°C. and add the ascitic fluid by means of a dispensing apparatus. Mix well, insert a dispensing apparatus and dispense. Dispense a layer of mineral oil approximately 6 mm. thick on each tube of medium by means of a third dispensing apparatus. Incubate for forty-eight hours at 37°C. followed by ninety-six hours at room temperature.

*Avery's dextrose-blood broth (51)*

Beef-infusion broth.....100 cc.
Dextrose, 20-per-cent solution..... 5 per cent or 5 cc. per 100 cc.
Rabbit blood, defibrinated..... 5 per cent or 5 cc. per 100 cc.

Procedure.—Add the dextrose solution and blood to sterile beef-infusion broth. Mix well, and dispense in 15-cc. centrifuge tubes, 5 cc. in each tube. Incubate a few tubes at 37°C. for forty-eight hours, and place the rest in the cold room.

Pneumococcus broth or any meat-infusion broth of suitable reaction (pH 7.2 to 7.8) may be used.

*Blood agar (slants and plates)*

Beef-infusion agar, 2 per cent.....900 cc.
Horse or sheep blood, defibrinated..... 50 cc.

Procedure.—Melt the agar and cool to 45°C. (The temperature must not be above this point since a higher temperature will darken the blood.) Add blood, and mix thoroughly. Insert a dispensing apparatus and dispense.

Slants: Dispense medium into sterile tubes of the size desired, and place at once on slant boards to harden.

Plates: Dispense into 150-by-19-millimeter tubes (sterilized in an inverted position in tin cans) and pour into sterile Petri dishes. Raise the cover slightly and hold at such an angle that the plate is exposed to the air as little as possible while the agar is being poured. Replace the cover, and tilt so that the medium covers the bottom of the dish evenly. Allow agar to harden. Place the plates in an inverted position in cans. Let stand for forty-eight hours at room temperature, leaving the covers off the cans. Examine for contaminants. Store in inverted position in uncovered cans in cold room.

*Blood agar, Bordet-Gengou, for B. pertussis (37)*

Bordet-Gengou base.....300 cc.
Defibrinated blood.....200 cc.

Procedure.—The procedure is the same as for routine blood agar.

*Blood agar, coagulated, for B. influenzae (slants and plates)*

Veal-infusion agar, 2.5 per cent with 5 per cent glycerin.....400 cc.
Defibrinated blood..... 5 per cent or 20 cc.

Procedure.—Melt the agar, and add the blood while the agar is at a temperature of 95°C. or above. Mix thoroughly, insert a dispensing apparatus and dispense in tubes or plates. Incubate for twenty-four hours at 37°C.

*Blood agar, coagulated, for B. pertussis (slants and plates)*

Beef-infusion agar, 2.5 per cent with 5 per cent glycerin.....1000 cc.
Defibrinated blood..... 5 per cent or 50 cc.

**Procedure.**—Procedure is the same as for coagulated-blood agar for *B. influenzae* except that the blood is added when the temperature of the agar is approximately 85°C.

*Blood agar, testicular, for the gonococcus (33)*

Testicular-infusion agar.....300 cc.
Defibrinated blood..... 5 per cent or 15 cc.

**Procedure.**—Prepare like routine blood agar, or add the blood to the hot agar, to give a chocolate-colored medium.

*Blood-extract agar for B. influenzae*

Veal-infusion agar, 2.5 per cent with
5 per cent glycerin.....100 cc.
Blood extract..... 10 per cent or 10 cc. per 100 cc.

**Procedure.**—Melt the agar. Add the blood extract and mix thoroughly. Dispense in tubes or plates as ordered. Incubate for twenty-four hours at 37°C.

*Carbohydrate, Andrade, ascitic-fluid beef-infusion agar*

Beef-infusion agar with indicator.....200 cc.
Carbohydrate, 10 per cent solution..... 10 per cent or 20 cc.
Ascitic fluid..... 10 per cent or 20 cc.

**Procedure.**—Melt the agar containing the indicator, and cool to 50°C. Add carbohydrate and serum (both previously warmed to 50°C.). Mix well, and insert a dispensing apparatus. Dispense in sterile tubes, and place in slanting position to solidify. Incubate at 37°C. for forty-eight hours followed by forty-eight hours at room temperature.

*Carbohydrate, Andrade, beef-infusion agar*

This medium is the same as the preceding except that the ascitic fluid is omitted.

*Carbohydrate-serum water (39, 41)*

Serum water with Andrade's indicator.....100 cc.
Carbohydrate, 10 per cent solution..... 10 cc.
or
20 per cent solution..... 5 cc.

**Procedure.**—Add to the serum-water base, containing 1 per cent Andrade's indicator, the correct amount of a sterile solution of the desired carbohydrate. Insert dispensing apparatus, and dispense in sterile 75-by-11-millimeter test tubes, either cotton plugged, or cotton plugged and corked. Incubate at 37°C. for forty-eight hours. Label each tube with the name of the carbohydrate used and the date the medium was combined. Send four tubes of each lot of medium to the antitoxin, serum and vaccine laboratories, to be inoculated by the bacterial-collection group with test organisms before the material is given out for use.

*Carbohydrate-peptone water (39)*

Peptone water with Andrade's indicator.....300 cc.
Carbohydrate, 10 per cent solution..... 30 cc.

**Procedure.**—The procedure is the same as for carbohydrate-serum water.

*Leptospira medium (52)*

Salt solution, 0.9 per cent.....800 cc.
Fresh rabbit serum.....100 cc.
Agar, 2 per cent, pH 7.4.....100 cc.
Rabbit hemoglobin (made by laking 1 part of defibrinated blood with 3 parts of distilled water).....10–20 cc.

**Procedure.**—Mix the agar and salt solution, and sterilize by steam at 121°C. for twenty minutes. Cool to 55°C. and add the serum, and sufficient hemoglobin to color a faint pink. Insert a dispensing apparatus, and dispense in 200-by-14-millimeter tubes, 6 to 7 cc. in each (45 to 50 mm. deep). For mass culture, dispense in 50-cc. Erlenmeyer flasks, 15 to 20 cc. in each. Incubate a few tubes from each lot at 37°C.

*Serum agar with 1 per cent dextrose (for slants)*

Beef-infusion agar, 2 per cent..... 1 liter
Horse serum.....10 per cent or 100 cc. per liter
Dextrose, 20 per cent solution..... 5 per cent or 50 cc. per liter

**Procedure.**—Melt the agar, and cool to 50°C. Add the serum and the dextrose solution, both previously warmed to 50°C. Mix well, and insert a dispensing apparatus, and dispense in tubes. Place at once on slant boards to solidify. Incubate for forty-eight hours at 37°C. followed by twenty-four hours at room temperature.

*Serum agar with 0.2 per cent dextrose (for plates)*

Beef-infusion agar, 1.5 per cent.....1 liter
Horse serum.....5 per cent or 50 cc. per liter
Dextrose, 20-per-cent solution.....1 per cent or 10 cc. per liter

**Procedure.**—The procedure is the same as for serum agar with 1 per cent dextrose. Dispense in Petri plates as blood agar is dispensed.

*Serum semisolid agar (53)*

Beef-infusion agar, 2.25 per cent.....1 part or 150 cc.
Horse serum.....5 parts or 750 cc.

**Procedure.**—Warm the bottle or flask containing the serum to 50°C. Add the serum to the previously melted agar (cooled to 50°C.) by means of a dispensing apparatus. Regulate the amount added by comparison with a control flask containing an amount of water equivalent to the total volume of medium desired (900 cc.). Mix the medium well, insert a dispensing apparatus and dis-

pense. Incubate for forty-eight hours at 37°C. followed by seventy-two hours at room temperature.

*Tellurite, ascitic-fluid beef-infusion broth*

Beef-infusion broth.....300 cc.
Ascitic fluid..... 60 cc.
Potassium tellurite, 1-per-cent solution 1 per cent or 3.6 cc. per 360 cc.
(Gives approximately 1:34,000 to 1:35,000 Te IV.) See note under tellurite solution.

Procedure.—Add the ascitic fluid and the tellurite solution to sterile beef-infusion broth, reaction pH 7.5 (+0.1 to +0.3). Mix well, and dispense as ordered. Incubate for forty-eight hours at 37°C.

*Tellurite, serum agar, with 0.2 per cent dextrose (43, 64)*

Beef-infusion agar, 1.5 per cent.....1 liter
Horse serum.....5 per cent or 50 cc. per liter
Dextrose, 20-per-cent solution.....1 per cent or 10 cc. per liter
Potassium tellurite, 1-per-cent solution....1 per cent or 10 cc. per liter
(Gives approximately 1:34,000 to 1:35,000 Te IV.) See note under tellurite solution.

Procedure.—Melt the agar, and cool to 50°C. Add serum, dextrose, and tellurite. (The procedure is the same as for serum agar with 1 per cent dextrose, except that the medium is not incubated.)

*Tissue, semisolid medium, for treponemata (Smith, Noguchi) (54, 55)*

Beef-infusion agar 2 per cent (pH 7.2–pH 7.4).....100 cc.
Sterility broth.....233 cc.
Ascitic fluid..... 66 cc.
Normal rabbit kidneys
Mineral oil or vaseline

Combine the melted agar and broth in a 500-cc. flask. Check reaction and adjust to from pH 7.2 to 7.4 if necessary. Sterilize by steam at 121°C. for twenty minutes.

Preparation of tissue (kidney fragments): Always choose a rabbit in good condition for this purpose. Kill by bleeding from the heart, in order to exsanguinate as completely as possible. Using aseptic precautions, and working as rapidly as possible, remove the kidneys, placing each in a short, wide-mouth tube (125 by 37 millimeters). Working with the tube held near a flame, and in slanting position, strip the capsule and cut each kidney into fragments the size of a small pea. Place the fragments in 200-by-14-millimeter tubes by means of sterile forceps. Shake fragments to the bottom of the tubes. Prepare the tissue just before dispensing the agar, so that it will not dry.

Procedure.—Add the ascitic fluid to the agar base, which has been cooled to approximately 55°C. Insert a dispensing apparatus and dispense the medium into 200-by-14-millimeter tubes, containing the fragments of kidney. (The column of medium should be from about 75 to 80 mm. high.) Add a layer,

at least 20 mm. deep, of sterile mineral oil, or vaseline. Incubate twenty-four hours at 37°C. and leave several days at room temperature. Keep two or three tubes in the incubator for two weeks as a further check.

*Loeffler's coagulated serum medium (56)*

Horse serum.....3 parts
Beef-extract broth, with dextrose.....1 part

Preparation of beef-extract broth with dextrose:

Distilled water.....1 kg.
Beef extract.....0.3 per cent or 3 grams per kg.
Peptone.....1 per cent or 10 grams per kg.
Sodium chloride.....0.5 per cent or 5 grams per kg.
Dextrose.....1 per cent or 10 grams per kg.

Mix the ingredients, and dissolve by warming over a flame. Do not adjust. Prepare just before filtering medium.

Procedure.—Mix serum and broth cooled to 55°C. and sterilize by filtration through a filter candle (pressure method) into sterile 4-liter bottles. Store the filtered mixture in the cold room until needed.

Prepare the medium for dispensing by siphoning it, with aseptic precautions, into several 2-liter flasks, and fit these with sterile dispensing apparatus. Dispense from 2 to 3 cc. of the medium into 125-by-13-millimeter tubes, being careful to prevent the formation of bubbles. Avoid unnecessary contamination of the tops of tubes, but do not flame.

To slant the medium, tip the tube gently back and forth until the serum-broth mixture runs freely up the side of the tube to the desired point. Avoid wetting the plugs. Place the tubes in layers in copper boxes, slanting the first layers on a narrow strip of wood in the bottom of the box and maintaining the desired inclination of the upper layers by means of narrow strips of paper.

Solidify and sterilize the medium in the autoclave by means of a mixture of steam and air under pressure. (A single period of fifty minutes at 100 to 105°C. is usually sufficient to insure sterility of medium.) Incubate for 24 hours at 37°C. followed by forty-eight hours at room temperature. Send five tubes of each batch to the diagnostic laboratories to be tested for ability to support the growth of freshly isolated strains of *B. diphtheriae*.

Corked tubes of this medium are prepared for distribution in individual outfits. The plugged and corked tubes are sterilized in paper-lined baskets. The medium is dispensed with aseptic precautions directly from the 4-liter bottle. As soon as tubes of solidified medium are removed from the autoclave the corks are pushed firmly into place.

*Petroff's egg medium (without dye) for B. tuberculosis (57)*

Glycerin, beef infusion.....1 part
Fresh-laid eggs.....2 parts

Special apparatus required: The following apparatus should be prepared and sterilized in steam at 121°C. for twenty minutes the day before the meat arrives:

1. Small, covered pail lined with a double thickness of cheesecloth large enough to be used for pressing meat.

2. Large, strong spoon wrapped in paper.

3. Tubing apparatus consisting of glass funnel lined with a double thickness of cheesecloth and covered with a small, pail cover. To the stem of the funnel is attached rubber tubing, glass filling tip and bell similar to those used on a dispensing apparatus for an inverted container. (See: fig. 14.)

4. A 2-liter Erlenmeyer flask containing a layer of glass beads.

Preparation of glycerin solution: The day before the meat arrives, dissolve 75 cc. of glycerin C.P. in 425 cc. of distilled water, making 500 cc. of a 15-per-cent solution. Sterilize by steam at 121°C. for thirty minutes.

Preparation of infusion:

Beef.....500 grams
Glycerin, C.P., 15-per-cent solution.....500 cc.

Whenever convenient it is planned to prepare beef for other media the day it is used for this medium. in order that a piece may be cut from the center of a large chunk. This is done to avoid, in some degree, gross initial contamination from outside sources.

Trim the meat on a piece of board thoroughly scrubbed and rinsed in boiling water, and frequently dip the knife used into boiling water. Also rinse the meat grinder thoroughly with boiling water, and collect the meat into the pail prepared for the purpose. Add the glycerin solution, cover, and infuse in the cold room overnight. Strain the infusion by twisting the cheesecloth, and pressing the bag thus formed against the sides of the pail with the sterile spoon.

Preparation of eggs: Wash thoroughly one dozen eggs in water, then in 5-per-cent phenol solution. Place on filter paper and allow to dry. Weigh the Erlenmeyer flask containing beads. Treat each egg as follows: Pass first one end then the other through the flame. Flame sharp-pointed forceps and chip the large end, removing enough of the shell so that the membrane exposed is about one centimeter in diameter. Take care not to break the membrane. Flame the forceps again and stab the small end of the egg while an assistant removes the plug of the Erlenmeyer flask and flames the mouth. Place the small end of the egg quickly over the mouth of the flask. Blow the contents of the egg into the flask taking care to blow from the cheeks. After the addition of each egg, flame the mouth of the flask, replace the plug and shake the flask well.

**Procedure for Combining the Parts.**—Weigh the flask containing the egg. Subtract original weight of flask from total weight with egg. By means of a sterile pipette, add one part of meat juice to two parts of egg. Flame the mouth of the flask, and pour the medium into the funnel-filter. Fill into 150-by-19-millimeter, 150-by-15-millimeter, or 125-by-13-millimeter tubes as ordered. Allowing for a short butt and a long slanted surface, slant on glass tubing, on a thick pad of cotton in the inspissator. Heat on three successive days as follows: first day, from 80 to 85°C. until all the medium is solidified; second and third days, 75°C. for one hour. Paraffin the plugs. Incubate at 37°C. for forty-eight hours.

This medium has been found very satisfactory for carrying stock cultures of *B. tuberculosis*.

## SECTION IV

## ADMINISTRATION OF THE DEPARTMENT

The variety of the work and the large size of the department, the staff of which consists of about forty people, necessitate organization into four groups (media, sterilization, glassware, and diagnostic outfits) each of which is in charge of an assistant who plans the work and oversees the details. These assistants are supervised by a bacteriologist with training and experience who is at the head of the department and is responsible for its conduct and output.

Contact between this department and the various research and routine laboratories is daily and continuous. A large proportion of the service rendered is in response to written requisitions. No media, whether kept in stock or made to order, are given out without requisitions. Special glassware is handled in the same manner, but the great bulk of routine glassware is distributed without requisitions, and no records are kept of it. Sterilization service is rendered without written requisitions, though the increasing activities of the various laboratories may soon make it advisable, in order to avoid misunderstanding, that the written requisition be adopted for this type of service also. Accurate records are kept in connection with the distribution of diagnostic outfits.

Within the department itself much use is made of bulletin boards in assigning the work. A daily list is posted for the media and sterilization groups; a weekly list for the glassware and the diagnostic-outfits group. A list of the glassware needed by the media group is also posted daily.

The glassware group is the largest in the department. It is divided into two subgroups, one being composed of workers who spend their entire time in the various activities connected with the preparation of glassware, and the other, of workers who spend half their time washing glassware and half in the preparation of diagnostic outfits. The entire personnel of the diagnostic-outfits group, with the exception of the assistant in charge, is composed of workers who spend half their time cleaning glassware. This arrangement permits all the workers to sit at their work part of the time and is proving satisfactory from every point of view.

The media group is entirely separate from the other groups of the

department, but as several of its members formerly worked in the glassware and diagnostic-outfits groups, they are able to help when an emergency in those groups arises. The men of the media group have, likewise, all had training in the operation of the sterilizers.

Copies of all media formulae are kept on file cards which may be removed by the workers and carried about with them while they are preparing the media. A duplicate set is kept, to which the workers do not have access. The guide cards used correspond to the divisions of the formulae into the groups of media mentioned in the preliminary discussion of the formulae.

Individual record cards are filled out by the workers for each medium prepared, and are filed for reference.

There is a separate guide card for each kind of medium. The sequence follows that used for the formulae, and duplicates of the formulae guide cards serve to separate the record cards into divisions corresponding to those of the formulae. A separate index file for the initial and the final reactions of all media is kept on the titration stand for handy reference.

A daily record card is placed on the bulletin board, on which each worker enters the work completed by him that day. This card is checked, before it is filed, against the daily list of work assigned. Several other records which are only of a temporary nature are also kept, such as a list of media in the incubator, a list of media to be tested by other departments and, most extensive of all, a card index of all materials in the media cold rooms.

The sterilization group consists of only two workers. One of them also operates the labeling machine, sometimes for an entire day at a time. When this occurs it is often necessary for one of the media workers to assist with the sterilization. This arrangement is of practical advantage in that it furnishes an opportunity to the media workers to keep in practice in operating the sterilizers. It is an example of the effort that is made to keep some degree of flexibility in a growing organization where the tendency is more and more toward division of labor.

In addition to frequent verbal reports, the bacteriologist in charge of the department receives written monthly reports from the assistants in charge of the different groups. In this way a complete general record is kept of the progress of the department.

# METHODS USED IN THE DIAGNOSTIC LABORATORIES

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# INTRODUCTION

The activities of the diagnostic laboratories include the examination of specimens for evidence of communicable diseases, research concerned with the etiology of these diseases and the improvement of methods for their diagnosis, supervision of approved local laboratories, and the issuing of registration numbers to laboratories handling pathogenic cultures.

In order to handle efficiently the mass of routine examinations, the procedures are limited to essentials, and shortcuts are taken wherever possible, the results being confirmed when necessary for control or for training of the staff. For example, as a routine, the results of the examination of cultures for diphtheria bacilli are based on morphology alone, while virulence tests are made when confirmation is desirable. This procedure furnishes a basis for elementary research and, as members of the staff show evidence of ability, opportunity is given them to undertake special investigations or research, either in the laboratory or in the field.

The supervision, inspection, and standardization of the approved laboratories[1] in the state has offered a basis for the evaluation of the technical procedures, and sound conclusions can be drawn in regard to their efficiency.

In order that the training of the staff may be well rounded, workers are usually promoted from the department for the preparation of media and glassware to the diagnostic laboratories and later to special research groups or other departments, for broader training. The more advanced research is taken up by the heads of groups and by those properly qualified, who are assigned to special groups.

The routine procedures have been arranged in sections which give methods for the examination of the following specimens: sputum for tubercle bacilli and for pneumococcus type differentiation; films for gonococci; cultures for diphtheria bacilli, including the determination of the virulence of microörganisms contained in them; stools, urine, and blood for organisms of the enteric-disease group; blood for the agglutination reaction with organisms of the enteric-disease group; blood and spinal fluid for the complement-fixation test for syphilis

[1] The number of approved laboratories varies; in December, 1926, 106 were approved.

and blood for the complement-fixation test for tuberculosis; tissue for anatomic study and heads of animals for evidence of rabies.

Methods for the examination of specimens from communicable diseases that occur less frequently in this state or from which specimens are infrequently submitted, and also procedures used for the study of exudates, discharges, etc., are grouped as "Miscellaneous Examinations." In this section also are outlined the technic for urine analysis, the cytological examination of blood, hemoglobin determination, and the examination of stomach contents, since examinations of this nature are made in connection with the routine physical examinations of members of the department staff and when certain special investigations are undertaken. Occasionally, they are made also for physicians who cannot arrange to have this type of work done elsewhere.

# SECTION I

## GENERAL REGULATIONS REGARDING SPECIMENS AND SLIDES

## CHAPTER 1

## RECEIVING AND RECORDING SPECIMENS, REPORTING RESULTS OF EXAMINATIONS, AND FILING SLIDES

Specimens for laboratory examinations are brought to the diagnostic department at 8:00 a.m. and at 10:30 a.m. daily except Sundays and holidays. The specimens received at 8:00 a.m. are those which were delivered to the laboratory between that time and 6:00 p.m. of the previous day. The number and kinds of specimens arriving during these hours are recorded by the night watchman. Cultures to be examined for diphtheria bacilli are placed in the incubator at from 35 to 37°C. All other specimens are stored in the cold room.

Specimens received at the laboratory during the day by special delivery or by express are delivered immediately to the diagnostic department.

### OPENING AND RECORDING SPECIMENS

As soon as the specimens are received in the diagnostic department, sort the containers according to the nature of the specimens submitted and record the number of each in a notebook kept for the purpose. Then distribute the specimens to the workers who make the various examinations. Before distributing the specimens received at 8:00 a.m., compare the number with the record made by the night watchman. Whenever a specimen is accompanied by a request for an unusual examination, refer the matter to the bacteriologist in charge of this work.

Open and number the specimens one at a time, in order to avoid the possibility of interchanging them. The first of each year, begin the laboratory number for each type of examination with "1" and number the specimens in serial order as they are opened. To avoid duplication or omission of numbers, always enter the serial number in a notebook or accession book before stamping it on the history blank, on the label on the specimen tube or jar, and on the mailing case. Each

morning put the new date in the notebook or accession book and follow it by the serial number, as

Dec. 14, 1925
2410
2411
2412

Stamp on the history blank the date the specimen was received. When a stamped number is indistinct, copy it in ink, just above. Never use a wax pencil to number specimens. Never change or obliterate a number. If an error occurs, refer it to the bacteriologist in charge, who will draw one line through the incorrect number, renumber it in ink, and initial the slip.

If a container is received without a specimen, make a note of this fact on the history blank, number it as usual, and send an explanatory letter to the physician.

When a letter is received which gives information additional to that on the history blank, fasten the two together and stamp both with the serial number. Have this additional information copied on the history blank by a clerk.

Compare the patient's name on the specimen label and on the history blank, and note on the history blank any discrepancy. If the patient's name does not appear on the specimen tube or jar, copy on the label the name given on the history blank, and stamp "No name on specimen," on the left margin of the history blank.

Before discarding the mailing case, make sure that the physician's name and address are on the history blank. If they are not given, copy the postmark or return address. If the patient's name and address appear only on the container, copy them on the left margin of the blank.

Whenever any information is copied on the original history blank, label it "copy" and give the source with the signature of the person responsible for it.

Read the history blank carefully so that none of the requested examinations will be omitted. If a specimen is to be examined in more than one group, cross-index it as follows: Make out a duplicate history blank for each type of examination to be made and give each a number in the appropriate series. For cross reference, indicate on the lower margin of each blank the other examinations that are being made and the numbers given the specimen in the other series.

If no history blank accompanies the specimen, or if the nature of the examination desired is not clearly indicated, consult the bac-

teriologist in charge, who will request the necessary information from the physician.

Since there is no provision for members of the staff to act as expert witnesses, refer all specimens accompanied by information indicating that the results of the examinations may be used in court action, to the head of the group who will attempt to arrange for such examinations in a laboratory doing this type of work.

Whenever an unsatisfactory specimen is received and the accompanying history indicates that the case is urgent, advise the physician by telephone or telegraph.

If there is evidence that a specimen has leaked, leave the jar or tube in the container and paste a label on this for the laboratory number. If the history blank is contaminated, place it in disinfectant and copy the information on a clean one. Make a note that the original blank was too badly soiled or contaminated for filing. Disinfect the hands at once after handling a contaminated history blank.

After all of the specimens have been opened, determine whether the number of specimens to which serial numbers have been assigned corresponds to the number of specimens received.

## DIRECTIONS FOR FORWARDING SPECIMENS NOT ROUTINELY EXAMINED IN THIS LABORATORY

Forward specimens accompanied by the information blanks sent out by the State Institute for the Study of Malignant Diseases, to that Institution. Notify the physician who sent the specimen that this has been done.

Forward specimens of animal tissue to the State Department of Farms and Markets, Bureau of Animal Industry, 122 State Street, Albany, New York. Notify the department by telephone that the specimen is being sent, and also the person submitting the specimen that it has been forwarded.

Forward insects to the Associate State Entomologist, Education Building, Albany, New York, and notify him of this fact by telephone.

## EXAMINATION OF SPECIMENS

Never discard a specimen until the examination has been completed, and the report submitted.

Record the steps of each procedure of an examination directly on the history blank or on the special record slip which is attached to the history blank.

Never make the first record elsewhere and later transfer it to the

record slip. When additional space on the back of the history blank is needed for recording the results of the examinations, stamp the serial number on a blank, pink slip and attach it to the original record by means of metal staples.

If the nature of the material submitted is not indicated on the history blank, add the information, if possible, after inspection of the specimen.

**Labeling of Slides for Stained Preparations.**—Write on the end of the slide, the serial number, preceded by a letter when necessary to designate the type of examination which is being made. Under this, insert the year and the number of the set to which the preparation belongs. For example, if a specimen to be examined for gonococci is received in 1926 and is given the serial number 1001 and if duplicate examinations are made, the slide belonging to the first set would be labeled as in figure 15.

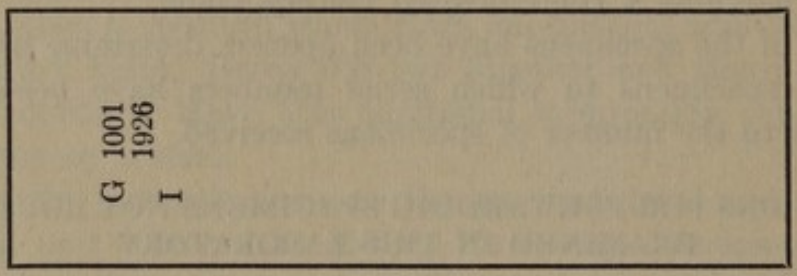



FIG. 15. SLIDE LABELED FOR STAINED PREPARATIONS

## REPORTING RESULTS OF EXAMINATIONS

When the examination of a specimen is finished, stamp the date it is completed and write the result in the space reserved for that purpose on the history blank. Enter the number of examinations made over the word "Examined," and sign in the space reserved for "Examiner."

The worker making each part of the examination signs in the place indicated but the "examiner" is responsible for the result reported.

In reporting the result of an examination, state whether pathogenic microörganisms, other parasites, or evidences of their presence have been found; that certain reactions have been obtained; or that the specimen was unsatisfactory for examination. Never state that the result was positive or negative.

For convenience, abbreviations may be used by the workers in entering the results of examinations on the history blanks. For example, "Tb" or "No Tb" may be written in the space reserved for the result of the examination instead of a statement that "Tubercle bacilli were found" or that "No tubercle bacilli were found." Any deviation from this usual routine is given in the description of the examination to which it applies.

Whenever it is necessary to telephone a physician relative to the tests that are being made, record the message on the history blank with the date, the time of day, and the signature of the person who gave it.

Except in an emergency, reports of examinations are telephoned only by persons especially authorized.

## THE FILING OF SLIDES

### PREPARATION OF SLIDES FOR FILING

Remove cedar oil from the slides by passing them successively through three Coplin jars containing xylol. Place one slide in each of the five grooves of the first jar; after several minutes transfer to the second and third in turn. Finally, drain, or dry them between clean blotting paper, using gentle pressure so that none of the material is removed from the slide.

The slides should then be clean and ready to be filed. If they remain sticky after this procedure, it is due to the condition of the xylol.

If the slides are sticky, discard the xylol used in jar I, mark jars II and III, I and II respectively, put fresh xylol in a clean jar and number it III. Then clean the slides a second time.

### FILING OF SLIDES

#### *General File*

File together in serial order the slides for one type of examination.

The slides are filed in a standard cabinet, each section of which is composed of twenty-two drawers (1$\frac{7}{8}$ by 3$\frac{1}{4}$ by 15$\frac{1}{4}$ inches).

To differentiate each type of examination, mark the drawers with labels of different colors on which are written the year, month, and the serial number of the slides the drawers contain. When slides for more than one month are filed in one compartment, make a note of this on the colored label and insert a guide card in the proper place in the drawer.

Preserve all the slides in this file for one year. The first of each month, remove all that are more than one year old and send them to the glassware department to be cleaned.

As one person is responsible for the filing of slides, never remove a slide from, or return it to, the files without his knowledge. Whenever a slide is removed, replace it by a substitution card on which write the

number of the slide, the date removed, and the name of the person to whom it is lent. Whenever a slide is broken, attempt to fit the pieces together with balsam and attach to another slide, then place the slide in an envelope, and write the serial number on the outside. File the envelope in the usual manner. Whenever a slide is lost, notify the person in charge of the group who will make a complete investigation.

*Special Files*

**For Specimens of Pathologic Tissue.**—File slides for anatomic examination in separate filing cases for permanent record.

**For Interesting Specimens.**—Keep indefinitely all slides made in the examination of specimens which are mentioned in the monthly reports as being of unusual interest. Remove them from the general file and place them in a section labeled "Permanent file for interesting specimens," substitution cards being used in the general file to account for their absence. Keep a record of these slides, noting the serial number, material and source of each, on 4-by-6-inch index cards.

## SECTION II

## EXAMINATION OF ROUTINE SPECIMENS

### CHAPTER I

### THE EXAMINATION OF SPUTUM FOR TUBERCLE BACILLI

The history blanks accompanying these specimens indicate that the sputum is to be examined for tubercle bacilli only.

The specimens which arrive during the day are counted and stored in the cold room until the following morning. Then they are opened with the specimens that have arrived during the night. Those which arrive on Sundays and holidays are treated in the same way.

#### OPENING AND RECORDING SPECIMENS

Open and record the specimens (See: specimens for examination, opening and recording), observing the amount and condition of the sputum in each jar. Whenever a small amount of sputum, less than 3 cc., is received, write on the lower margin of the history blank "Insufficient material for sterilization." When the amount of material received is insufficient for two stained preparations, write on the lower margin of the history blank "Insufficient material sent," and report immediately to the bacteriologist in charge. Whenever sputum of a bloody or serous character is received, make a note of this fact also.

#### STERILIZATION OF SPECIMENS

Do not sterilize specimens under the following circumstances:

1. When the physician asks for an early report or that the report be telephoned or telegraphed.
2. When very little material is sent.
3. When a request for pneumococcus typing or for a cultural study is made.
4. When sputum is of a bloody or serous character.

Sterilize all other specimens of sputum in the inspissator. Before putting the specimen jars in the inspissator, place them in wire baskets with a weight on the corks to prevent their being blown out. (Flat pieces of iron about $\frac{5}{8}$ of an inch thick may be used for the purpose.) Place the history blanks in the inspissator at the same time so that

they will be sterilized before they are handled by the clerk. Heat for two hours after the water in the outside chamber has begun to boil.

The thermometer in the inside chamber then registers approximately 90°C.

## PREPARATION OF SPECIMENS FOR STAINING

When the specimens are cool, make two complete sets of stained preparations. Use new slides or the unused sides of slides that have been used once before, and write on them the serial number, the year, and the number of the series. When the ends are not etched, draw lines through old serial numbers and dates.

The specimens that are opened and sterilized on Saturday are left in the inspissator after heating until Monday morning when they are prepared as described above.

Remove the cork from the specimen jar[1] with an awl, which has been heated in the flame, and place it, contaminated side up, on cotton soaked in disinfectant. Then, with a straw about 4 inches long, of the type designed for use at soda fountains, take up a small amount of the sediment by plunging the straw to the bottom of the jar, with the finger over the upper end of the straw, removing it for an instant, replacing it, and then transferring the material to the slide. As each preparation is completed, discard the straw into a dish of disinfectant. Place the preparations, while still moist, on an electric plate kept at approximately 90°C. When they are almost dry, use the side of a fresh straw to spread the sediment smoothly over the slide, taking care to leave a space of at least an inch at the numbered end. Fix by replacing the slides on the electric plate until dry. Replace the cork, and flame the awl before opening another jar.

To make films from specimens which have not been inspissated, select yellowish, cheesy particles, when present, and spread the material smoothly on the slide with a wire loop.

Since some of the specimens cannot be inspissated, when all the preparations have been made, wash the diamond-point pencil, the awl, and the working space on the table with a disinfectant.

## STAINING SPECIMENS

As soon as the preparations are cool, stain them as follows, completing the first series before starting the second:

Place the slides, film side up, on the electric plate, kept at approxi-

[1] A new type of sputum container with a screw cap is being adopted, which is easier to open.

mately 90°C. Cover them with carbolfuchsin and allow them to remain for five minutes after the stain has begun to steam. Do not allow the slides to dry during this process, but add fresh stain from time to time as it is needed. When they have steamed for five minutes, discard the excess stain, decolorize them with acid alcohol, and counterstain with aqueous methylene blue.

For a control, stain with each set a preparation from a specimen known to contain tubercle bacilli. Mark this slide with its serial number and the word "Control" and write across the same end the date and the number of the series (I or II) with which it was stained.

## EXAMINATION OF STAINED PREPARATIONS

Different workers examine the two series, each writing, under his signature on the history blank, the number of the series he has examined.

**Procedure.**—Whenever acid-fast bacilli are found on only one slide, or when less than eight are found on both, examine duplicate preparations.

It is sometimes advisable to make the duplicate preparation from the sediment after centrifugalizing the specimen. It may be desirable also to centrifugalize specimens when no tubercle bacilli are found in the first preparations and the history blank indicates the desirability of special examination, as when the results of the tests do not agree with the clinical evidence.

When tubercle bacilli cannot be found in very thin preparations, make duplicate preparations for confirmatory examination. Do not report that no tubercle bacilli are found until the entire surface of each film has been examined under the microscope. Spend a period of at least five minutes in this search.

## REPORTING RESULTS

Record the results of the examinations in the place reserved for that purpose on the history blank as follows:

### *Routine Reports*

Report: "Tb," when several tubercle bacilli are found on both slides.

Report: "No Tb," when no tubercle bacilli are found.

When the results of the examinations are unsatisfactory, indicate the appropriate statement on the slip designed for these reports.

*Special Reports*

When no tubercle bacilli are found in a specimen that is bloody or serous, report the result "Unsatisfactory." Send a letter to the physician stating the facts and requesting that further specimens from the patient be submitted.

When so few acid-fast bacilli are found that a definite report cannot be made, send a special letter to the physician stating the facts and requesting that further specimens from the patient be submitted.

## CHAPTER 2

## DIFFERENTIATION OF PNEUMOCOCCUS TYPES

Pneumococcus type differentiation may be attempted with the following specimens: sputum from the deeper air passages; secretions collected on swabs, when sputum is not obtainable, as is frequently the case with children; urine; blood submitted in a Keidel tube; spinal fluid; pleural and other exudates.

It is especially important that all material should be examined as soon after collection as possible. Members of the staff assigned to the work make the examination promptly after the receipt of the specimen.

The specimen is given a serial number and the time of receipt and the other data are recorded in the usual manner.

### SPUTUM

If sufficient material is received, give a portion of the specimen to the bacteriologist in charge of the routine examination of sputum for tubercle bacilli so that it will be cross-indexed and examined by that group also.

#### *Microscopic Examination*

Make Gram and capsule stains of the sputum, marking the slides in the usual way. If the specimen appears to be saliva or post-nasal discharge, and the stained preparations show many contaminating organisms with few or no Gram-positive cocci in pairs, it is advisable to obtain another specimen immediately. If this cannot be done, begin the tests at once, since the organism may eventually be isolated.

### METHODS AVAILABLE FOR TYPE DIFFERENTIATION

The Krumwiede method should be used for the examination of all specimens which are albuminous and of sufficient amount. By this method, the result, if definite, may be reported within an hour from the time the specimen is received. If the specimen is not satisfactory for this test, or if the results show no reaction or one of doubtful significance, the mouse method may be employed and, if the case is urgent, the Avery cultural method as well. The former is preferred, but Avery's method, if the specimen is satisfactory, will give more prompt and equally reliable results.

### *Krumwiede's Method (58)*

Transfer from 3 to 10 cc. of sputum, depending on the amount available, to a 7-by-1-inch Pyrex test tube and immerse it in boiling water for several minutes or until a rather firm coagulum results. Break this up with a glass rod or pipette and add sufficient 0.85-percent salt solution to make about 1 cc. of fluid. Immerse the tube in boiling water again, for a few minutes, shaking it several times during the heating. Then centrifugalize the extract at high speed for from five to fifteen minutes and layer 0.3 cc. of the clear supernatant fluid on an equal amount of each of the three undiluted type antipneumococcus sera. To do this, tilt the agglutination tubes containing the sera, and allow the extract to flow slowly down the sides.

If the specimen is satisfactory for the test and contains type-I, -II, or -III pneumococci, a ring of precipitate will usually be formed at the surface of contact of the two fluids in the tube containing the homologous serum.

If the reaction does not occur immediately, plug the tubes loosely, without shaking, and incubate them at from 50 to 55°C. Observe after five minutes', and again after fifteen minutes' incubation. Then shake the tubes gently and reincubate for not more than one hour.

After shaking, a definite clouding throughout, or a precipitation, will usually appear in the homologous serum. If definite precipitation is obtained by Krumwiede's method in any one of the type sera, it is not necessary to confirm the reaction by animal inoculation or cultural tests.

### *Mouse Method (59)*

**Preparation of the Specimen.**—If the sputum is very fluid and has no firm portions, inoculate the mouse directly. Otherwise, take up a firm portion, consisting of not more than 0.5 cc., in a sterile glass syringe without a needle and wash it three times in sterile salt solution. Grind the washed sputum in a sterile mortar, adding from 1.5 to 2.5 cc. of broth during the process. Before inoculating the mouse with this dilution, inoculate a blood-agar plate, which may be fished later, if necessary, see figures 16 and 17.

**Inoculation of a Mouse.**—Inoculate a mouse intraperitoneally with from 0.5 cc. to 1 cc. of the diluted, washed sputum. Mark the animal with picric acid, record the area which has been colored, put the mouse into a clean, pint fruit jar, one-fourth full of shavings, and cover the jar with wire netting. Furnish the animal with food and water, and attach a tag on which are written the serial and department numbers.

**Exploratory Puncture.**—Within from four to eight hours, puncture the peritoneum of the mouse with a sterile needle attached to a syringe

and withdraw one or two drops of exudate. Spread this on two slides, marked "E" to indicate exploratory puncture, and make Gram and capsule stains. If microscopic examination shows as many Gram-positive cocci as may be found in an 18-hour broth culture chloroform the mouse and proceed with the test.

If only a few organisms are found, leave the mouse until the following morning and if it is not dead then, make a second exploratory punc-

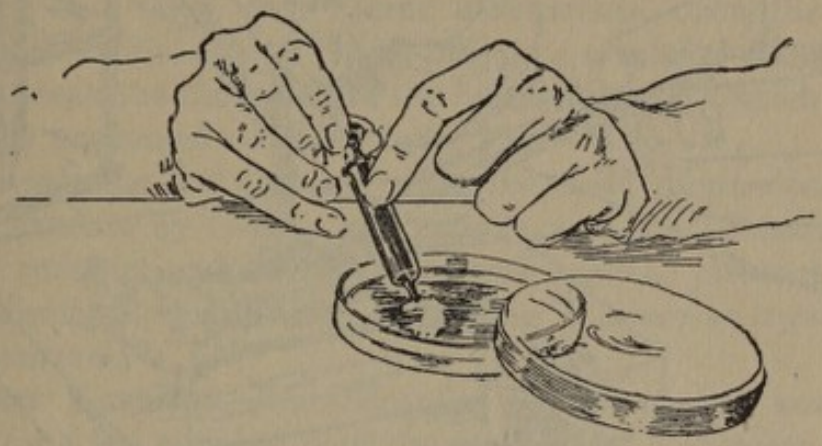


FIG. 16. WASHING SPUTUM IN SALT SOLUTION

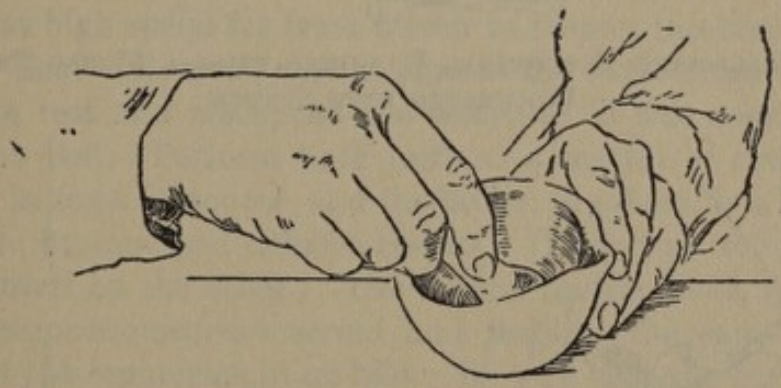


FIG. 17. GRINDING AND EMULSIFYING SPUTUM IN BROTH BEFORE INOCULATION IN THE PERITONEUM OF A MOUSE

ture. If the stained preparations made at this time do not show sufficient growth, leave the mouse, and report on the results of Avery's cultural method, or on the growth on the plates inoculated with the sputum.

**Autopsy.**—Since it is desired to recover the organisms in pure culture, carefully observe sterile precautions throughout the autopsy.

When the skin has been laid back, make a short, longitudinal opening in the abdominal wall. Take a loopful of the peritoneal exudate and streak half a blood-agar plate. Also make two dried preparations

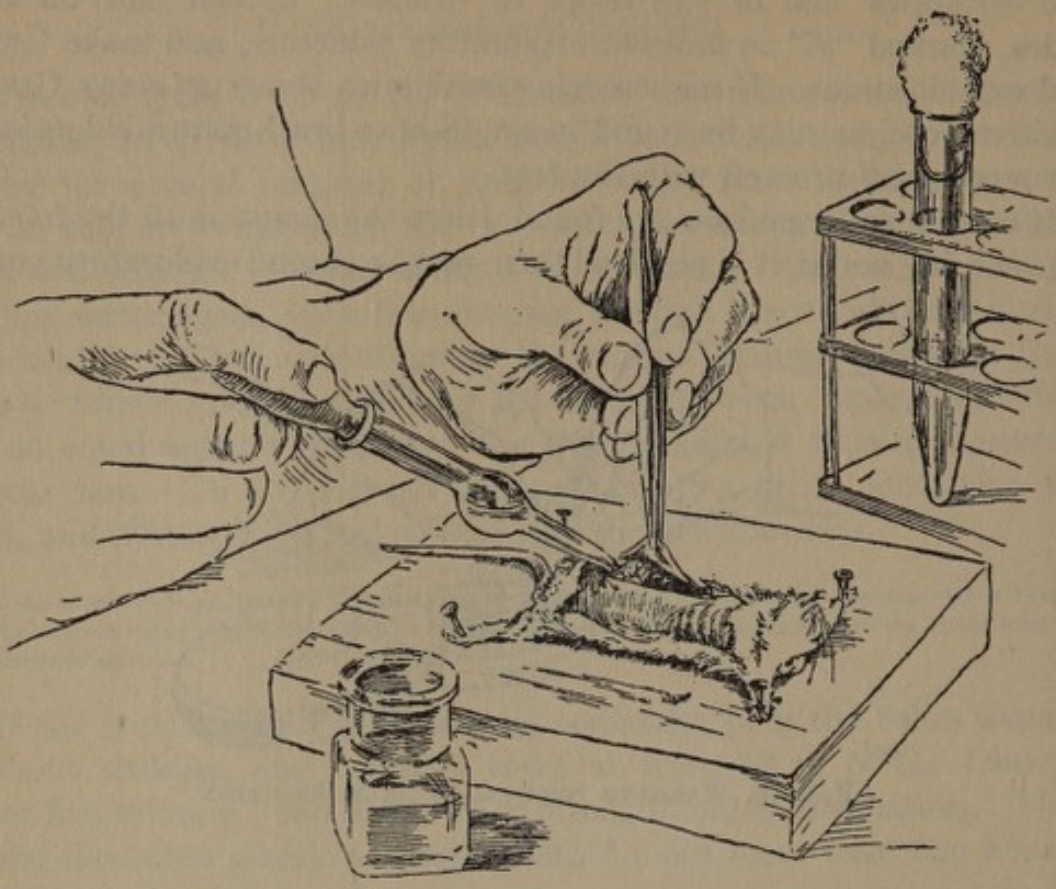


FIG. 18. COLLECTING PERITONEAL WASHINGS FROM A MOUSE PREVIOUSLY INOCULATED WITH SPUTUM

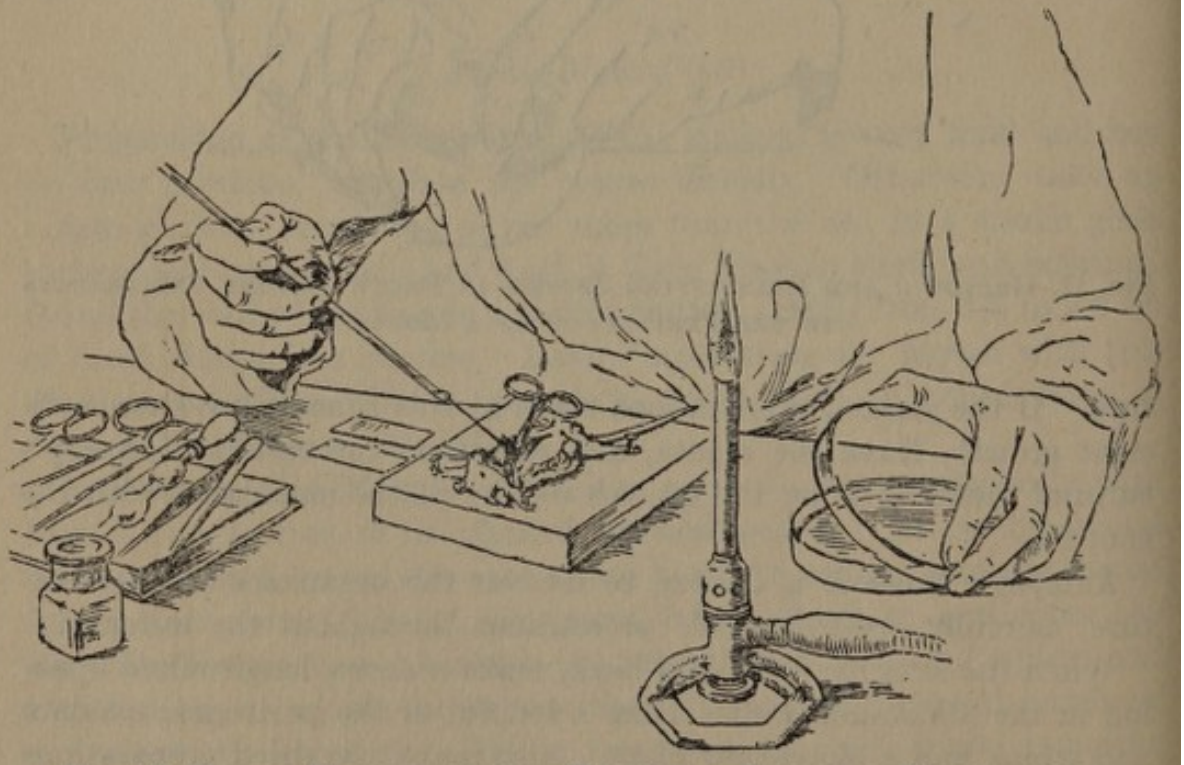


FIG. 19. TAKING BLOOD FROM THE HEART FOR CULTURAL EXAMINATION

for Gram and capsule stains, if they have not been made immediately before from an exploratory puncture. Mark these slides "P," for peritoneal exudate. Then enlarge the opening in the abdominal wall and note if the exudate is sticky, suggesting the presence of type-III pneumococci or *B. mucosus capsulatus* (Friedlander's bacillus). Using a bulb pipette, wash the peritoneum thoroughly with from 3 to 4 cc. of 0.85-per-cent salt solution, and put the washings in a centrifuge tube, figure 18. Then, with sterile instruments, open the thoracic cavity, and, from the heart's blood, inoculate a tube of pneumococcus broth and streak the other half of the blood-agar plate which has been used for the peritoneal exudate, figure 19.

After completing the autopsy, stain the dried preparations of the peritoneal exudate by Gram's and capsule methods. Examine them, noting the presence and size of the capsule and the presence of other microörganisms, especially those suggesting *B. mucosus capsulatus*, or *B. typhi murium*.

**Peritoneal Washings.**—Centrifugalize the peritoneal washings at low speed for a few minutes, pour the supernatant suspension of organisms into a second centrifuge tube, and discard the sediment, which contains cellular debris from the peritoneum. Centrifugalize the suspension at high speed for from fifteen to twenty minutes or until it is perfectly clear. Remove with a pipette the supernatant fluid for a precipitation test and resuspend the sediment in salt solution for an agglutination test. Perform both tests as a matter of routine, since the former, in some instances, and the latter, in others, has been found to give more prompt and definite results. (Each test acts, also, as a valuable control on the other.) Use for the routine tests, types-I, -II, and -III antipneumococcus serum and test, at the same time, the solubility of the organisms in ox bile. Be sure that the serum is clear each time before it is used, since the presence of a precipitate would obscure the reaction. If there is a precipitate present, centrifugalize the serum, under aseptic precautions, or filter it through sterile filter paper in a sterile funnel, as the serum, which contains no preservative, easily becomes contaminated. Also be sure that the salt solution used in making the suspensions and serum dilutions is free from sediment.

**Precipitation Test.**— Using the three types of serum undiluted and diluted, as determined by standardization for the agglutination test, set up the test as follows:

Tube 1—0.3 cc., serum, type I (undil.) + 0.3 cc., supernatant peritoneal washings

Tube 2—0.3 cc., serum, type I (dil.) + 0.3 cc., supernatant peritoneal washings
Tube 3—0.3 cc., serum, type II (undil.) + 0.3 cc., supernatant peritoneal washings
Tube 4—0.3 cc., serum, type II (dil.) + 0.3 cc., supernatant peritoneal washings
Tube 5—0.3 cc., serum, type III (undil.) + 0.3 cc., supernatant peritoneal washings
Tube 6—0.3 cc., serum, type III (dil.) + 0.3 cc., supernatant peritoneal washings

Add the supernatant fluid slowly to each tube, as described in the Krumwiede method, and look for the formation of a ring. Observe again at the end of fifteen minutes' heating in a water-bath at 37°C. Then shake the tubes gently and return them to the water-bath.

**Agglutination Test.**—Add slowly to the pneumococcus sediment in the centrifuge tube enough sterile 0.85-per-cent salt solution to make a suspension corresponding in density to an 18-hour broth culture. Set up the test as described above, using this suspension in place of the supernatant fluid and adding a seventh tube containing 0.1 cc. of sterile, ox bile and 0.4 cc. of suspension, and thoroughly shake the racks containing the tubes.

As a control, whenever agglutination or precipitation tests are made, test each type serum with the homologous antigen, using 0.3 cc. of each.

*Preparation of the Antigen for Precipitation Test.*—Transfer about 0.5 cc. of stock cultures of types-I, -II, and -III pneumococci from solid medium to tubes of pneumococcus broth, and incubate at from 35 to 37°C. for from eighteen to twenty-four hours. The following day, transfer the whole broth culture to a flask containing 200 cc. of pneumococcus broth, and incubate from forty-eight to seventy-two hours, until a very heavy culture is obtained. To this flask add from 2 to 3 grams of egg albumen, previously dissolved in a small amount of water. Heat in a water-bath until a heavy coagulum is formed. Break up the coagulum with a pipette, and transfer the suspension to centrifuge tubes. Centrifugalize at high speed for from ten to fifteen minutes to throw down the coagulum. Remove 0.9 cc. of supernatant fluid and layer 0.3 cc. over each of the three type sera. If a ring is immediately formed in the homologous serum, and no rings in the other two type sera, indicating that the antigen is satisfactory, pipette it into 20-cc. bottles. Add, as a preservative, enough of a 5-per-cent aqueous phenol solution to make a final dilution of from 0.3 to 0.5 per cent of phenol.

**Interpretation of Reaction.**—Record the readings for both precipitation and agglutination tests after fifteen minutes', thirty minutes',

and one hour's incubation, using the following symbols. If the reaction is not definite, record the results again after two hours' incubation.

4+ = complete flocculation; supernatant fluid clear; all large clumps
3+ = supernatant fluid clear or nearly clear; very definite clumping
2+ = supernatant fluid not clear; definite clumping
\+ = supernatant fluid not clear; very small clumps definitely visible to the unaided eye
± = questionable reaction
− = supernatant fluid uniformly turbid, no clumping

If precipitation and agglutination reactions are obtained in both the undiluted and diluted pneumococcus sera of types I, II, or III, record the result as pneumococcus, type I, II, or III.

After incubation of the precipitation test, a fibrin web may appear in all the tubes, and care must be taken not to confuse this with a specific reaction. Some cross agglutination may occur in the undiluted sera, but should not occur in the dilutions, since these were chosen because of their specificity.

If agglutination is obtained in the undiluted type-II serum only, report the reaction as atypical type II.

With such an atypical strain, agglutination may not occur until after the 30-minute reading, and the precipitation reaction with type-II serum may fail entirely to occur.

If no specific precipitation or agglutination reaction occurs, but the bile clears, make a confirmatory test. Then, if the tests agree, report the result, pneumococcus, type IV.

Whenever agglutination is obtained in the undiluted type-I or undiluted type-III serum only, make control tests to determine whether satisfactory serum and serum dilutions were used. If this reaction is indicated with type-I serum at the end of thirty minutes' incubation, repeat the test immediately, using a fresh dilution of the serum. Use, in these confirmatory tests, a homologous, standard, pneumococcus-broth culture as well as the culture under investigation. If the standard culture does not agglutinate in both the diluted and the undiluted serum, repeat the test with serum from another horse. If advisable, pass the culture through another mouse to increase the agglutinability. If, after all these tests, the culture still agglutinates in the undiluted serum only, record the results pneumococcus, type IV. Refer all irregular reactions to the bacteriologist in charge of the group, who may suggest further control tests.

Whenever there is an indication of the presence of more than one type of pneumococcus, plate the culture and make an agglutination test with each subculture isolated, in an effort to isolate the types.

Contaminating organisms, especially streptococci, may cause irregular agglutination reactions, but they are not dissolved by the bile. Such cultures should be plated and the pneumococci, if present, obtained in pure culture.

**Confirmatory Tests.**—If the results of the tests of the peritoneal washings are not definite, they should be confirmed on the next day by the following tests with the broth culture from the heart's blood of the mouse.

*Microscopic Examination.*—To determine the purity of the culture, make a dried preparation and stain it by Gram's method.

*Bile-Solubility Test.*—Add 0.6 cc. of the broth culture to 0.1 cc. of sterile, ox bile.

*Agglutination Test.*—Perform an agglutination test. If the broth cultures are not pure, treat them as described under Avery's cultural test and make a precipitation test.

*Fermentation Reaction.*—If an additional differential test is desired, inoculate a tube of Andrade, inulin, serum-water medium.

Pneumococci ferment inulin and produce coagulation. Streptococci do not ferment this carbohydrate.

If the heart's-blood culture is not pure, test the cultures isolated from the sputum plates, as described above. Also fish typical pneumococcus colonies from the plate inoculated at autopsy for further study, if necessary.

### *Avery's Cultural Method (60)*

Inoculate approximately 5 cc. of Avery's medium with 0.2 cc. of diluted, washed sputum, washed and diluted as described under the mouse method, or, if the specimen is fluid, with an equal amount of undiluted sputum. Mix well and incubate at from 35 to 37°C. for from five to seven hours. After incubation, make a slide preparation and stain it by Gram's method. If a growth of Gram-positive diplococci, comparable to an 18-hour broth culture is shown, centrifugalize the Avery culture for a few minutes to throw down the blood cells, inoculate a blood-agar plate from the supernatant fluid, and then proceed with the test as follows:

Transfer to a sterile, centrifuge tube about 3 cc. of the supernatant growth, avoiding the blood cells which have settled to the bottom, add 1 cc. of sterile ox bile and incubate the mixture at from 35 to 37°C. for thirty minutes to dissolve the pneumococci. Then centrifugalize the solution at high speed for twenty minutes to throw down cells and contaminating organisms and, with the clear, supernatant, bile solution, make a precipitation test as previously described.

If a definite reaction is not obtained by Avery's method, fish typical colonies from the plates previously inoculated with the sputum or Avery's culture, to pneumococcus broth, and identify the cultures as previously described.

## SECRETIONS COLLECTED ON SWABS

Inoculate a blood-agar plate and make two dried preparations, moistening the swab if the material on it is very dry. Stain one by Gram's method and one by Hiss' capsule method. If the swab is moist, emulsify the material in Avery's medium, and then store the swab in the cold room until a report is made. If the specimen is not fresh, immerse the swab entirely in Avery's medium. If, after incubation, good growth of pneumococci is obtained, follow Avery's cultural method. If the culture is grossly contaminated, inoculate a mouse with from 0.5 cc. to 1 cc. of the Avery culture before the bile has been added and carry out the technic previously described.

When satisfactory results cannot be obtained by either of these methods, fish the original plate inoculated from the swab.

## URINE (61)

In some instances when an immediate diagnosis, or confirmation of a previous diagnosis, is desired, a specimen of urine may be tested for specific precipitation reactions. The unconcentrated urine may be tested first and the concentrated later, if necessary.

**Unconcentrated.**—Centrifugalize the fresh specimen at high speed and with the clear, supernatant fluid, perform a precipitation test as described under the mouse method. Incubate the test at from 35 to 37°C.

The reaction may occur immediately or in an hour, but bacterial growth is likely to obscure later readings.

**Concentrated.**—Add two or three drops of acetic acid to about 25 cc. of urine, preferably from a 24-hour specimen, and concentrate by boiling over a free flame to approximately 5 cc. Then filter through paper and add to the filtrate from eight to ten volumes of 95-per-cent alcohol. Collect the precipitate by centrifugalization and dry it rapidly by attaching the container to a vacuum pump, if available, to remove the excess alcohol. Then extract the residue with 2 or 3 cc. of 0.85-per-cent salt solution to redissolve the specific substance. Centrifugalize the extract and with the clear, supernatant fluid perform a precipitation test as previously described. Incubate in a water-bath at 37°C. for two hours.

## BLOOD

Centrifugalize 10 cc. of a blood culture, containing a pure growth of Gram-positive cocci, at low speed to throw down the blood cells.

Make an agglutination test as described under the mouse method, using the supernatant broth culture.

## SPINAL FLUID

Perform a precipitation test on the supernatant fluid after centrifugalization. If large numbers of Gram-positive cocci in pairs are present, suspend the sediment in broth and inoculate both a mouse and a tube of Avery's medium. If there are sufficient cocci present, make a bile-solubility test and an agglutination test with at least the type-I serum. Always confirm such tests, however, by a study of the organism in pure culture. If a stained preparation shows no diplococci, make a routine cultural examination only.

## PLEURAL AND OTHER EXUDATES

If a special request for pneumococcus type differentiation accompanies a specimen of pleural exudate, centrifugalize the fluid at high speed for thirty minutes. Make slide preparations of the sediment and stain by Gram's method and Hiss' capsule method. If Gram-positive cocci in pairs are found, follow Avery's method of determining their type. If possible, perform a precipitation test with the supernatant fluid. Always inoculate blood plates with the sediment, for a general cultural examination.

## SPECIAL INFORMATION

### *Protection Test*

A protection test with mice may be made whenever confirmation of agglutination and precipitation reactions is desired. In routine diagnostic work, however, these tests are seldom required. All such tests should be referred to the bacteriologist in charge of the antitoxin, serum, and vaccine laboratories.

### *Standardization of Immune Serum*

In order to avoid cross agglutination and also prezone reactions, it is practically always necessary to use diluted, as well as undiluted, sera in the tests. A dilution of the type-II serum, especially, should never be omitted as it is necessary for the differentiation of atypical type-II strains.

**Procedure.**—Do not use serum dilutions that have been kept for more than forty-eight hours.

The serum is standardized by the pneumococcus-immunization group and the agglutinating titer indicated on the label of the bottle. Every bottle obtained for diagnostic tests, however, is restandardized according to the following procedure.

Test the undiluted serum and two or three dilutions of it with broth cultures of the standard pneumococcus, types I, II, and III.

Those strains, originally obtained from the bacterial collection, may be preserved in semisolid medium in the cold room and transferred as often as needed for standardization tests.

Record the results of the test on a special card, see figure 20.

PNEUMOCOCCUS SERUM STANDARDIZATION

Pneumococcus serum type..... Horse No. ..... Date tested.....

| CULTURE | | TYPE I | TYPE II | TYPE III |
|---|---|---|---|---|
| Serum dilutions | | | | |
| Period of incubation | 15 min. | | | |
| | 30 min. | | | |
| | 2 hrs. | | | |

Dilution to be used in test:.....

Examiner:.....

FIG. 20. RECORD CARD

## REPORTING RESULTS

Report: "The presence of pneumococcus, type I, II, or III is indicated," when a definite reaction is obtained by either the Krumwiede, Avery, or mouse method.

Report: "No pneumococci were isolated." If another organism is found which might be the incitant of the disease, report its presence.

Report: "Pneumococcus, type I, II, II atypical, III, or IV was isolated." (The isolation of other pathogenic microörganisms may be reported.)

As soon as pneumococcus, type I, is indicated, notify the bacteriologist in charge who will send the report by telephone or telegraph.

If the physician desires serum, have it sent immediately.

## CHAPTER 3

# THE EXAMINATION OF EXUDATES FOR GONOCOCCI

After the mail has been sorted, the specimens to be examined for gonococci are placed in a properly labeled drawer until the workers are ready to examine them, unless information on the history blank indicates that the specimen has been collected from the eyes or that a telegraphed report is desired, in which instances the proper examinations are made at once.

### OPENING AND RECORDING SPECIMENS

Open and record the specimens just before preparing them for examination. (See: specimens for examination, opening and recording.) Stamp the serial number on each history blank. If there are paper labels on the slides, stamp the serial number on them as well. Write with a diamond-point pencil on each of the two slides submitted, the serial number, the year, the letter "G," and number the slides 1 and 2 respectively.

### STAINED PREPARATIONS

**Fixing and Staining Specimens.**—Fix the preparations in the flame and stain them by Gram's method.

**Examination of the Preparations.**—First examine the controls on the end of the slide. If the staphylococci are characteristically Gram positive and the *B. coli* characteristically Gram negative, examine the discharge.

The nuclei of the pus cells should have retained some of the purple color and the cytoplasm should be stained by the counterstain. Each specimen should be examined by two persons independently.

Do not report that no gonococci are found until the entire surface of each film has been examined under the microscope. Spend a period of at least five minutes in this search.

### REPORTING RESULTS

Record the results of the examinations in the place reserved on the history blank as follows:

*Routine Reports*

Report: "Gc," when Gram-negative intracellular micrococci having the morphology of gonococci are found.

Report: "No Gc," when Gram-negative intracellular micrococci are not found. When feasible, report the number of pus cells per field. When a stained preparation shows many pus cells and no morphologically typical organisms, send the form letter which states this fact and suggests that another specimen be submitted.

When the results of the examinations are unsatisfactory, indicate the appropriate statement on the slip designed for these reports.

*Special Reports*

When only extracellular organisms resembling gonococci are found, report the result "Unsatisfactory" and note the fact on the report slip.

When atypical intracellular and extracellular Gram-negative micrococci are found, follow the same procedure.

# CHAPTER 4

## THE EXAMINATION OF CULTURES FOR DIPHTHERIA BACILLI

Since the results of the examination of cultures for diphtheria bacilli must be reported as soon as possible, detailed directions are given relative to the times when the examinations must be made. All cultures for this examination must be incubated for a minimum period of eighteen hours before making a report that no diphtheria bacilli are found. A preliminary examination may be made at the end of ten hours, however.

If diphtheria bacilli are found, send the report immediately. When films prepared from exudates are submitted, examine them and if morphologically typical diphtheria bacilli are present, submit the report. If the organisms are atypical or no diphtheria bacilli are seen in such preparations, however, notify the physician that this examination is not entirely satisfactory and that a definite report cannot be made until a culture is examined. If two films are submitted, stain one by Gram's method, since some of the anaerobic bacteria associated with Vincent's angina, which are Gram negative when stained with methylene blue, may slightly resemble diphtheria bacilli.

The following schedule for examining cultures has been found convenient:

| TIME OF ARRIVAL | PRELIMINARY EXAMINATION | INCUBATION PERIOD | FINAL EXAMINATION | INCUBATION PERIOD |
|---|---|---|---|---|
| 5:00 a.m. | 3:00 p.m. | 10 hours | 8:00 a.m. | 27 hours |
| 11:00 a.m. | | | 8:00 a.m. | 21 hours |
| 6:00 p.m. | | | 1:00 p.m. | 19 hours |
| 11:00 p.m. | 1:00 p.m. | 14 hours | 8:00 a.m. | 33 hours |

On Saturdays, Sundays, and holidays, the cultures received at 6:00 p.m. may be given a preliminary examination during the following morning and those received at 11:00 p.m. of the previous day and at 5:00 a.m. of the same day may be given a preliminary examination at noon.

## RECEIVING, OPENING, AND RECORDING SPECIMENS

Place specimens which arrive at the laboratory outside of working hours in the incubator with a note indicating the hour of arrival. In the morning, count the specimens received during the night and then open them, giving each a serial number. Return the specimens to the incubator in case the incubation period has not been completed. Open and number the specimens received during the day before incubating them. Under the following conditions, inoculate tubes of Loeffler's culture medium from the swab that has been returned with the culture, and write on the accompanying history blank that another culture has been made and the reason for preparing it:

If the medium is contaminated or otherwise unsatisfactory;
If the culture tube is badly broken;
If the swab has been left in the medium;
If the specimen has been more than four days in transit;
If a swab only is received.

After each specimen tube has been numbered, place it in the proper position in a rack which holds ten specimens, to facilitate handling. Arrange the tubes so that those with numbers ending in "1" occupy the first place at the left, in front, and the others follow in order from left to right.

Stamp the hour that the specimen was received on the lower-left corner of the history blank, and the words "Preliminary examination" on the left margin of the blanks accompanying specimens received at 5:00 a.m. and 11:00 p.m. since specimens received at these times are given a preliminary examination.

Before incubating cultures received at 5:00 a.m. and 11:00 p.m., make an "R" on each tube with a red pencil so that it may be easily identified for the second incubation period.

## STAINED PREPARATIONS

**Preparing Specimens.**—Make two complete sets of stained preparations for all but preliminary examinations. Prepare on one slide, films from three specimens that have been given consecutive serial numbers. Write on the etched end of the slide the first serial number, a dash and the last two figures of the third serial number; the year, and the number of the set (See: fig. 21), placing nearest this end the film having the lowest number.

When a slide is used for preparations from two cultures instead of three, write the serial numbers with a plus sign instead of a dash.

If used for one film only, place an "X" after the serial number. When slides are used for cultures inoculated from a swab, write the word "Swab" below the serial number. Mark with an "R" those used for the reëxamination of cultures. In the case of preliminary examinations, write the letter "P" in place of "R."

Just before preparing the cultures for examination, remove the specimen tubes from the incubator, place them at one side of the working space and place an empty rack at the other. Beginning with the first specimen tube in the rack, make the preparations in order as previously described. Be careful to pass the loop lightly over the entire surface of the slant and to spread the material evenly on the slide. If too much of the growth has been used and the film is thick, flame the loop and add another drop of water. Keep the film well isolated on the slide and flame the loop after the preparation of each specimen.

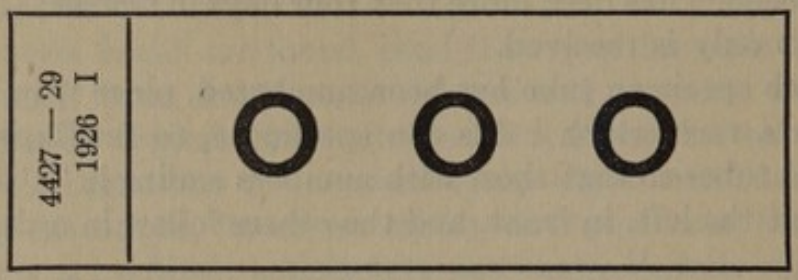



FIG. 21. SLIDE FOR STAINED PREPARATIONS

As soon as a specimen tube has been replugged, place it in the empty rack in a position corresponding to the one it occupied in the rack from which it was taken. After the preparations have been allowed to dry, fix them by passing the slides slowly, film side down, through the flame of a Bunsen burner, and set them aside to cool.

**Staining Specimens.**—When the slides are cool, stain with Loeffler's alkaline methylene blue. If the microörganisms have been grown on artificial media for a long time, stain for from three to five minutes.

Contrary to the general rule, the surplus stain from these slides may be used again if filtered.

After staining, arrange the sets in serial order on separate trays.

**Examination of the Preparations.**—Before examining a stained preparation, read carefully the information on the corresponding history blank. Place racks and culture tubes near by so that the growth and the appearance of the medium may be noted.

Whenever very few diphtheria-like bacilli are found, examine a third stained preparation. If the morphology of the bacilli found is atypical, preparations stained by Gram's and Albert's methods may

be of assistance in making the examination. In all cases, however, base the report on the results obtained with the methylene-blue stain. Do not report that no diphtheria bacilli are present until the preparations have been examined under a microscope for at least one minute. In all cases where there is the least uncertainty relative to the identity of the organisms seen in a culture, test their virulence for guinea pigs (62).

The slides are examined at 8:30 a.m., 1:30 p.m., and at 3:00 p.m., each set by a different person. Since set I only is kept on file it is examined by the person responsible for the results. For the preliminary examination of cultures received at 5:00 a.m. and 11:00 p.m., only one set of stained preparations is made, unless diphtheria bacilli are found, in which case another stained preparation is made, examined by a second worker, and the report is sent immediately. Those in which no diphtheria bacilli are found, however, are examined by one person and his signature is placed after the words "Preliminary examination" on the margin of the blank.

One person only examines specimens on Sundays and holidays and reports the results of examinations of first cultures for diagnosis. In all other cases, the results of examinations are reported only when there is not the least doubt concerning the identity of the organisms in the preparations. On the next working day, a second person examines all of the preparations made on Sundays or holidays and reports the results, after consultation if necessary, on the specimens containing organisms of questionable significance.

## REPORTING RESULTS

### *Routine Reports*

Indicate the results of the examination as follows in the place reserved on the history blank:

Report: "Bd," when morphologically typical diphtheria bacilli are found.

Report: "No Bd," when morphologically typical diphtheria bacilli are not found.

When the results of the examinations are unsatisfactory, due to the presence of atypical organisms, medium being dried, liquefied, contaminated, frozen, etc., indicate the condition as outlined on the proper report form. (See: reports, typing of.)

### *Special Reports*

Send special reports in the following instances:

1. When organisms resembling, but not typical of, diphtheria bacilli are present, even though in small numbers, in a first culture from a case which the physician has diagnosed clinically as diphtheria, report: "Diphtheria bacilli were found," and state that the virulence of the organisms will be tested.

When organisms resembling *B. hofmanni* are found in a culture having a similar history, report: "The organisms were atypical and the isolation and identification of these organisms will be attempted."

2. When organisms are found whose morphology is not perfectly typical of *B. diphtheriae* but yet are considered to be such, report the presence of diphtheria and notify the physician that a virulence test will be made.

3. When the medium is contaminated, liquefied, or dried, and diphtheria bacilli are found in the culture inoculated from the swab, report their presence.

When the medium is contaminated, liquefied, or dried, and no diphtheria bacilli are found in the swab culture, indicate the condition as outlined on the proper report form.

When it is necessary to report two successive cultures from one patient "Unsatisfactory" because of the presence of a contaminating, encapsulated organism, send a letter to the physician, recommending the use of a warm, saline irrigation before another culture is taken.

4. When the swab has been left in the tube of medium and no diphtheria bacilli are found in the original or in the culture inoculated from the swab, report the specimen "Unsatisfactory," and send a letter requesting the physician, in the future, to remove the swab after inoculating the medium.

5. When the swab only is received, report the result of the examination of the culture prepared from it.

6. When no diphtheria bacilli are found in a culture that has been delayed in transit for more than four days, send the report "Unsatisfactory."

## VIRULENCE TESTS

Virulence tests are made in the following instances:

1. On cultures from cases clinically diagnosed as diphtheria and from contact carriers three months after date of onset or exposure (63). Cultures from cases and contact carriers of less than three

months' duration are tested only when the physician or health officer gives a valid reason for requesting the examination. Otherwise, send a letter stating the time when virulence tests are performed and explain that exceptions to this rule are made only when an adequate reason is given.

2. On cultures from noncontact carriers which include carriers who are inmates of state institutions where there is no diphtheria.

3. On first cultures from cases clinically diagnosed as diphtheria when *B. hofmanni* or organisms resembling, but not typical of, diphtheria bacilli are found.

4. On cultures reported unsatisfactory because of the presence of atypical organisms.

Report the result of such tests only when virulent diphtheria bacilli are isolated or the physician has been notified that the test will be made.

### *Numbering Specimens*

Number as miscellaneous specimens all cultures from which the organisms are to be isolated, using the back of the original history blank for this purpose. Cross-index the serial numbers. Note on the lower margin of the history blank that a virulence test is being attempted. In case insufficient data are given on the history blank, send a letter requesting the necessary information.

### *Isolation of the Microörganism*

**Plating the Specimen.**—Suspend a representative loopful of the culture to be examined in a tube of ascitic-fluid broth containing tellurite (64), and inoculate with two or three loopfuls of this suspension, a series of two dextrose-serum-agar plates containing tellurite. Incubate the inoculated media from eighteen to twenty-four hours: then inoculate the medium in another plate from the surface growth on the fluid medium. Whenever unable to isolate the organisms on dextrose-serum agar containing tellurite, plate the specimens on dextrose-serum agar without the tellurite. Whenever it is necessary to determine the purity of a culture, use the solid medium without the tellurite.

For purposes of control, plate daily a pure culture of *B. diphtheriae* on the medium containing tellurite. Also, every time the medium is prepared, inoculate one plate of it with *B. pyocyaneus*, *B. subtilis*, and two cultures of *B. diphtheriae*. (If the medium is satisfactory *B. pyocyaneus* and *B. subtilis* are entirely inhibited and a good growth of *B. diphtheriae* is obtained.) Use one plate of dextrose serum agar without tellurite, for an additional control. Control the ascitic-fluid broth containing tellurite in a similar manner.

**Transferring the Original Culture.**—Following the inoculation of the broth, make a transfer of the original culture to Loeffler's serum medium. Store it in the cold room with the original culture for use in case the first attempt to isolate the organisms proves unsuccessful.

Before plating such a transfer, make sure, by examining a stained preparation of it, that there are apparently enough diphtheria bacilli present to make their isolation possible.

Transferring for several days will often increase the relative number of these organisms and make isolation possible although the original culture contained very few diphtheria bacilli.

**Fishing Colonies.**—There is considerable variation in the appearance of colonies of *B. diphtheriae* on dextrose-serum agar containing tellurite. A typical colony is small and granular with a dark center and an irregular outline. Some strains grow more luxuriantly, however, presenting a similar granular surface, but assuming a more even contour and regular outline.

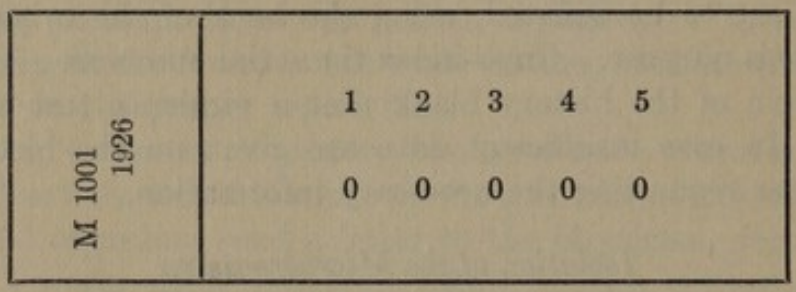



FIG. 22. NUMBERING OF FILMS

Fish characteristic colonies to Loeffler's serum medium. Use a separate rack for the cultures from each specimen. When no characteristic colonies can be found on the plates, fish a few of the various types present, in case the organisms seen in the original stained preparation resemble diphtheria bacilli in morphology only and are not similar to them in colony formation.

### *Identification of the Microörganism*

**Morphologic Examination of the Cultures Isolated.**—After the cultures have been incubated, make stained preparations with Loeffler's alkaline methylene blue. If no growth is obtained after twenty-four hours' incubation, extend the incubation period to forty-eight hours. Prepare films from five or six of the cultures on one slide and number them as illustrated in figure 22.

Examine these preparations to determine if diphtheria bacilli have been isolated and if the cultures are pure.

If the cultures isolated differ slightly in morphology, choose the one containing organisms most typical of diphtheria bacilli. In case this culture shows evidence of being nonvirulent, test the others by the intracutaneous method only. Complete the test, however, with the first culture chosen.

If none of the cultures contain organisms which resemble *B. diphtheriae* nor the organisms on which the original report was based, fish the plate inoculated from the pellicle on the culture in fluid medium. If the organisms are not isolated by this procedure, reëxamine the original plates or repeat the tests with the growth on the tube of Loeffler's medium inoculated with the original culture.

**Selection of Cultures for Animal Inoculation.**—If a pure culture of an organism morphologically typical of *B. diphtheriae* is obtained, test its virulence for guinea pigs. Should there be sufficient growth on one of the tubes of Loeffler's medium, inoculate a guinea pig intracutaneously. If there is insufficient growth for this purpose, make a transfer on Loeffler's serum. If the organism isolated resembles those found in the original culture, but is not typical of *B. diphtheriae*, test its fermentation reactions to determine whether it is necessary to test its virulence in guinea pigs.

Describe on the record blanks the organisms which are used in the test.

**Fermentation Reactions.**—When it is necessary to test the fermentation reaction of a culture, inoculate one tube each of dextrose-, saccharose-, and dextrin-serum-water medium. When satisfactory dextrin cannot be obtained, use dextrose and saccharose only.

If a pure culture of an organism resembling *B. diphtheriae* proves to be nonvirulent, it is desirable to test its fermentation reactions so that a tentative identification of the organism may be made for the benefit of the staff.

Incubate the media containing carbohydrates at from 35 to 37°C. for forty-eight hours, and record the readings as follows after twenty-four and forty-eight hours:

++ = acid and coagulation
\+ = acid
± = very slight acid
− = no change

Table 1 shows the reactions of *B. diphtheriae*, *B. hofmanni*, and *B. xerosis*.

In case the organisms isolated from cultures selected for virulence tests do not confirm the results of the morphologic examinations, refer the matter to the bacteriologist in charge of the group who will arrange to have all of the stained preparations reëxamined by all the workers connected with the test.

Test the virulence of cultures which show the fermentation reactions of *B. diphtheriae* as well as of those having the fermentation reactions of *B. hofmanni* and *B. xerosis* isolated from first cultures accompanied by a history of diphtheria.

## PERFORMING AND RECORDING THE TESTS

### *Intracutaneous Test (65)*

To determine the purity of the culture to be used for the intracutaneous test, make a stained preparation from it, marking the slide "Intracutaneous," below the serial number and date.

TABLE 1

*Reactions of B. diphtheriae, B. hofmanni, and B. xerosis*

| CULTURE | DEXTROSE | SACCHAROSE | DEXTRIN |
|---|---|---|---|
| *B. diphtheriae* | ++ | − | ++ |
| *B. hofmanni* | − | − | − |
| *B. xerosis* | ++ | ++ | − |

If the culture is pure, suspend one loopful, using a loop approximately 2 mm. in diameter for the purpose, in 10 cc. of 0.85-per-cent salt solution, and adjust to the turbidity of a standard prepared by diluting 2 cc. of barium sulfate standard No. 2 with 1 cc. of salt solution.

Use for the test two light-colored, preferably white, guinea pigs, one of which has received previously an immunizing dose of about 500 units of diphtheria antitoxin subcutaneously. Prepare the animals for inoculation and inject 0.2 cc. of the suspension intracutaneously in corresponding positions on both animals. (See: fig. 7.) Test no more than four cultures on one set of animals which weigh approximately 400 grams each.

Stamp on the history blank the outlines of two guinea pigs and write on the outline the numbers of the animals used, their color markings and indicate the site of the tests. (See: fig. 23.)

Record the reactions of the animals at the end of twenty-four, forty-eight, and seventy-two hours.

RECORD OF VIRULENCE TEST

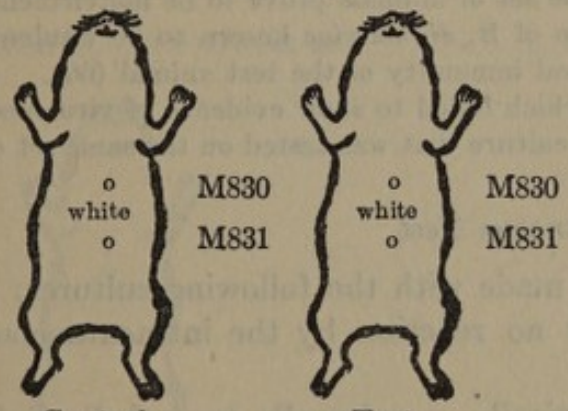
Type of organism in original film.....

.....

.....

| PLATED | FISHED | PLATED | FISHED |
|---|---|---|---|
| | | | |
| | | | |
| | | | |
| | | | |

| SUGAR REACTION | | | | INTRACUTANEOUS TEST | | | | |
|---|---|---|---|---|---|---|---|---|
| Date | Dextrose | Saccharose | Dextrin | Date | Pig No. | 24 hrs. | 48 hrs. | 72 hrs. |
| | | | | | | | | |
| | | | | | | | | |
| | | | | | | | | |
| | | | | | | | | |




Control
No. 192

Test
No. 196

Laboratory number M..... Exam. completed.....

Examiner.....

Result of examination.....

.....

.....

.....

.....

Specimen opened by..... Original film made by.....

FIG. 23. HISTORY BLANK FOR DIPHTHERIA

(Blue paper)

If the organisms injected are virulent diphtheria bacilli, there should be at the site of inoculation on the guinea pig unprotected by antitoxin, redness and slight induration at the end of twenty-four hours and a marked necrotic area after from forty-eight to seventy-two hours. On the animal protected by antitoxin, there should be no reaction at the site of inoculation other than the injury caused by the needle.

Record the results of the intracutaneous tests as follows:

4+ = very marked necrosis
3+ = necrosis with purple concentric area, oedema, and inflammation
2+ = purplish discoloration with oedema and inflammation
\+ = brownish discoloration with slight oedema and inflammation

Report the results of cultures which incite definite lesions after forty-eight hours. If no reaction is obtained in forty-eight hours, inoculate a tube of toxin broth. After two days' incubation, test for the presence of toxin by the subcutaneous method.

Should all the cultures tested on one set of animals prove to be nonvirulent, inoculate the guinea pigs with a strain of *B. diphtheriae* known to be virulent, as a control against the possible natural immunity of the test animal (66).

On the record blanks for cultures which failed to show evidence of virulence, record the serial number of a virulent culture that was tested on the same set of animals.

*Subcutaneous Test*

Subcutaneous inoculations are made with the following cultures:

Those which give doubtful or no reaction by the intracutaneous method.

Those which are not morphologically or culturally typical *B. diphtheriae*, that is (*a*) those which in pure culture resemble the organism on which the report of the morphologic examination was based, if they give the fermentation reactions of diphtheria bacilli, (*b*) all cultures resembling *B. diphtheriae* in any respect, if isolated from first specimens from clinical cases of diphtheria.

Before inoculating an animal, make a stained preparation from the culture in toxin broth, and examine it for purity. Mark the slide with the serial number, the year, and the word "Subcutaneous."

Inject 1 cc. of the toxin-broth culture subcutaneously into a light-colored guinea pig weighing from 250 to 300 grams. Make a record of the test on the form (See: fig. 24), stamping the form on a separate blank slip if necessary. Indicate the number and color of the guinea pig used, in a manner similar to that shown in figure 23.

Observe the animal daily. If it dies, make an autopsy as soon as possible and record the findings in the place reserved for that purpose on the slip. Make cultures and stained preparations from the site of inoculation for microscopic examination. Mark the slides with the serial number, the year, and the word "Recovered."

If the death of the animal is due to the inoculation of virulent diph-

---

Laboratory number.....

Date of Subcutaneous Test.....

Laboratory number..... Guinea pig number.....

Date of Autopsy.....

Site of inoculation.....

Adrenals.....

Pleural fluid.....

Site of inoculation: Direct film..... Culture.....

Heart's blood: Direct film..... Culture.....

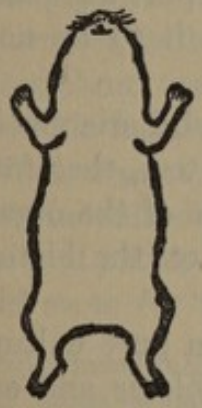


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FIG. 24. RECORD BLANK FOR VIRULENCE TEST (SUBCUTANEOUS) (Pink paper)

theria bacilli, there should be induration, congestion, and necrosis at the site of inoculation, and congestion of the adrenals. There may or may not be an increase in pleural fluid. Diphtheria bacilli should be present in the stained preparation and in the culture made with material taken from the site of inoculation.

Whenever questionable results are obtained, repeat the test, using two guinea pigs, one of which has received about 500 units of antitoxin twenty-four hours

previously. If no reaction or a doubtful reaction is obtained on an animal inoculated intracutaneously, and the animal inoculated subcutaneously dies, repeat the subcutaneous and also the intracutaneous tests, using two guinea pigs for each, one of each pair having previously received about 500 units of diphtheria antitoxin.

Send only a preliminary report to the physician at this time, stating that, to date, the results of the tests are inconclusive. Send a final report when the confirmatory tests are completed.

If the animal is alive and appears well on the fifth day after the injection, report the culture nonvirulent and discharge the animal.

## REPORTING RESULTS

Before reporting a virulence test, consult the records to determine if subsequent cultures from the patient have been received, as the results of the morphologic examinations of these may alter the wording of the report. Use the following forms in writing reports:

1. "Virulent diphtheria bacilli were isolated," when the microörganisms isolated incite lesions in guinea pigs typical of those produced by diphtheria toxin.

2. "The organisms isolated proved to be nonvirulent," when the organisms isolated resemble those on which the original report was based and the intracutaneous and subcutaneous tests prove it to be nonvirulent.

3. "We have been unsuccessful in isolating the diphtheria bacilli (or atypical organisms), in the culture. The virulence test, therefore, could not be performed. If you still wish the virulence of the organisms tested, please send another specimen, and write on the history blank accompanying it, 'For special attention.'"

4. "The organisms in the specimen, when isolated in pure culture, proved not to be diphtheria bacilli," when the morphologic and cultural reactions are not characteristic of *B. diphtheriae* and no virulence test was made.

If, when a virulence test is completed, the physician has not supplied the information desired, send another letter requesting it.

# CHAPTER 5

# THE ISOLATION OF TYPHOID, PARATYPHOID, AND DYSENTERY BACILLI FROM SPECIMENS OF FECES, URINE, AND BLOOD

## OPENING AND RECORDING SPECIMENS

When recording specimens to be examined for organisms of the enteric-disease group (See: specimens for examination, opening and recording), stamp both the front and the back of the history blank with the serial number, in the place indicated, and copy the required information on the back of the history blank, figure 25.

## ISOLATION OF THE MICROÖRGANISM

### *Plating Specimens*

**Feces.**—When specimens are submitted in outfits containing 30-per-cent glycerin, inoculate the plates directly. When the specimens are submitted with no preservative, suspend them in 30-per-cent glycerin, using about ten parts of liquid to one of solid, and allow the suspension to settle for ten minutes before inoculating the plates.

If no organism of the enteric-disease group is isolated from the plates first inoculated with the latter type of specimen, inoculate another series of plates from the suspension in glycerin after it has stood in the cold room for forty-eight hours.

Specimens in glycerin (67) received after 12:00 m. on Saturdays or on Sundays and holidays may be placed in the cold room until the following working day, except in the case of specimens which have been in transit for seven days or longer and first specimens for diagnosis.

For each specimen, arrange six plates on the table in the following order according to the kind of medium used:

1. Endo's agar
2. Endo's agar
3. eosin blue
4. eosin blue
5. brilliant green, "strong"
6. brilliant green, "weak"

Using a loop about 6 mm. in diameter, place one loopful of the upper layer of the suspension on plates 1, 3, and 5. Then inoculate all of them (See: plating methods) in the order arranged. For purposes of

Laboratory number..... Specimen opened by.....

Name of patient..... Specimen plated by.....

Character of material..... Specimen fished by.....

Sugar reactions of fishings from: Ag. test made by.....

| DATE FISHED | ENDO | BRILLIANT GREEN | EOSIN BLUE | BLOOD AGAR |
|---|---|---|---|---|
| | | | | |
| | | | | |
| | | | | |
| | | | | |
| | | | | |

| RESULT OF MACROSCOPIC SLIDE AGGLUTINATION TEST | MORPHOLOGY | MOTILITY |
|---|---|---|
| | | |
| | | |
| | | |
| | | |
| | | |
| | | |

Remarks:

RESULT OF MACROSCOPIC TUBE AGGLUTINATION TEST

| ANTISERUM | DATE | DILUTIONS | | | | | NEGATIVE CONTROL | POSITIVE CONTROL |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |
| | | | | | | | | |
| | | | | | | | | |
| | | | | | | | | |
| | | | | | | | | |

AGGLUTINATION TEST (*Widal*)

| CONTROLS | TYPHOID | | | | PARA A | | | | PARA B | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1-20 | 1-40 | 1-80 | | 1-20 | 1-40 | 1-80 | | 1-20 | 1-40 | 1-80 | |
| | | | | | | | | | | | | |
| | | | | | | | | | | | | |
| | | | | | | | | | | | | |

FIG. 25. HISTORY BLANK (BACK) FOR ENTERIC DISEASES

(Green paper)

comparison and to insure the use of satisfactory media, inoculate one plate of Endo's, one of eosin methylene blue, and one of each strength of brilliant-green agar with pure cultures of *B. typhosus*, *B. dysenteriae* Shiga, and *B. coli*. Dilute the material used for the inoculation of these plates sufficiently to insure the development of isolated colonies. When desired, use a specimen of feces known to contain *B. typhosus* as an additional control for the media.

If, due to miscalculation, there are insufficient plates to arrange in the usual order, note this fact on the history blank.

**Urine.**—Pipette 2 cc. of the specimen into a tube containing 8 cc. of beef-infusion or extract broth. Incubate for from eighteen to twenty-four hours. If there is evidence of bacterial growth,[1] inoculate four plates of media by the following procedure: Place one loopful on a plate containing "strong" brilliant-green medium and, with a plating rod, spread the material over plates containing "weak" brilliant-green, Endo's, and eosin-methylene-blue media. If the broth appears clear, reincubate it and inoculate the plates the following day.

**Blood.**—If the blood is sent in a Keidel, blood-culture outfit, proceed as directed under blood cultures, inoculating, in addition, one plate of Endo's agar, and one plate of blood agar. In case a pathogenic microörganism other than one of the enteric-disease group is isolated, cross-index it with a number in the miscellaneous series and give it to the bacteriologist in charge of that group, who will make the tests necessary for its identification.

When blood is received in glycerin, pipette it into a bottle containing 50 cc. of dextrose-beef-infusion broth for enrichment. After two days' incubation, inoculate media in plates from these cultures, using one plate of Endo's agar and one of blood agar.[1] If, after twenty-four hours' incubation, these plates show no growth of bacteria, incubate them for forty-eight hours more. In case no growth appears, but stained preparations from broth cultures, after six days' incubation, show bacteria to be present, plate the broth again.

Blood clots (68, 69) from all specimens submitted for the microscopic agglutination test are cultured unless the specimens are submitted from persons who are not ill. Do not plate such specimens directly, but break up the clot with the forceps used for the purpose and transfer it to a bottle containing 50 cc. of dextrose-beef-infusion broth. Then proceed as with the blood in glycerin. If the history indicates the possibility of undulant fever, follow the procedure outlined under undulant fever.

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[1] If there is evidence of an abundant growth in the enrichment broth, dilute the culture by making a suspension of proper density in broth before plating.

### *Fishing Colonies*

After incubation, examine the plates under a microscope, or hand lens if preferred. Fish typical or suggestive colonies (See: table 3) to slants of triple-sugar Andrade agar, making a deep puncture into the butt and then carrying the needle along the surface of the slant. In addition to the date and serial number, enter on the label of the tube the type of medium from which the colony was fished, and the number of the culture. Use a separate rack for the cultures fished from each specimen.

**Recording Reactions of Cultures.**—After from eighteen to twenty-four hours' incubation, record the reactions obtained in the triple-sugar slants, noting the number of cultures fished from each kind of medium and the reaction of each, making use of the following symbols:

+ = acid in butt, colorless slant, no gas; (*B. typhosus, B. dysenteriae*)
⊕ = acid and gas in butt, colorless slant; (paratyphoid-enteritidis group)
‡ = acid in butt and slant, no gas; (cocci and some strains of *B. dysenteriae* and *B. typhosus*)
‡g = acid and gas in butt, acid slant; (*B. coli* and other cultures that ferment lactose or saccharose with the production of acid and gas)

### *Macroscopic Slide-Agglutination Test*

For the macroscopic slide-agglutination test, place a loopful of a specific dilution (See: immune serum, standardization of, for typhoid agglutination) of the immune serum on a glass slide and carefully emulsify in it a very small amount of the growth to be tested, then tilt the slide back and forth for a few seconds.

The bacilli of the homologous species show definite macroscopic agglutination, while the bacilli of other species are unchanged, the drops remaining homogeneous in appearance.

If agglutination is obtained in any immune serum, control this reaction by testing the same colony or culture in a corresponding dilution of serum from a normal animal of the same species as that from which the immune serum was obtained. Always control the reaction of each immune serum used by testing it with the homologous culture.

Stain by Gram's method the preparations made in these tests whenever a morphologic study of the culture is necessary.

Test by the macroscopic slide-agglutination method:

1. In typhoid immune serum;
   a. All cultures giving + reactions.
   b. All cultures giving ⊕ reactions, if the gas formation is questionable.
   c. All cultures giving ± reactions with growth characteristic of this group of organism.
2. In polyvalent-dysentery immune serum;
   a. All cultures giving + reactions and not agglutinated in typhoid immune serum.
   b. All cultures giving ± reactions with growth characteristic of this group of organisms.
   c. All cultures giving ⊕ reactions, if the gas formation is questionable and they are not agglutinated in typhoid immune serum.
3. In paratyphoid A and paratyphoid B immune sera;
   a. All cultures giving ⊕ reactions.

A report may be based on the macroscopic slide agglutination of typical colonies or cultures from specimens from patients or carriers from whom our records show that the organism indicated has been previously isolated.

*Macroscopic Tube-Agglutination Test*

**Preliminary Examination of Morphology and Motility.**—Transfer to beef-infusion, or extract broth each culture which is to be tested by the tube-agglutination method and after two hours' incubation, examine a hanging drop and note the motility. Also examine a Gram-stained preparation of each culture tested by the tube-agglutination method to be sure that this reaction is not due to the presence of contaminating cocci.

**Performing the Test.**—Select the immune sera to be used in this test according to the schedule given in table 2.

Set up the tests in clean 11-by-75-millimeter tubes, putting no more than one specimen in a rack. Use 0.3 cc. each of a broth culture corresponding in density to barium sulfate standard No. 2, and of the two lowest specific dilutions of the immune serum. For each culture tested, prepare a negative control consisting of 0.3 cc. each of salt solution and of culture, and for a control of the sera combine 0.3 cc. of the two lowest specific dilutions of each serum used, and 0.3 cc. of a broth

culture of the homologous organism. Do not use serum that has been diluted for more than one week.

After the cultures and serum dilutions have been combined, flame the tubes and plug them with cotton. Incubate the tests for two hours at from 35 to 37°C. and then allow them to stand in the cold room overnight before recording the reactions.

TABLE 2

*Schedule indicating the immune sera to be used in the macroscopic tube-agglutination test*

| CHARACTERISTICS OF CULTURES | | RESULT OF SLIDE-AGGLUTINATION TEST IN IMMUNE SERUM | TEST BY MACROSCOPIC TUBE AGGLUTINATION IN IMMUNE SERUM |
|---|---|---|---|
| Motility | Reaction in triple-sugar agar | | |
| Motile or nonmotile..... | + | Agglutination in typhoid | Typhoid |
| Motile or nonmotile..... | + | Agglutination in polyvalent dysentery | Monovalent dysentery (Shiga, Flexner, and Mt. Desert) |
| Motile..... | + | No agglutination in typhoid | Typhoid |
| Nonmotile..... | + | No agglutination in typhoid; no agglutination in polyvalent dysentery | Typhoid; polyvalent dysentery |
| Motile or nonmotile..... | ⊕ | Agglutination in paratyphoid A | Paratyphoid A |
| Motile or nonmotile..... | ⊕ | Agglutination in paratyphoid B | Paratyphoid B |
| Motile or nonmotile..... | ∓* | Agglutination in polyvalent dysentery; agglutination in typhoid | Polyvalent dysentery; typhoid |

* Pure culture Gram-negative bacilli.

**Interpretation of Agglutination Reactions.**—Read the tests in the morning and record the degree of agglutination as follows:

4+ = complete agglutination; supernatant fluid clear; all large clumps
3+ = supernatant fluid clear or nearly clear; very definite clumping
2+ = supernatant fluid not clear; definite clumping

+ = supernatant fluid not clear; very small clumps definitely visible to the unaided eye
± = questionable reaction
− = suspension uniformly turbid; no clumping

For the identification of an organism, a 3+ reaction must be obtained in at least one specific dilution of the immune serum.

After reading the agglutination tests and recording the results on the record blank, discard the tubes into a pail containing disinfectant.

### *Special Tests*

*B. dysenteriae.*—Before reporting an organism *B. dysenteriae,* test its fermentation reactions in maltose, mannite, saccharose, and lactose, Andrade peptone-water medium (See: table 3). Record the reactions after incubation for one, two, and five days.

*B. paratyphosus* B.—Since there is so much cross agglutination among organisms of this group, when a Gram-negative bacillus gives the reaction typical of *B. paratyphosus* in triple-sugar Andrade agar and agglutinates in paratyphoid-B immune serum, a further study of the culture is necessary before making a report.

Inoculate maltose, mannite, saccharose, and lactose, Andrade peptone-water media and record the reactions after incubation for one, two, and five days. If after twenty-four hours, the sugar reactions are typical of *B. paratyphosus,* carry out an absorption test with paratyphoid-B immune serum. If such a culture fails to absorb, report it as belonging to the enteritidis group. If none of the carbohydrates is fermented, test the culture for agglutination in serum immune to the Morgan bacillus.

Cultures which do not ferment these carbohydrates in forty-eight hours and are definitely agglutinated in serum immune to the Morgan bacillus may be reported without being tested by the absorption method.

If *B. fecalis alkaligenes, Streptococcus viridans, Streptococcus hemolyticus, Staphylococcus aureus,* or any other microörganism of known pathogenic significance is present in large numbers, refer the results to the bacteriologist in charge of the group, who will, if necessary, send a special report.

### *Procedure Followed When Atypical Microörganisms Are Isolated*

If the history indicates that a specimen is from a case of enteric disease and organisms other than *B. coli* are present in large numbers,

which are not agglutinated in the immune sera used, refer to the bacteriologist in charge of the group for advice relative to the extent of the further study that should be made. Except in these instances, use the procedures outlined as follows:

**Cultures Which Ferment Dextrose with the Formation of Gas.**—If an organism with the reaction of *B. paratyphosus* in triple-sugar agar fails to agglutinate in the macroscopic slide-agglutination test in paratyphoid immune sera, send report No. 3.

When there is any doubt about the formation of gas, transfer the culture to dextrose broth in a Durham fermentation tube. If no gas is produced, test further as described in the following paragraph.

**Cultures Fermenting Dextrose without the Formation of Gas.**—When an organism with characteristic morphology and staining properties gives the reaction of *B. typhosus* in the triple-sugar medium, but fails to agglutinate in the typhoid or polyvalent-dysentery immune serum, transfer the culture at once from the original triple-sugar slant to two tubes of gelatin-agar semisolid medium. In addition to the serial number and the date of isolation, write the patient's last name on the tube. Seal the transfers with paraffin and, after incubation, store them at room temperature. Keep these cultures for further study, if necessary.

If all the cultures from one specimen which give typical sugar reactions fail to agglutinate in the typhoid or polyvalent-dysentery immune sera, inoculate maltose, mannite, lactose, and saccharose, Andrade peptone-water media, dextrose broth in a fermentation tube, and gelatin, and determine if the cultural characteristics in these media correspond to those of *B. typhosus* or *B. dysenteriae*.

Since organisms freshly isolated from the body do not always agglutinate readily, transfer the culture on beef-extract agar, daily for one week and make agglutination tests at the end of that period, using a suspension of the growth in salt solution, and dilutions of typhoid and polyvalent-dysentery immune sera ranging from 1:100 through the highest dilutions used routinely. If the motility of the organisms is questionable, make a flagella stain.

If the culture cannot be identified by these cultural and serological tests, send special report No. 6.

In case a similar organism has been previously isolated from the same patient, the culture need not be transferred and a final report may be made after the 48-hour reading of the carbohydrate reactions and after testing the agglutinability in dilutions of serum ranging from 1:100 through the highest final dilution used routinely.

### *Absorption Tests*

Occasionally, when the results of agglutination tests are indefinite or when cross agglutination is obtained, it is possible to identify the organism by absorption tests.

**Procedure.**—Use the following procedure, which is based on the work of Krumwiede and Cooper (70):

Inoculate beef-infusion agar in pint Blake bottles with culture homologous to the serum which is to be used. After eighteen hours' incubation, suspend the growth from each bottle in from 5 to 10 cc. of 0.85-per-cent salt solution. Pipette each suspension into a graduated centrifuge tube and centrifugalize at high speed for one hour. Remove the supernatant fluid and add the proper amount and dilution of serum as determined by standardization.

Incubate the mixture for three hours at 45°C., inverting the tubes occasionally to insure contact of serum and organisms. Leave them in the cold room overnight and centrifugalize at high speed for one hour, or until the supernatant fluid is clear. Prepare dilutions of 1:100, 1:200, 1:400, 1:500, 1:1000 and combine 0.3 cc. of each with 0.3 cc. of a broth culture of the organism homologous to the serum used. Incubate the tests at from 35 to 37°C. for two hours and leave them in the cold room overnight before reading the reactions.

The identification of a culture by the absorption test depends on its ability to alter the serum so that the agglutination reaction with the homologous culture is not obtained.

For control purposes make tests, at the same time, with sera which have not been subjected to absorption with their homologous cultures.

## STANDARDIZATION OF IMMUNE SERUM

Whenever serum with a new serial number is obtained from the serum-production group, determine its agglutinating titer and the dilution and dose factor necessary for complete absorption.

**For Agglutination Tests.**—Test the sera in dilutions from 1:100 through 1:32,000 with the stock cultures of *B. typhosus*, *B. paratyphosus* A, and B, and each of the three strains of *B. dysenteriae*.

Record the result of the standardization tests on 4-by-6-inch cards. Also determine the lowest dilution of serum (1:10 to 1:500) which, when tested by the macroscopic slide method, agglutinates the homologous organism but not the heterologous organisms of this group.

**For Absorption Tests.**—A serum dilution of 1:50 is usually found satisfactory for absorption tests. If this does not give satisfactory results, other dilutions may be tested.

**Procedure.**—Inoculate infusion agar in two pint Blake bottles with the culture homologous to the serum being standardized, and incubate for from eighteen to twenty-four hours. Suspend the growth in 0.85-per-cent salt solution, and distribute it in three graduated centrifuge tubes. Centrifugalize at high speed for one hour or until the volume of packed organisms remains constant. Remove all the supernatant fluid with a capillary pipette and add serum dilutions to each tube so that the packed organisms represent one part in ten of the total volume in the first tube, one part in twenty in the second tube, and one part in forty in the third tube.

It is sometimes necessary to test with larger or smaller amounts of bacteria than those mentioned.

Incubate the tubes in a water-bath at 45°C. for three hours, inverting them occasionally to insure contact of organisms and serum. Leave the tubes in the cold room overnight and then centrifugalize at high speed for one hour, or until the supernatant fluid is clear. Prepare dilutions of 1:100, 1:200, 1:400, 1:500, and 1:1000, and combine 0.3 cc. of each with 0.3 cc. of a broth culture of the homologous organism. Incubate the tests at 37°C. for two hours and leave them in the cold room overnight before reading. Select as a standard for use in routine tests one and one-half to three times the smallest amount of packed organisms which completely absorbs the serum in a dilution which is at least twenty times as concentrated as the titer.

## TESTING DIFFERENTIAL MEDIA

The plates of differential media are prepared in the media department. Whenever a fresh lot of medium is obtained, it is tested as described under plating specimens.

In the case of brilliant-green agar, the amounts of dye necessary must be determined for each new lot of medium. Six plates each of agar containing 0.1, 0.2, 0.3, 0.4, and 0.5 per cent of a 0.1-per-cent aqueous solution of brilliant-green dye are prepared in the media department.

**Procedure.**—Inoculate each set of plates containing different percentages of dye with stock strains of *B. coli*, *B. typhosus* (Bender and Pfeiffer strains); *B. dysenteriae* (Shiga type); and specimens from cases of typhoid fever or persons known to be carriers of *B. typhosus*, if possible. After incubation, observe the growth, and select for use, as a "weak"-dye agar, that dilution which best inhibits the growth of *B. coli* without materially affecting the growth of typhoid bacilli. Choose as a "strong"-dye agar a dilution which inhibits the growth of

*B. coli* completely or nearly so, and the growth of *B. typhosus* not more than 50 per cent. Record the results of the standardization on a 4-by-6-inch card, which is then given to the media department.

## REPORTING RESULTS

Record the result in the place reserved on the history blank for this purpose, as follows:

1. "*B. typhosus*"; "*B. paratyphosus* A," or "*B. paratyphosus* B"; "Morgan's bacillus has been isolated"; "*B. dysenteriae* Shiga," or "*B. dysenteriae* of the mannite-fermenting group (Flexner, etc.) has been isolated," when Gram-negative bacilli which give all the reactions typical of any one of these organisms were isolated.

2. "An organism belonging to the enteritidis group has been isolated," when Gram-negative bacilli are isolated which have the cultural characteristics of *B. paratyphosus* and are agglutinated by, but fail to absorb, paratyphoid-B immune serum.

3. "No *B. typhosus*, no *B. paratyphosus*, no *B. dysenteriae*," when none of these organisms is isolated. If no growth of bacteria is obtained from a cultural examination of urine, add this statement to the report.

4. "No growth of bacteria obtained," when no growth is obtained from a blood culture.

5. "Preliminary report—An organism has been isolated which belongs to the paratyphoid-enteritidis group. This culture is being studied further and a final report will be made later," when it is necessary to make a report before the tests have been completed to identify fully an organism of this group.

6. "(Unsatisfactory C-1.)" A special report is sent when Gram-negative bacilli are isolated which give a + reaction in triple-sugar Andrade agar and fail to agglutinate in typhoid or polyvalent dysentery immune serum.

7. "In addition to the agglutination test, a cultural examination has been made and (name of the organism) was isolated," when one of the organisms of the enteric-disease group is isolated from the clot of a specimen of blood submitted for the agglutination test.

8. "Unsatisfactory," when the specimen has proved unsatisfactory for examination.

For cases not covered by the report form, make an explanatory note.

## CARE OF STOCK CULTURES

Cultures of *B. typhosus*, *B. paratyphosus* A, and B, and *B. dysenteriae* (Shiga, Flexner, and Mt. Desert types) are kept as stock cultures to be used as controls.

Transfer these cultures daily on beef-extract agar and triple-sugar Andrade agar. If the cultural reactions or the agglutination tests indicate that any of the cultures may be contaminated, follow the procedure for the isolation of a pure culture, fishing the colonies to triple-sugar Andrade agar. The cultural and morphologic characteristics having been found typical, make agglutination tests, using typhoid, paratyphoid A, and B, and monovalent-dysentery immune sera in the two specific dilutions as determined by standardization. Whenever the purity of a culture is tested, record the procedure on 4-by-6-inch cards.

TABLE 3

*Characters and reactions by which the typhoid, paratyphoid, and dysentery bacilli may be identified*

| | COLONY CHARACTERISTICS ON DIFFERENTIAL MEDIA | | | FERMENTATION REACTIONS | | | | | | MORPHOLOGY | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Brilliant-green agar | Endo's agar | Eosin methylene-blue agar | Dextrose | Mannite | Maltose | Lactose | Saccharose | Triple-sugar slants | Motility | Gram stain |
| *B. typhosus* | Colorless by direct light, translucent, greenish by transmitted light, border undulated, grape-leaf character marked | Similar, less translucent, grape-leaf character not so marked | Colorless, translucent | A | A | A | 0 | 0 | + | Motile | Gram-negative |
| *B. paratyphosus*, A and B | Similar to *B. typhosus*, except for rounded edges and absence of grape-leaf character | Similar | Colorless, translucent | AG | AG | AG | 0 | 0 | ⊕ | Motile | Gram-negative |
| *B. dysenteriae* (Shiga) | No growth | Colorless, translucent, border regular, no granulation, no grape-leaf character | Similar to those on Endo's agar | A | 0 | 0 | 0 | 0 | + | Not motile | Gram-negative |
| *B. dysenteriae* (Mt. Desert) | | | | A | A | 0 | 0 | 0 | + | Not motile | Gram-negative |
| *B. dysenteriae* (Flexner) | | | | A | A | A | 0 | Weak A* | + | Not motile | Gram-negative |
| *B. coli (communis)* | Greatly inhibited; pinkish, regular border | Pink or red, nearly opaque under low power, granular and brownish border | Colorless at edge, centers dark blue almost black, opaque, border regular | AG | AG | AG | AG | 0 | ± g | Motile | Gram-negative |

+, = acid in butt, colorless, slant, no gas; ⊕, = acid and gas in butt, colorless in slant; ± g, = acid and gas in butt, acid in slant; A = acid; G = gas; 0 = no change.

* Variable.

## CHAPTER 6

# THE AGGLUTINATION TEST FOR TYPHOID FEVER, PARATYPHOID FEVER, AND DYSENTERY

Test specimens of blood submitted for the agglutination reaction, with cultures of *B. typhosus* only, unless a special request is made, or the history indicates that a test with *B. paratyphosus* A, and B, or with *B. dysenteriae* might be of value (71).

### OPENING AND RECORDING SPECIMENS

Open the specimens of blood to be tested for the agglutination reaction. Give each a serial number and record the necessary information on the history blank as described under specimens for examination, opening and recording. In addition, stamp the serial number in the place assigned to it on the back of the history blank, see figure 25.

### AGGLUTINATION TESTS WITH CULTURES OF *B. TYPHOSUS*

**Preparation of the Culture.**—Late every afternoon transfer the stock culture of *B. typhosus* which is on agar to beef-extract broth to be used for the tests the following morning. Incubate this culture at a temperature of about 25°C. which is usually maintained on top of the 37°C.-incubator.

Before using the culture for the tests, dilute it with broth until its density corresponds to that of the barium sulfate standard No. 0.5 which is prepared by adding 0.5 cc. of barium sulfate standard No. 10 to 9.5 cc. of beef-extract broth.

**Preparation of the Culture Controls.**—To determine the motility and the agglutinability of the culture, prepare hanging drops of the organisms in 0.85-per-cent salt solution and typhoid immune serum in a dilution determined to be specific by standardization, and mark the slides, "T/−" for the motility control, and "T/+" for the control of agglutinability.

**Preparation of Color Controls (72).**—Use dilutions of defibrinated sheep blood as color standards for the dilutions of specimens of dried blood.

Once each week, obtain 5 cc. of defibrinated sheep blood from the serum-diagnosis group. Add 0.25 cc. of a 1:20-dilution of formalin

(40-per-cent formaldehyde) as a preservative and store it in the cold room.

Pipette 0.04 cc. of the defibrinated sheep blood into the first depression of a porcelain, dilution plate, and allow it to dry, usually overnight, before diluting. Prepare the color standard in three dilutions, 1:10, 1:20, and 1:40, as follows: Add 0.36 cc. of distilled water to the dried blood and allow it to stand until dissolved. Then pipette 0.04 cc. and 0.12 cc. of distilled water into the second and third depressions respectively and add to each 0.04 cc. of the 1:10-dilution. Prepare a second set of color controls by diluting the defibrinated sheep blood without drying.

**Preparation of the Dilutions of Dried Blood.**—Prepare the dilutions of dried blood in a porcelain, dilution plate. Before diluting each specimen, write its serial number on the porcelain plate opposite the row of depressions in which it is to be diluted. If two drops of blood are received, use the better one for the test.

If the drops are small, it may be necessary to use both.

Prepare three dilutions of the blood, 1:10, 1:20, and 1:40 as follows: With a Pasteur pipette which is held at the same angle throughout the procedure to insure the use of drops of equal size, place one drop of 0.85-per-cent salt solution in the second depression of the porcelain plate and three drops in the third depression. Then add sufficient salt solution to the drop of dried blood to make an approximate 1:10-dilution and allow it to stand for at least ten minutes before mixing so that an even suspension is made. Mix the specimen with a wire loop and transfer to the first depression on the porcelain plate. Before making the higher dilutions, compare the color of the 1:10-dilution of the specimen with that of the defibrinated sheep blood and add more salt solution if necessary. With the pipette previously used, transfer one drop of this dilution to the second and third depressions, thus making the 1:20- and 1:40-dilutions.

After the dilutions have been made, cover the porcelain plate with another plate or piece of glass to prevent evaporation.

**Preparation of the Dilutions of Serum (73).**—When 1 cc. or more of clotted blood is received in a tube, separate the serum and the clot by centrifugalization.

Using aseptic precautions, draw off the serum for the agglutination tests and give the clot to the bacteriologist in charge of the isolation of the incitants of the enteric diseases, for cultural tests.

Using a 0.2-cc. pipette, prepare 1:10-, 1:20-, and 1:40-dilutions of the serum in 11-by-75-millimeter tubes.

### AGGLUTINATION TESTS WITH CULTURES OF *B. PARATYPHOSUS*

When it is necessary to make agglutination tests with *B. paratyphosus* A, and B, make the test also with *B. typhosus*, as a control.

Since broth cultures of *B. paratyphosus* A, and B are not made daily, transfer the cultures as soon as it is decided to make the test and incubate them at from 35 to 37°C. until the density corresponds to that of the standard, which usually requires about two hours. For control purposes, prepare hanging drops of the cultures of *B. typhosus*, *B. paratyphosus* A, and B in specific dilutions of the antiserum for each and in 0.85-per-cent salt solution. Dilute the specimen and prepare the hanging drops as described under hanging drops for the test.

### AGGLUTINATION TESTS WITH CULTURES OF *B. DYSENTERIAE*

When a request for an agglutination test with cultures of *B. dysenteriae* is received, test with three cultures of *B. dysenteriae* (Shiga, Flexner, and Mt. Desert), as well as with *B. paratyphosus* A, and B, and *B. typhosus*. If such an examination is to be made, obtain 24-hour agar-slant cultures of the three types of *B. dysenteriae* from the group making cultural examinations for organisms of the enteric diseases. Make broth cultures from these and from the two cultures of *B. paratyphosus* and incubate them at from 35 to 37°C. until the density corresponds to that of the standard, which usually requires about two hours.

Prepare the control tests in a manner similar to that previously described and test each culture in salt solution, the homologous serum, and the heterologous sera. Use polyvalent-dysentery immune serum as the homologous serum for the cultures of *B. dysenteriae*. Since cultures of *B. dysenteriae* are often agglutinated in normal serum, test these organisms also in normal human serum in the same dilution as used for the specimen under investigation.

### HANGING DROPS FOR THE TEST

**Preparation.**—Prepare hanging drops (74) of the three dilutions of the specimen being tested by transferring one loopful of each to a cover glass and adding one loopful of the broth culture of *B. typhosus*. The final dilutions are then 1:20, 1:40, and 1:80 in the case of both dried blood and serum.

Beginning with the highest, transfer the serum dilutions in succession without flaming the loop between; but when transferring the culture be sure to flame the loop after each transfer.

Mark on the slides with a wax pencil the last two digits of the serial number, the strength of the dilution, and a letter to indicate the culture used, see figure 26. Incubate these tests for one hour at from 35 to 37°C.

**Examination.**—After incubation, first examine the hanging drops prepared for control purposes and then examine the specimens and record the results on the history blanks. The organisms in the dilutions of heterologous sera should be actively motile[1] and distributed more or less evenly over the field, while those in the homologous sera should be definitely clumped and have lost their motility.

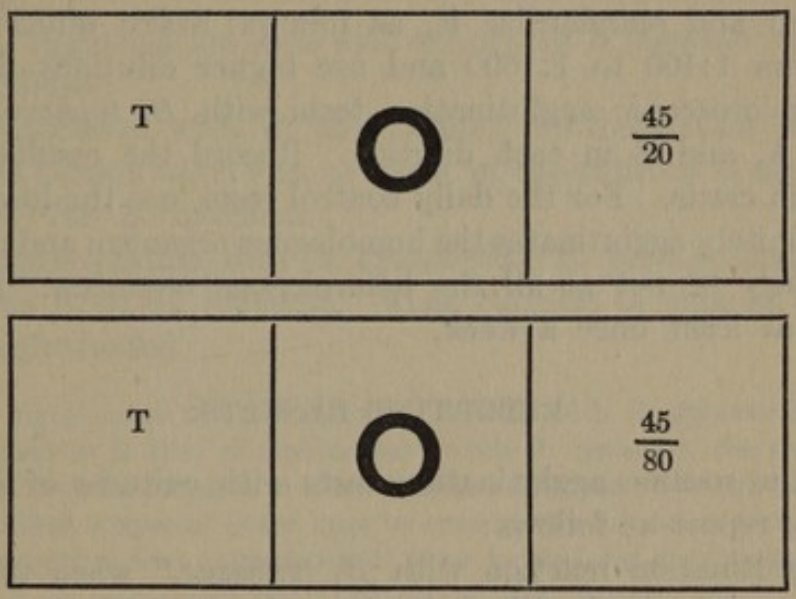


FIG. 26. SLIDES FOR AGGLUTINATION TESTS

Use the following symbols for recording the degree of agglutination:

4+ = complete agglutination; all large clumps; no motility
3+ = nearly all bacteria agglutinated; very definite clumping; a few free organisms; little or no motility[1]
2+ = majority of organisms agglutinated; definite clumping; some motility[1]
\+ = definite small clumps; many motile organisms[1]
± = an occasional small clump; almost all organisms motile[1]
− = all organisms motile;[1] no clumps

Whenever a stronger reaction is obtained in one of the higher dilutions than in the lower, repeat the test to be sure that a technical error has not been made.

[1] In case motile organisms are being tested.

Also, whenever the same degree of agglutination (less than a 3+ reaction) is obtained in all three dilutions, repeat the test, using fresh dilutions, if possible.

Whenever the lytic action of a serum is so strong as to interfere with the agglutination, inactivate part of the serum by heating at 55°C. for thirty minutes and repeat the test. In the case of dried blood such a reaction may necessitate reporting the examination unsatisfactory.

## STANDARDIZATION OF IMMUNE SERUM

Obtain the serum used in the control tests from the serum-production group and standardize it, as follows: Make dilutions of each serum from 1:100 to 1:1600 and use higher dilutions if necessary. Prepare microscopic agglutination tests with *B. typhosus*, *B. paratyphosus* A, and B in each dilution. Record the results on special 4-by-6-inch cards. For the daily control tests, use the lowest dilution which definitely agglutinates the homologous organism and agglutinates very slightly or not at all the heterologous organism. Make fresh dilutions at least once a week.

## REPORTING RESULTS

When the routine agglutination tests with cultures of *B. typhosus* are made, report as follows:

1. "Agglutination reaction with *B. typhosus*," when the following reactions are obtained with *B. typhosus* in dried blood or serum;

| 1:20 | 1:40 | 1:80 |
|---|---|---|
| 3+ | 3+ | + |
| or | or | or |
| stronger | stronger | stronger |
| or 3+ or 4+ | 2+ | 2+ |

2. "Partial agglutination," when the following reactions are obtained with *B. typhosus* in dried blood or serum;

| 1:20 | 1:40 | 1:80 |
|---|---|---|
| 3+ | 2+ or + | + or weaker |
| or 2+ | 2+ | 2+ or weaker |

3. "No agglutination with *B. typhosus*," (75) when no agglutination is obtained or when the reactions are weaker than those described under 2.

When the agglutination tests with cultures of *B. paratyphosus* are made, report as follows:

1. "Agglutination with *B. paratyphosus* A (or *B. paratyphosus* B)," when a culture of one of these organisms is agglutinated.

(Based on the same reactions as with *B. typhosus*.)

2. "Partial agglutination with *B. paratyphosus* A (or *B. paratyphosus* B)," when a culture of one of these organisms is partially agglutinated.

(Based on the same reactions as with *B. typhosus*.)

3. "No agglutination with *B. paratyphosus* A, *B. paratyphosus* B, or *B. typhosus*," when cultures of none of these organisms are agglutinated.

When the agglutination tests with cultures of *B. dysenteriae* are made, report as follows:

1. "Agglutination with *B. dysenteriae*," no distinction being made between the strains when one, or more, of the cultures is agglutinated, as indicated for *B. typhosus*.

2. "No agglutination with *B. dysenteriae*, *B. paratyphosus* A, or *B. paratyphosus* B, or *B. typhosus*," when cultures of none of the organisms are agglutinated.

Since the significance of the agglutination test with *B. dysenteriae* is not so well established as is that of similar tests with *B. typhosus*, the results of the test should always be brought to the attention of the bacteriologist in charge of the group so that a special letter may be sent to the physician explaining these facts and suggesting that a specimen of feces be sent for confirmatory tests.

## CLEANING OF UTENSILS

Place the slides with the cover glasses still attached, in a pan containing a solution of washing soda, and boil for five minutes before sending them to the glassware department to be cleaned.

Allow the porcelain plates to stand in 1-per-cent crude carbolic solution for at least ten minutes and then wash with soap and water. Rinse thoroughly and dry on a clean towel.

Remove the rubber bulbs from Pasteur pipettes, place the pipettes in a small jar and cover them with disinfectant. After letting them stand for twenty-four hours in this solution, send them to the glassware department to be cleaned.

## CARE OF STOCK CULTURES

If the cultural characteristics, motility, or agglutination reactions of the cultures of *B. typhosus* or *B. paratyphosus* A, or B indicate that any of the cultures may be contaminated, follow the special procedure

for the isolation of pure cultures. If the reactions in the triple-sugar medium are typical, make a broth culture from one of the colonies fished and on the following day perform agglutination tests, using all three immune sera in the specific dilutions as previously determined and described under standardization of immune serum. If any irregularity is encountered, consult the bacteriologist in charge of the group, who will make an investigation. Keep a record of these tests on 4-by-6-inch cards. Follow the same procedure whenever a new culture is obtained from the bacterial collection. The cultural and agglutinating characteristics having been found typical, make two subcultures of each kind of organism on beef-extract agar from the Andrade agar culture. Transfer these twice each week, Monday and Thursday, inoculating two agar slants and one Andrade triple-sugar slant. (The latter is made to determine the purity of the cultures.) Use one set of the agar cultures for the daily broth transplants and keep the other set for the transplants on the following Monday or Thursday, so that there will always be one set of unopened cultures available.

# SECTION III

# COMPLEMENT-FIXATION TESTS

## CHAPTER I

## THE COMPLEMENT-FIXATION TEST FOR SYPHILIS

The complement-fixation test for syphilis, as performed at the state laboratory for the physicians and institutions of New York State, is a modification of the original Wassermann test. Changes in technic have been based upon the valuable material which has accumulated through years of a steadily increasing volume of work, as well as upon the results of experimental studies. The modifications made have been shown to insure greater accuracy.

### OPENING AND NUMBERING SPECIMENS

The specimens, usually consisting of approximately 5 cc. of clotted blood or spinal fluid, are received in the outfits provided by the laboratory. Follow the methods described under specimens for examination, opening and recording.

**Procedure.**—Before stamping the serial number on each specimen tube, history blank, and container, stamp the number on the accession sheet.

The accession sheet is later used for recording the readings of the complement-fixation tests.

Place the specimen tubes consecutively as stamped in racks with holes for ten tubes, a specimen with serial number ending in "1" being put in the first place at the left and so on in order, so that a specimen with a number ending in a given figure may always be found in a certain position in the rack. If a specimen tube is found broken on arrival, number an empty tube correspondingly and substitute it in the rack.

After the specimens and histories have been numbered, compare the data on each history blank with those on the corresponding tube. If the name of the patient as given on the specimen tube does not agree with that on the history blank, note that fact on the lower-left margin of the blank; if the patient's name does not appear on the specimen tube, copy on the tube label the name given on the history blank, and stamp "No name on tube" on the blank.

Since specimens of spinal fluid are not prepared in the same manner as blood specimens, place them in separate racks as soon as the data on each spinal-fluid tube and history blank have been compared, and substitute in the racks containing the blood specimens empty tubes numbered correspondingly and labelled "Spinal fluid."

For the guidance of workers performing the tests, prepare a list each day, containing the first and last serial numbers of the specimens received, the serial numbers of spinal fluids, specimens on which telegraphed or "rush" reports are requested, specimens which are being retested, and those which for any reason are unsatisfactory for examination.

## PREPARATION OF SPECIMENS

### *Blood Serum*

If a blood specimen is collected in the laboratory, slant the tube before coagulation occurs in order to obtain the maximum amount of serum. When the blood has coagulated, rim the clot with a clean, sterile wire or clean, sterile glass rod, and place the tube in the cold room for several hours, or preferably overnight, to permit separation of the serum.

Follow the above procedure whenever it is desired to separate serum from coagulated blood, unless it is necessary to test the serum immediately. In that case separate the serum in the same manner as that to be used for complement.

Arrange the specimen tubes in three-tube or six-tube trunnion carriers, counterbalance them and centrifugalize to deposit the cells. Using a capillary pipette fitted with a rubber bulb, transfer the clear serum from each specimen to a clean tube numbered correspondingly.

If any serum is found to be hemolyzed, bile stained, or to show marked discoloration, note the fact on the corresponding history blank. If a serum is badly hemolyzed, list it among those unsatisfactory for examination and discard it.

When the serum has been pipetted from each specimen, place the racks containing clots and clear serum in parallel rows and compare the number on the clot tube with that on the tube containing the clear serum of each specimen to see that the tubes are in corresponding positions in the racks. If any specimen is found out of order, note the fact on the history blank, and obtain a new portion of serum from the original clot.

Inactivate specimens of blood serum in a water-bath at 55°C. for one-half hour on the day they are to be tested. If a serum is to be re-

tested twenty-four hours or more after the original half-hour's inactivation, reinactivate it for five minutes at 55°C.

In case the temperature of the inactivating bath should rise to above 56°C., obtain a new portion of each serum from the original clot if possible. List the specimens of which a new portion cannot be obtained, as they will be reported unsatisfactory unless they give complete fixation in the test.

### *Spinal Fluid*

Note on the back of the history blank the appearance of each spinal fluid as to turbidity, xanthochromia, presence of hemoglobin, blood cells, or sediment. Centrifugalize all spinal fluids for one-half hour, withdraw the supernatant fluid of each to a clean tube and record its appearance. If, on macroscopic examination, a fluid contains no blood cells but shows a yellow coloration, determine by the benzidin test whether the color is due to hemoglobin.

Inactivate specimens of spinal fluid in the same manner as blood specimens.

Except in cases in which immediate reports are requested, specimens of blood and of spinal fluid for the complement-fixation test for syphilis are not examined until the next working day following their arrival at the laboratory. As soon as the preparation of each lot of specimens is completed, at noon or night, the tubes are plugged with cotton and stored in the cold room.

## PREPARATION OF REAGENTS

In addition to the serum or spinal fluid to be tested, four reagents are employed; a suspension of sheep, red blood cells; antisheep amboceptor; complement; and antigen.

Each of these reagents, except antigen, is kept in the cold room at from 3 to 6°C. when not in use, and complement, since it deteriorates rapidly, is kept in a pan of ice water while in use.

For preparing the suspension of red blood cells, and for making all dilutions, physiological salt solution, 0.85-per-cent sodium chloride, is used.

### *Suspension of Sheep Red Blood Cells*

The suspension of red blood cells is prepared from defibrinated or citrated sheep blood collected not more than forty-eight hours previously.

**Procedure.**—Before washing defibrinated blood, filter it through absorbent cotton. Place from 6 to 7 cc. of filtered, defibrinated blood in a 30-cc. centrifuge tube, fill the tube with salt solution and mix by

drawing up once in a 50-cc. volumetric pipette. If the blood has been collected in citrate solution, one part of blood in one part of 2-per-cent sodium citrate in salt solution, place 15 cc. of blood in each centrifuge tube, and add salt solution to fill the tube. Counterbalance the tubes in the metal centrifuge cups and centrifugalize for fifteen minutes at about 1400 r.p.m. Draw off the supernatant liquid, replace with fresh salt solution, mix and centrifugalize as before. Repeat this process once more, allowing for the final centrifugalization fifteen minutes at about 1800 r.p.m. in order to pack the cells. Test the supernatant liquid for serum albumin with concentrated nitric acid. If any cloud appears, wash the cells once more. After the final washing, the supernatant liquid should be clear and colorless; if the liquid is tinged with hemoglobin, do not use the cells.

The first washing may be done on the day before the cells are to be used. In this case the salt solution should be drawn off after the washing and the tubes plugged before being placed in the cold room.

If graduated tubes are used, read and record the volume of the packed cells in each before removing the supernatant salt solution of the final washing. If ungraduated tubes are used, carefully mark the level of the packed cells in each by pasting on a small label so that its upper edge coincides exactly with the level of the packed cells. Draw off the salt solution and transfer the cells to a graduate, using as little salt solution as possible. Invert the tubes on filter paper to drain, and determine the quantity of packed cells which was contained in each by measuring the liquid required to replace the cells. To do this add salt solution to each tube from a graduated pipette till the lowest point of the meniscus is on a level with the upper edge of the label. Note the quantity required and add a correction of 0.1 cc. for the approximate amount of moisture remaining in each tube after partial drying.

To prepare the 5-per-cent suspension, add sufficient salt solution to the washed cells to make the entire volume twenty times that of the packed cells.

To prepare sensitized cells mix equal parts of the 5-per-cent suspension and the standard dilution of amboceptor. Pour the diluted amboceptor on to the cells and pour back and forth from one flask to another at least three times to mix thoroughly.

### *Antisheep Amboceptor*

The blood serum of an animal that has been immunized to washed, sheep, red blood cells is used for antisheep amboceptor.

For the purpose of economy a large animal rather than a rabbit is

used for the production of amboceptor, as large amounts of this reagent are required. (For the method of immunization see "Production of antisheep amboceptor in a mule," by Ruth Gilbert, N. Y. State Jour. Med., 1922, *22*, 286 (76).)

**Procedure.**—When an amboceptor of satisfactory titer has been obtained, take a large bleeding. When the blood has coagulated, separate and measure the clear serum, and inactivate it. Then mix with an equal quantity of pure, sterile, neutral glycerin (77), and bottle. Label the bottles, cork tightly and seal with a glycerin-glue mixture.

### *Complement*

Use for complement the pooled serum of at least three, and preferably six or more guinea pigs, bled not more than twenty-four hours previously.

To avoid chylous serum, do not bleed the animals until several hours after feeding.

Guinea pigs suffering from an infection of any sort may not yield serum satisfactory for complement, but the serum of used animals, for example those previously employed for antitoxin standardization, has proved perfectly satisfactory.

When used guinea pigs or small, normal guinea pigs, from 300 to 500 grams in weight, are to be bled for complement, bleed them to death, but when large, normal guinea pigs, weighing from 500 to 800 grams, are available, take from 5 to 10 cc. of blood from the heart.

Collect the blood in sterile Petri plates. When it is firmly coagulated, place it in the incubator at 37°C. for thirty minutes, then rim each clot and leave the blood in the cold room for one-half hour or longer. Pipette the serum from each specimen into a separate tube, as the individual guinea-pig sera are subjected to certain preliminary tests before being pooled. Centrifugalize the serum for the minimum time necessary to deposit the cells, usually eight or nine minutes. Withdraw the clear serum into clean tubes and cork the tubes before placing them in the cold room.

The clots may be centrifugalized also, and serum from all the clots, if not too hemolyzed, may be pooled and tested as a separate specimen. If it proves satisfactory by preliminary tests, this portion is later mixed with the rest of the complement.

### *Antigens*

Two antigens are used, an alcoholic extract of the acetone-insoluble lipoids of calf- or beef-heart tissue, prepared by the method of Bordet and Ruelens (78), and a cholesterinized extract of dried ether-ex-

tracted beef-heart tissue, prepared by a method similar to that of Neymann and Gager (79).

Fresh, normal, unincised hearts are employed, and, as experience has shown that there is a variation in the quality of antigen prepared from different hearts, the tissues from several hearts are used in each antigen.

**Acetone-Insoluble Antigen.**—Remove the fat, blood vessels, pericardium, and endocardium, and cut up the muscle into cubes about one inch in diameter. Then grind the heart muscle in a meat chopper. Weigh the ground tissue, and for each 100 grams add 125 cc. of 95-per-cent alcohol.

The purpose of the alcohol is to coagulate the proteins and not to dissolve the fatty substances or lipoids, and, as the alcohol is in comparatively small proportion and is immediately diluted with the tissue juice, it exerts but slight dissolving power.

Let the mixture stand at room temperature for several days, then filter, spread the residue on trays in a thin layer and dry in the incubator for a day. To the dried tissue add 200 cc. of acetone for each 100 grams of tissue originally used. Mix and leave at room temperature for one week, shaking for a minute or two once a day, then filter. Add 200 cc. of fresh acetone for each 100 grams of tissue originally used, mix, and filter the next day. Remove the tissue to trays, and dry in the incubator for one day, then pulverize it finely in a mortar. To the ground tissue add 200 cc. of 95-per-cent alcohol for each 100 grams of tissue originally used. Mix and allow to extract at room temperature for ten days, shaking for a minute or two three times a day. Then remove the alcoholic extract by filtration through soft filter paper, and store in the dark at room temperature.

**Cholesterinized Antigen.**—Cut and grind the heart muscle, as in preparing tissue for the acetone-insoluble antigen. Then spread it in the thinnest possible layer on a clean, smooth surface, such as a glass plate or stone shelf. Dry before an electric fan for five or six hours, then turn the tissue with a spatula and allow the other side to dry before the fan overnight. Break up the resulting dry sheet of tissue and grind it again. Spread this powder on a tray and dry it in an incubator at 37°C. for three or four days, then pulverize it finely in a mortar.

Extract the dry, pulverized tissue with successive additions of ether, in the cold room, until the ether is practically water clear, filtering off the ether and replacing it with fresh ether daily. After the final ether extraction, allow the tissue to dry on a tray until the odor of ether is not evident.

To prepare the alcoholic extract of the dried, ether-extracted tissue, add 10 cc. of absolute alcohol per gram of tissue and boil for one hour under a reflux condenser. After cooling to room temperature, filter the extract through soft filter paper. In case a precipitate forms after standing, refilter before adding cholesterin.

Cholesterinize 100- or 200-cc. portions of this extract as needed, by adding 0.4 gram per 100 cc. Weigh the cholesterin[1] accurately on an analytical balance, place in a clean bottle, and add the measured quantity of alcoholic extract. Shake the mixture, and, if necessary, crush large particles of the cholesterin with a glass rod. Stopper the bottle tightly and place in the water-bath at 37°C. until the cholesterin is completely dissolved. Leave at room temperature for twenty-four hours and filter, if a precipitate is then evident.

## STANDARDIZATION OF REAGENTS

As the total volume of the test is approximately 0.5 cc., one-tenth that of the original Wassermann test, the reagents are standardized to conform to this amount. For convenience, they are diluted so that quantities of 0.1 cc., or 0.2 cc., may be pipetted in the final test.

### *General Directions*

For measuring quantities of fluid less than 0.1 cc., as in titrations of amboceptor and complement, use a pipette of 0.2-cc. capacity graduated to 0.01 cc. For measuring quantities of from 0.1 cc. to 1 cc., use a pipette of 1-cc. capacity graduated to 0.1 cc.[2]

An exception is made in pipetting sensitized cells because the rapid addition of 0.2 cc. of cells to each of a series of tubes is facilitated by the use of a 2-cc. pipette graduated to 0.1 cc.[3]

For quantities of from 1 cc. to 10 cc., use a 2-cc., 5-cc., or 10-cc. pipette. For quantities of between 10 cc. and 50 cc., use a 10-cc. pipette, but for quantities of 50 cc. or more, use a graduated cylinder.

[1] The cholesterin furnished by some manufacturers has been found less satisfactory than that procured from other firms. C. P. Pfanstiehl Cholesterol is used.

[2] For measuring 0.1-cc. quantities of antigen and complement, pipetting machines, manufactured by Hipple of the University of Wisconsin, have been found to provide increased accuracy and speed. The machines are run by electricity and are accurately adjusted so that, with each stroke of a piston, exactly 0.1 cc. is delivered.

[3] A machine adjusted to deliver 0.2-cc. quantities may also be used for pipetting sensitized cells provided that the material is agitated sufficiently to keep the cells in suspension.

In adding the salt solution to equalize the volume of liquid in the different tubes of titrations and complement-fixation tests, use a dropping bottle.

For accuracy, take a minimum of 0.1 cc. of fluid as the basis for any dilution. When only a small amount of a high dilution is required, as in titrating amboceptor, make a 1:100-dilution and prepare from that the higher dilution desired. Mix the diluted reagent thoroughly by pouring back and forth from one flask to another at least three times, or, if there are only a few cubic centimeters of it, by drawing up in a clean 5-cc. or 10-cc. pipette and expelling at least three times.

In carrying out titrations or tests for which the directions are given in tabular form, pipette the reagents in the order indicated beginning at the left. If the first reagent is to be measured in quantities of less than 0.1 cc., pipette it to the bottom of each tube. Pipette the other reagents from the top, and shake the rack after the addition of each reagent. Add the salt solution in such a way as to wash down any reagents which may have adhered to the side of the tube.

Shake the suspension of sheep, red blood cells thoroughly before taking each pipetteful, and before withdrawing the portion for preparing sensitized cells, in order to obtain a representative sample. During the water-bath incubation of titrations or tests to which the cells have been added, shake the racks sufficiently to keep the cells in suspension. Do not shake the racks so continuously or violently as to cause foaming of the liquid.

Record the readings of all titrations and all necessary data concerning them, directly on the cards designed for each. Indicate the result in each tube of the titration by means of the same symbols which are used to denote corresponding degrees of inhibition of hemolysis in complement-fixation tests. Although in reading complement-fixation tests less than 10-per-cent inhibition is disregarded, in recording results of titrations use the symbol "±," which represents slight fixation, to indicate perceptible inhibition to 20-per-cent inhibition of hemolysis.

### *Suspension of Sheep Red Blood Cells*

To see if the 5-per-cent suspension of sheep cells is of approximately standard density, dilute a 0.5-cc. quantity to 200 cc. with salt solution, in a volumetric flask, and determine its turbidity in a candle turbidimeter. If the turbidity lies between 220 and 230, consider the suspension to be satisfactory. If the turbidity does not lie between these values, make the necessary adjustments, and determine that the turbidity of the adjusted suspension is satisfactory, before use.

*Antisheep Amboceptor*

For the test of the first, trial bleeding, dilute a sample of the hemolytic serum 1:100. For tests of later bleedings, prepare higher dilutions, depending upon the titer previously recorded.

As it is inadvisable to use for hemolytic amboceptor a serum which has any appreciable agglutinating effect, test the agglutinating, as well as the hemolytic, properties. To test the agglutinating properties, add 0.1 cc. of each dilution of the serum, and 0.3 cc. of salt solution to tubes containing 0.1 cc. of a 5-per-cent suspension of sheep cells, and incubate for fifteen minutes at 37°C. If no agglutination is evident at that time, make a second reading after the test has stood overnight in the cold room. Discard any serum which causes agglutination in the highest dilution which is hemolytic in 0.05 cc.

TABLE 4

*Amboceptor titration*

| TUBE NUMBER | AMBOCEPTOR | COMPLEMENT (1:15) | SHEEP CELLS (5 PER CENT) | SALT SOLUTION | INCUBATION |
|---|---|---|---|---|---|
| | *cc.* | *cc.* | *cc.* | *drops* | |
| 1 | 0.10 | 0.1 | 0.1 | 3 | Incubate for fifteen minutes in the water-bath at 37°C. |
| 2 | 0.09 | 0.1 | 0.1 | 3 | |
| 3 | 0.08 | 0.1 | 0.1 | 3 | |
| 4 | 0.07 | 0.1 | 0.1 | 3 | |
| 5 | 0.06 | 0.1 | 0.1 | 3 | |
| 6 | 0.05 | 0.1 | 0.1 | 4 | |
| 7 | 0.04 | 0.1 | 0.1 | 4 | |
| 8 | 0.03 | 0.1 | 0.1 | 4 | |
| 9 | 0.02 | 0.1 | 0.1 | 4 | |
| 10 | 0.01 | 0.1 | 0.1 | 4 | |

In determining the hemolytic titer, if there is no amboceptor of known titer on hand and there is, consequently, no standardized complement to use in titrating the new amboceptor, make the preliminary titrations with complement, diluted 1:15. Use 0.1 cc. of this dilution, which amount represents at least two units, and usually an excess, of complement.

Perform the titration of amboceptor as shown in table 4.

Determine from the average of titrations with at least five different specimens of complement, diluted 1:15, the lowest dilution of amboceptor which gives complete hemolysis in 0.05 cc. Adopt this dilution temporarily as containing two amboceptor units in 0.1 cc. and use it for titrating complement.

After the complement unit is determined as described under titration of pooled complement, determine the standard amboceptor unit accurately by titrations on at least five different days with complement diluted to contain two units in 0.1 cc.

Definition: The standard amboceptor unit is arbitrarily regarded as the least quantity required to give complete hemolysis of 0.1 cc. of a 5-per-cent suspension of sheep cells in the presence of 2 units of complement after incubation for fifteen minutes in a water-bath at 37°C.

For convenience, adopt as the standard dilution of amboceptor that which contains two standard units in 0.1 cc. After one amboceptor has been standardized by the procedure described, standardize all new amboceptors directly with complement diluted to contain two units in 0.1 cc.

TABLE 5

*Test of individual guinea-pig serum for hemolytic activity*

| TUBE NUMBER | GUINEA-PIG SERUM (1:10) | SALT SOLUTION | SENSITIZED CELLS | INCUBATION |
|---|---|---|---|---|
| | *cc.* | *drops* | *cc.* | |
| 1 | 0.04 | 3 | 0.2 | Incubate for fifteen minutes |
| 2 | 0.03 | 4 | 0.2 | in the water-bath at 37°C. |
| 3 | 0.02 | 4 | 0.2 | |

*Complement*

On the day the guinea pigs are bled, that is, the day before the serum is to be used for complement, the sera of the individual animals are subjected to the following tests, to prevent the inclusion in the pooled complement of guinea-pig sera which are deficient in hemolytic activity, which contain excessive amounts of natural antisheep amboceptor, or which show, to a marked degree, the property of combining with antigen when no syphilitic serum is present. The testing of individual sera for nonspecific fixability with antigen is of especial importance when only a few guinea pigs are bled, since the presence in the complement of one serum possessing this property may delay hemolysis of the antigen controls. As guinea-pig sera have more frequently been found to fix nonspecifically with the cholesterinized antigen than with the noncholesterinized antigen, it is sufficient to make this preliminary test with the cholesterinized antigen only.

**Preliminary Test of Hemolytic Activity.**—Test each guinea-pig serum for hemolytic activity as indicated in table 5.

Record the least amount of the diluted serum which gives complete hemolysis.

Include in the pooled complement only the guinea-pig sera which give complete hemolysis in 0.04 cc., or less, of the 1:10-dilution.

**Test for Natural Antisheep Amboceptor.**—Test each guinea-pig serum for natural antisheep amboceptor, including with each group of tests a control of the cells suspension, as indicated in table 6.

TABLE 6

*Test of individual guinea-pig serum for natural antisheep amboceptor*

| TUBE NUMBER | GUINEA-PIG SERUM (1:10) | SHEEP CELLS (5 PER CENT) | SALT SOLUTION | INCUBATION |
|---|---|---|---|---|
| | *cc.* | *cc.* | *cc.* | |
| 1 | 0.1 | 0.1 | 0.3 | Incubate for fifteen minutes in the water-bath at 37°C. |
| 2 | None | 0.1 | 0.4 | |

TABLE 7

*Test of individual guinea-pig serum for nonspecific fixability*

| TUBE NUMBER | CHOLESTERINIZED ANTIGEN (DILUTION IN USE) | GUINEA-PIG SERUM (1:20) | FIXATION* | SENSITIZED CELLS | SECONDARY INCUBATION |
|---|---|---|---|---|---|
| | *cc.* | *cc.* | | *cc.* | |
| 1 | 0.2 | 0.1 | Fix for two hours in the refrigerator at from 3 to 6°C. | 0.2 | Incubate in the water-bath at 37°C. until hemolysis is complete |

* In the routine complement-fixation tests, the period for fixation is four hours at from 3 to 6°C., but in these preliminary tests, the complement and antigen are exposed for only two hours at from 3 to 6°C., it having been found that guinea-pig sera which are markedly affected by exposure with the antigen for four hours are usually perceptibly affected in the shorter period. A sample of each serum is tested also with fixation for one-half hour at 37°C., since, as explained under performing the test, certain supplementary tests with cholesterinized antigen are fixed at 37°C.

Centrifugalize each tube to deposit the cells, and record the degree of hemolysis in each.

The hemolysis is due to natural antisheep amboceptor in the guinea-pig serum providing the cells control shows no hemolysis.

Include in the pooled complement only guinea-pig sera containing no natural amboceptor, or a very slight amount, according to this test.

**Test for Nonspecific Fixability.**—Test each guinea-pig serum for nonspecific fixability as indicated in table 7.

Record the time required for complete hemolysis with each guinea-pig serum.

Include in the pooled complement only the sera with which the sensitized cells are hemolyzed in less than ten minutes.

**Titration of Pooled Complement** (80).—On the day the complement is to be used, pool the specimens of guinea-pig serum found suitable in the preliminary tests, dilute a sample of the pooled serum, not less than 0.5 cc., 1:40, and titrate its hemolytic activity as shown in table 8.

TABLE 8

*Complement titration*

| TUBE NUMBER | COMPLEMENT (1:40) | SALT SOLUTION | SENSITIZED SHEEP CELLS | INCUBATION |
|---|---|---|---|---|
| | *cc.* | *drops* | *cc.* | |
| 1 | 0.15 | 3 | 0.2 | Incubate for fifteen minutes in the water-bath at 37°C. |
| 2 | 0.14 | 3 | 0.2 | |
| 3 | 0.13 | 3 | 0.2 | |
| 4 | 0.12 | 3 | 0.2 | |
| 5 | 0.11 | 3 | 0.2 | |
| 6 | 0.10 | 4 | 0.2 | |
| 7 | 0.09 | 4 | 0.2 | |
| 8 | 0.08 | 4 | 0.2 | |
| 9 | 0.07 | 4 | 0.2 | |
| 10 | 0.06 | 4 | 0.2 | |
| 11 | 0.05 | 4 | 0.2 | |
| 12 | 0.04 | 4 | 0.2 | |

In the complement titration, use sensitized cells prepared from portions of the 5-per-cent sheep-cell suspension and the standard dilution of amboceptor prepared for the day's work.

The sensitized cells prepared for the complement titration may be used also for any complement-fixation tests of which the fixation period is completed within the next two hours.

Prepare the sensitized cells required for the complement-fixation tests fixed for four hours in the cold room at from 3 to 6°C. from the diluted amboceptor remaining, not more than ten or fifteen minutes before it is time to add them to the first set of tests. As practically all the tests done with four hours' fixation are completed within a period of one and one-half or two hours, use the same lot of sensitized cells for all.

Definition: The unit of complement is arbitrarily regarded as the least quantity required to give complete hemolysis of 0.1 cc. of a 5-per-cent suspension of

sheep cells sensitized with two units of antisheep amboceptor, after incubation for fifteen minutes in a water-bath at 37°C.

Dilute the complement for use in complement-fixation tests, and titrations of amboceptor or antigen, so that two units are contained in 0.1 cc. For example, if the unit is 0.1 cc. of a 1:40-dilution, prepare a 1:20-dilution for tests, or if the unit is 0.11 cc., prepare a 1:18.2-dilution.

After the complement is diluted to contain two units in 0.1 cc., make a control titration of amounts graded from 0.1 to 0.01 cc., to determine if it will give complete hemolysis in 0.05 cc. but not in 0.04 cc.

### *Antigen*

Antigen, before use in complement-fixation tests, is titrated for lytic, anticomplementary, and antigenic properties, the anticomplementary and antigenic properties being determined from titrations with at least twenty different specimens of pooled complement, during a period of at least four weeks. The dilution of optimum sensitivity, within the range which is antigenic but not lytic or anticomplementary, is then determined by titrations and tests of syphilitic sera.

**Procedure.**—Dilute the acetone-insoluble antigen as follows: Evaporate to dryness one part of antigen, by pipetting the antigen on a watch glass of about six-inch diameter or in an evaporating dish and placing it in front of an electric fan. Then suspend the dried residuum in two and one-half parts of distilled water. For example, if 3 cc. of antigen is evaporated to dryness, suspend the dried residuum in 7.5 cc. of distilled water. Add the first cubic centimeter slowly with a 0.2-cc. pipette and mix as thoroughly as possible with a glass rod after the addition of each 0.2 cc. The remainder of the water may be added rapidly, but make sure that the suspension is thoroughly mixed. Add the appropriate amounts of this suspension to the amounts of 0.85-per-cent salt solution necessary to make the dilutions desired. Mix well by shaking.

Dilute the cholesterinized antigen as follows: Pipette the antigen to the bottom of a 1-liter beaker. Place the required amount of 0.85-per-cent salt solution in another liter beaker. Pour the salt solution on to the antigen as rapidly as possible and mix thoroughly by pouring back and forth from one beaker to the other, several times.

In titrations of cholesterinized antigens, make a comparison of dilutions prepared by both slow and rapid mixture of extract and salt solution, and then strictly adhere to the more favorable method.

**Titration of Hemolytic Properties.**—Extracts of dried heart tissue

are occasionally hemolytic in low dilutions, and before being titrated for antigenic or anticomplementary properties must be titrated to determine the lowest dilution which will exclude hemolytic substances.

**Procedure.**—For the first titration of hemolytic activity, dilute a sample of the antigen, 1:10, as a no more concentrated dilution would be used in any case, and test higher dilutions if necessary.

Perform the titration as illustrated in table 9.

TABLE 9

*Titration of hemolytic properties of antigen*

| TUBE NUMBER | ANTIGEN (1:10) | COMPLEMENT (1:10) | SHEEP CELLS (5 PER CENT) | SALT SOLUTION | INCUBATION |
|---|---|---|---|---|---|
| | *cc.* | *cc.* | *cc.* | *drops* | |
| 1 | 0.4 | 0.1 | 0.1 | 0 | Incubate for fifteen minutes in the water-bath at 37°C. |
| 2 | 0.2 | 0.1 | 0.1 | 3 | |
| 3 | 0.1 | 0.1 | 0.1 | 5 | |
| 4 | None | 0.1 | 0.1 | 6 | |
| 5 | None | None | 0.1 | 7 | |

TABLE 10

*Titration of anticomplementary properties of antigen*

| TUBE NUMBER | ANTIGEN | COMPLEMENT (2 UNITS) | SALT SOLUTION | FIXATION* | SENSITIZED SHEEP CELLS | SECONDARY INCUBATION |
|---|---|---|---|---|---|---|
| | *cc.* | *cc.* | *drops* | | *cc.* | |
| 1 | 0.4 | 0.1 | None | Fix for four hours in the refrigerator at from 3 to 6°C. | 0.2 | Incubate for fifteen minutes in the water-bath at 37°C. |
| 2 | 0.2 | 0.1 | 1 | | 0.2 | |
| 3 | 0.1 | 0.1 | 2 | | 0.2 | |

* Cholesterinized antigen is also titrated with one-half-hour fixation at 37°C. since supplementary tests with cholesterinized antigen are made by this method in certain cases, as described under performing the test.

Centrifugalize the tubes to deposit the cells, and record the degree of hemolysis in each. Make a second reading after the test has stood in the cold room overnight.

If there is any hemolysis in tubes 1 to 3 and none in the control tubes 4 and 5, titrate higher dilutions to determine that which is not hemolytic.

**Titration of Antigenic and Anticomplementary Properties.**—Beginning with the lowest dilution of antigen which is not hemolytic in 0.4 cc., titrate the antigen for anticomplementary properties as shown in table 10, and for antigenic properties as shown in table 11.

Place with the titrations for the secondary incubation, a control of the sensitized cells, containing 0.2 cc. of sensitized cells and 0.3 cc. of salt solution.

Leave the titrations and cells control in the cold room overnight, to allow the cells to settle, and then record the results.

Approximately the same results may be obtained if the titrations are centrifugalized and read immediately.

At the time the titrations are read, the control of the sensitized cells should show no hemolysis. In the titration of anticomplementary properties, all three tubes should show complete hemolysis. If any of these tubes shows the slightest inhibition of hemolysis, higher dilutions are titrated. In the titration of antigenic properties, the first three tubes should show fixation, that is, inhibition of hemolysis, and the fourth tube, which is the control of the syphilitic serum, should show complete hemolysis.

TABLE 11

*Titration of antigenic properties of antigen*

| TUBE NUMBER | FOUR-PLUS SYPHILITIC SERUM | ANTIGEN | COMPLEMENT (2 UNITS) | SALT SOLUTION | FIXATION* | SENSITIZED SHEEP CELLS | SECONDARY INCUBATION |
|---|---|---|---|---|---|---|---|
| | *cc.* | *cc.* | *cc.* | *drops* | | *cc.* | |
| 1 | 0.02 | 0.1 | 0.1 | 2 | Fix for four hours in the refrigerator at from 3 to 6°C. | 0.2 | Incubate for fifteen minutes in the water-bath at 37°C. |
| 2 | 0.02 | 0.05 | 0.1 | 3 | | 0.2 | |
| 3 | 0.02 | 0.025 | 0.1 | 4 | | 0.2 | |
| 4 | 0.02 | None | 0.1 | 4 | | 0.2 | |

* Cholesterinized antigen is also titrated with one-half-hour fixation at 37°C. since supplementary tests with cholesterinized antigen are made by this method in certain cases, as described under performing the test.

If the fixation is complete with the smallest amount of a given dilution of antigen tested, make further titrations to find the highest dilution of which 0.1 cc. will give complete fixation.

Since the least amount which is antigenic, that is, which fixes complement in the presence of syphilitic serum, varies with the syphilitic serum used, it must be determined from the average of titrations with different four-plus sera.

**Determination of Optimum Dilution of Antigen.**—Compare different dilutions of antigen within the range which is antigenic, but not lytic or anticomplementary, in titrations of "four-plus" syphilitic sera as indicated in table 12, to determine the dilutions of antigen giving marked or complete fixation with the least amount of syphilitic serum.

Compare different dilutions of antigen within the range of greatest sensitivity, as above determined, in complement-fixation tests of partially reacting syphilitic sera, as indicated in table 13, and select as

optimum the dilution giving the greatest degree of fixation in these tests.

The dilution of antigen found to be optimum with fixation at from 3 to 6°C. is used also for tests fixed at 37°C., providing it gives complete fixation with syphilitic serum known to give a four-plus reaction and is not anticomplementary in four times the test dose, with fixation at 37°C.

**Comparative Trial of New Antigens.**—Compare a new antigen, in its optimum dilution, with a previously standardized antigen, in parallel tests of at least five hundred human sera, including not fewer than two hundred from treated cases of syphilis, and not fewer than one hundred from presumably nonsyphilitic cases. If the results show close agreement, consider the new antigen to be satisfactory for use.

TABLE 12

*Titration of syphilitic serum*

| TUBE NUMBER | FOUR-PLUS SYPHILITIC SERUM | | ANTIGEN | COMPLEMENT (2 UNITS) | SALT SOLUTION | FIXATION | SENSITIZED SHEEP CELLS | SECONDARY INCUBATION |
|---|---|---|---|---|---|---|---|---|
| | Undiluted | Diluted (1:10) | | | | | | |
| | *cc.* | *cc.* | *cc.* | *cc.* | *drops* | | *cc.* | |
| 1 | 0.05 | None | 0.1 | 0.1 | 1 | Fix for four hours in the refrigerator at from 3 to 6°C. | 0.2 | Incubate for fifteen minutes in the water-bath at 37°C. |
| 2 | 0.02 | None | 0.1 | 0.1 | 2 | | 0.2 | |
| 3 | None | 0.1 | 0.1 | 0.1 | None | | 0.2 | |
| 4 | None | 0.05 | 0.1 | 0.1 | 1 | | 0.2 | |
| 5 | None | 0.02 | 0.1 | 0.1 | 2 | | 0.2 | |
| 6 | None | 0.01 | 0.1 | 0.1 | 2 | | 0.2 | |
| 7 | 0.05 | None | None | 0.1 | 3 | | 0.2 | |
| 8 | 0.02 | None | None | 0.1 | 4 | | 0.2 | |
| 9 | None | None | 0.1 | 0.1 | 2 | | 0.2 | |
| 10 | None | None | 0.4 | 0.1 | None | | 0.2 | |

After a cholesterinized antigen has been standardized as described, titrate portions of the same extract subsequently cholesterinized in the same dilution as the cholesterinized antigen in use, and carry out only a brief series of complement-fixation tests to determine if the reactions with the new cholesterinized portion agree with those given by the antigen in use.

**Controls of Antigen in Use.**—For control of the stability of the antigen as well as the adjustment of the other reagents, include a titration of both the acetone-insoluble and the cholesterinized antigen, in the dilutions used, with each day's complement-fixation tests.

## THE COMPLEMENT-FIXATION TEST

### *Performing the Test*

Perform the test of blood serum with a control test of a syphilitic serum known to give a four-plus reaction and controls of the anti-complementary properties of the antigen as illustrated in table 13.

TABLE 13

*Complement-fixation test with blood serum*

| TUBE NUMBER | PATIENT'S SERUM | FOUR-PLUS SYPHILITIC SERUM | ANTIGEN | COMPLEMENT (2 UNITS) | SALT SOLUTION | FIXATION* | SENSITIZED SHEEP CELLS | SECONDARY INCUBATION |
|---|---|---|---|---|---|---|---|---|
| **Back row** | | | | | | | | |
| | *cc.* | *cc.* | *cc.* | *cc.* | *drops* | | *cc.* | |
| 1 | 0.05 | None | None | 0.1 | 3 | Fix for four hours in the refrigerator at from 3 to 6°C. | 0.2 | Incubate for fifteen minutes in the water-bath at 37°C. |
| 2 | 0.02 | None | None | 0.1 | 4 | | 0.2 | |
| 3 | None | 0.05 | None | 0.1 | 3 | | 0.2 | |
| 4 | None | 0.02 | None | 0.1 | 4 | | 0.2 | |
| **Front row** | | | | | | | | |
| 1 | 0.05 | None | 0.1 | 0.1 | 1 | Fix for four hours in the refrigerator at from 3 to 6°C. | 0.2 | Incubate for fifteen minutes in the water-bath at 37°C. |
| 2 | 0.02 | None | 0.1 | 0.1 | 2 | | 0.2 | |
| 3 | None | 0.05 | 0.1 | 0.1 | 1 | | 0.2 | |
| 4 | None | 0.02 | 0.1 | 0.1 | 2 | | 0.2 | |
| 5 | None | None | 0.1 | 0.1 | 2 | | 0.2 | |
| 6 | None | None | 0.4 | 0.1 | None | | 0.2 | |

* Four hours' fixation in the cold room at from 3 to 6°C. is used as a routine with both the acetone-insoluble and the cholesterinized antigen, as this method has generally proved more sensitive than fixation at 37°C. Occasionally, however, syphilitic sera have been found to give definite fixation at 37°C. which give slight or no fixation at from 3 to 6°C. As this type of reaction has been observed more frequently in early primary cases than in advanced cases of syphilis, supplementary tests are made with cholesterinized antigen with fixation for one-half hour in the water-bath at 37°C. on sera from untreated cases with history of a recent initial lesion if they fail to give definite fixation at from 3 to 6°C. (81, 82).

Perform the test of spinal fluid as shown in table 14, including the same controls of syphilitic serum and antigen as are shown in table 13.

When a large number of specimens is to be examined, it is necessary to test them in groups of a limited number. In practice, tests of eighteen specimens, a control test of syphilitic serum and antigen controls, as illustrated, are included in each set. The tests with the acetone-insoluble and the cholesterinized antigens are performed independently by different workers, as agreement in the results of two separate tests supplies a check on technical accuracy.

*Preparation of Color Standards (83)*

As an aid in making uniform readings, prepare from the reagents in use each day, color standards containing known proportions of hemolyzed and unhemolyzed cells.

TABLE 14

*Complement-fixation test with spinal fluid*

| TUBE NUMBER | SPINAL FLUID | ANTIGEN | COMPLEMENT (2 UNITS) | SALT SOLUTION | FIXATION | SENSITIZED CELLS | SECONDARY INCUBATION |
|---|---|---|---|---|---|---|---|
| | | | Back row | | | | |
| | *cc.* | *cc.* | *cc.* | *drops* | | *cc.* | |
| 1 | 0.4 | None | 0.1 | None | Fix for four hours in the refrigerator at from 3 to 6°C. | 0.2 | Incubate for fifteen minutes in the water-bath at 37°C. |
| 2 | 0.2 | None | 0.1 | 2 | | 0.2 | |
| | | | Front row | | | | |
| 1 | 0.2 | 0.1 | 0.1 | 1 | Fix for four hours in the refrigerator at from 3 to 6°C. | 0.2 | Incubate for fifteen minutes in the water-bath at 37°C. |
| 2 | 0.1 | 0.1 | 0.1 | 2 | | 0.2 | |

TABLE 15

*Preparation of color standards*

| TUBE NUMBER | INHIBITION OF HEMOLYSIS | HEMOGLOBIN SOLUTION | | SHEEP CELLS (5 PER CENT) | COMPLEMENT (2 UNITS) | ANTIGEN | SALT SOLUTION |
|---|---|---|---|---|---|---|---|
| | | Undiluted | Diluted (1:10) | | | | |
| | *per cent* | *cc.* | *cc.* | *cc.* | *cc.* | *cc.* | *cc.* |
| 1 | 100 | None | None | 0.1 | 0.1 | 0.1 | 0.2 |
| 2 | 95 | None | 0.05 | 0.095 | 0.1 | 0.1 | 0.155 |
| 3 | 75 | 0.025 | None | 0.075 | 0.1 | 0.1 | 0.2 |
| 4 | 50 | 0.05 | None | 0.05 | 0.1 | 0.1 | 0.2 |
| 5 | 25 | 0.075 | None | 0.025 | 0.1 | 0.1 | 0.2 |
| 6 | 10 | 0.09 | None | 0.01 | 0.1 | 0.1 | 0.2 |

Centrifugalize 10 cc. of the 5-per-cent sheep-cell suspension in a graduated tube. Remove the supernatant liquid and add sufficient distilled water to bring the volume to 9 cc. When the cells are completely laked, add 1 cc. of 8.5-per-cent salt solution to make the hemoglobin solution isotonic. Dilute a small amount of the hemoglobin

solution, 1:10, with 0.85-per-cent salt solution, and prepare standards as indicated in table 15. Wipe dry with a bit of cotton the outside of the pipettes used to measure the hemoglobin solution and the cells suspension, so that only the indicated amount of each of these reagents will be delivered. Centrifugalize the standards representing 50-per-cent or more inhibition and use the others with the cells in suspension.

*Reading and Recording Results*

When hemolysis is complete in the antigen controls, which is usually the case after fifteen minutes' incubation at 37°C., read each test which shows complete hemolysis in the controls containing patient's serum or spinal fluid without antigen. If the controls of any individual test are not hemolyzed at this time, reincubate the test at 37°C.

Compare tubes showing less than 50-per-cent inhibition with the standards containing cells in suspension; centrifugalize tubes showing 50-per-cent or more inhibition and compare with the centrifugalized standards. If not convenient to centrifugalize the tubes immediately, place them in ice water. If the patient's serum is tinged with hemoglobin, make controls containing 0.05 cc. and 0.02 cc. of serum and 0.1 cc. each of antigen, complement, salt solution, and 5 per cent of cells. Centrifugalize the serum controls and allow for the hemolysis shown by these controls when estimating the percentage inhibition in the test.

Record in the space indicated for each antigen on the accession sheet, the degree of fixation in each tube according to the following scale:

| *Percentage of inhibition of hemolysis* | *Degree of fixation*[4] |
|---|---|
| None (or less than 10 per cent) | None, − |
| 10–20 per cent | Slight, ± |
| 25–45 per cent | Partial, + |
| 50–70 per cent | Marked, 2+ |
| 75–95 per cent | Nearly complete, 3+ |
| Complete (or more than 95 per cent) | Complete, 4+ |

After the reactions have been read by the examiner responsible, they are read by an assistant, the examiner checking the result.

In the case of specimens which are anticomplementary, that is, which show delayed hemolysis in the tubes without antigen, record, under "Remarks," the time required for hemolysis of the controls. If

[4] For convenience, the plus signs are omitted, and merely the symbols −, ±, 1, 2, 3, and 4 are recorded on the accession sheet.

the controls do not hemolyze within an hour, note the fact above the reading, and record, under "Remarks," the degree of fixation in the controls.

Repeat tests of anticomplementary specimens, providing they are not hemolyzed or apparently contaminated. In case such specimens give complete fixation in the usual amounts, include on repetition smaller amounts, see method of testing diluted serum, table 12. Repeat also any tests showing evidence of technical inaccuracy, as well as tests in which there is a marked discrepancy between the results with the two antigens, or in which the reaction is not in agreement with the results of previous examinations.

*Reporting Results*

Follow the general directions for reporting results under methods of reporting results.

Compute the result to be reported with each antigen by finding the average of the fixation obtained with the two amounts of serum or spinal fluid, counting "±" as ½, "+" as 1, "2+" as 2, etc., and record in the "Report" column on the accession sheet.

To illustrate, if the reaction is read "3+" in one amount and "+" in the other, the result reported would be "2+." If the average is not a whole number, the next higher value is given to a figure having a fraction equal to one-half or more. For example, if the reaction is read "3+" in one amount and "2+" in the other, the average, which is 2½, would be reported "3+." The only exception to this rule is in the case of tests read "4+" in one amount and "3+" in the other, the average, although 3½, being reported "3+" since fixation is not actually complete.

Report the specimen anticomplementary if twenty minutes or more are required for hemolysis in the controls and no more than partial fixation is obtained in the tubes containing antigen, or if the controls are not hemolyzed within an hour.

When a specimen showing delayed hemolysis in the controls shows slight or partial fixation at the time that the controls are hemolyzed, or when a specimen which is anticomplementary in the usual amounts, even after an hour's incubation, gives marked fixation in smaller amounts, which are not anticomplementary, a statement of the result should accompany the report. In such instances, the age and appearance of the specimen must be considered, as a hemolyzed or contaminated specimen may give an unreliable result.

If a satisfactory result is obtained with both antigens, indicate on the history the fixation with each antigen as follows:

"Cf 4+" (or "Cf 3+, 2+, +, or ±" depending on the degree of fixation), when complement fixation is obtained.

"No cf," when no complement fixation is obtained.

If the specimen is unsatisfactory for examination, or if a satisfactory result can not be obtained with both antigens, indicate on the accession sheet and also on the history blank the nature of the report to be sent according to the report form.

### *Interpretation of Reactions*

A reaction of marked intensity, 2+, 3+, or 4+ is rarely obtained with the noncholesterinized antigen except in cases of syphilis. The reaction with the cholesterinized antigen, which is generally but not invariably more sensitive, serves as a control and affords an opportunity for a comparative study of the reactions, which is of value, particularly in doubtful cases, and in cases receiving antisyphilitic treatment.

In special instances, a partial (+) reaction obtained with the noncholesterinized antigen may be given practically the same interpretation as a reaction of more marked intensity. In general, a slight (±) or partial (+) reaction should not be considered significant unless there is definite clinical evidence that the case is one of syphilis.

In every instance, the results of the test should be interpreted in the light of the clinical data.

## CHAPTER 2

# THE COMPLEMENT-FIXATION TEST FOR TUBERCULOSIS

### OPENING AND NUMBERING SPECIMENS

Follow the directions for opening and numbering specimens given under the complement-fixation test for syphilis. Since each specimen received for the complement-fixation test for tuberculosis is examined also by the test for syphilis, give each specimen a syphilis serial number as well as a tuberculosis serial number, and record the syphilis serial number in the "Remarks" column on the accession sheet.

If a specimen is received with a tuberculosis history blank, stamp the tuberculosis number in the space designated for the serial number on that blank, stamp the syphilis number in the space designated on a syphilis blank, copy the data from the tuberculosis blank on the syphilis blank, and cross-index the two. If a specimen is received with a syphilis history blank, stamp the syphilis number on that blank and the tuberculosis number on a tuberculosis blank, copy the data from the syphilis blank on the tuberculosis blank and cross-index the two.

Refer all histories which are received with specimens for the complement-fixation test for tuberculosis to the worker in charge of the group, so that if further data are needed for our records they may be requested before the examination is completed.

### PREPARATION OF SPECIMENS

Specimens are prepared as for the complement-fixation test for syphilis.

In the case of sera which are not to be tested immediately, as well as standard immune serum, heat for one-half hour at from 54 to 56°C. on three successive days, but inactivate bovine serum for one hour at 58°C. before testing. Repeat the fractional sterilization at from 54 to 56°C. after each time a bottle or tube containing serum is opened. If there is a large amount of serum, dispense in small quantities in order to avoid repeated sterilization.

Bovine sera are inactivated by heating for one hour at 58°C. If a bovine serum which has once been inactivated at 58°C. is to be retested, it is reinactivated in the same manner as human serum, by heating for five minutes at from 54 to 56°C.

## PREPARATION OF REAGENTS

In addition to the serum to be tested, four reagents are employed: a suspension of sheep, red blood cells; antisheep amboceptor; complement; and antigen. A standard immune serum, although not actually a reagent, is necessary for standardization of antigen and for purposes of control.

For washing the red blood cells, 0.85-per-cent salt solution is used. For preparing the suspension of the washed, red blood cells, for the dilution of all reagents and to equalize the volume in the various tubes of tests and titrations, a buffered solution is used, 0.85-per-cent sodium-chloride solution buffered to a pH value of 7.6 by the addition of 10 per cent of Sörensen's borate-buffer mixture (84).

Each of the reagents, except antigens prepared from alcoholic extracts, is kept in the cold room at from 3 to 6°C. when not in use, and complement is kept in a pan of ice water while in use.

### *Suspension of Sheep Red Blood Cells*

The suspension of red blood cells is prepared from citrated sheep blood collected not more than forty-eight hours previously. The blood is washed and the 5-per-cent suspension of cells is prepared as for the complement-fixation test for syphilis, except that the suspension is made in buffered salt solution.

### *Antisheep Amboceptor and Complement*

These reagents are prepared as for the complement-fixation test for syphilis.

### *Antigens*

The antigens are prepared from a virulent strain of the tubercle bacillus of human origin (161). The two antigens used routinely are a dialyzed, distilled-water extract of the tubercle bacillus, and a purified and concentrated antigen prepared from an aqueous extract or a culture filtrate. So far as yet determined, the purified and concentrated antigens prepared from aqueous extracts and culture filtrates react similarly, and may be used interchangeably if indicated in the records.

**Cultures.**—Use for antigen, glycerin-broth cultures of the tubercle bacillus grown to the point of optimum growth, that is, until the pellicle just covers the surface of the medium, which requires from three to four weeks. Sterilize the broth cultures by heating in an Arnold sterilizer for twenty minutes.

To separate the bacillary residue, filter the sterilized culture through soft filter paper until perfectly clear, and sterilize the culture filtrate three days in an Arnold sterilizer. Transfer the bacillary residue to a Buchner funnel and wash rapidly with large quantities of distilled water, using suction. Continue the suction until the organisms are dry and then desiccate over sulfuric acid for two or three days. Store the dried organisms in glass-stoppered bottles sealed with paraffin.

**Dialyzed Distilled-Water Extract.**—Extract 1 gram of the washed and dried tubercle bacilli for one hour with 100 cc. of distilled water under a reflux condenser. Free the extract of sediment by centrifugalization or filtration through soft paper; sterilize the filtrate by heating for fifteen minutes in an Arnold sterilizer, or for thirty minutes on three successive days at from 54 to 56°C. and dialyze in sterile collodion bags in sterile, neutral, distilled water in the cold room. Use precautions to avoid contamination of the extract throughout the filtration and dialysis.

The procedure has been to dialyze 500 cc. of extract in about three liters of distilled water, changing the water twice daily. The removal of lytic and anticomplementary properties, which is the purpose of the dialysis, may possibly be accomplished in less than five days, depending upon the permeability of the membranes used.

**Purified and Concentrated Antigen.**—1. To one liter of a dialyzed aqueous extract prepared as above described, add normal horse serum in the proportion of one part of serum to twenty parts of extract and precipitate the globulins with $CO_2$ gas. Remove the globulin precipitate and extract with successive portions of absolute alcohol until no antigenic properties can be detected in the extracts. Then combine the alcoholic extracts and concentrate *in vacuo* to 100 cc.

The number and period of the alcoholic extractions necessary cannot be exactly prescribed.

Add 500 cc. of absolute alcohol to the globulins precipitated from one liter of the aqueous extract, and extract at room temperature, changing the alcohol on the third, tenth, twentieth, and thirtieth days. Make further extractions, if necessary, for removal of the antigenic substances.

2. Dialyze the culture filtrate to remove lytic and anticomplementary properties: three days in running tap water is usually sufficient. Then prepare the purified and concentrated antigen from the dialyzed filtrate in the same manner as from the dialyzed aqueous extract.

3. Extract 1 gram of the washed and dried tubercle bacilli for one

hour with 100 cc. of culture filtrate, under a reflux condenser. Free the extract of sediment by centrifugalization or filtration through soft paper; sterilize the filtrate by heating for thirty minutes on three successive days at from 54 to 56°C. and dialyze in sterile collodion bags to remove lytic and anticomplementary properties. Then prepare the purified and concentrated antigen from the dialyzed filtrate in the same manner as from the dialyzed aqueous extract.

TABLE 16

*Complement titration**

| TUBE NUMBER | COMPLEMENT | | | BUFFERED SALT SOLUTION | SENSITIZED SHEEP CELLS | INCUBATION |
|---|---|---|---|---|---|---|
| | Dilution number | Amount pipetted | Actual amount of serum present | | | |
| | | cc. | cc. | cc. | cc. | |
| 1 | 1 | 0.1 | 0.004 | 0.2 | 0.2 | Incubate for |
| 2 | 2 | 0.1 | 0.0036 | 0.2 | 0.2 | one hour in |
| 3 | 3 | 0.1 | 0.0032 | 0.2 | 0.2 | the water- |
| 4 | 4 | 0.1 | 0.0029 | 0.2 | 0.2 | bath at 37° |
| 5 | 5 | 0.1 | 0.0026 | 0.2 | 0.2 | C. |
| 6 | 6 | 0.1 | 0.0023 | 0.2 | 0.2 | |
| 7 | 7 | 0.1 | 0.0021 | 0.2 | 0.2 | |
| 8 | 8 | 0.1 | 0.0019 | 0.2 | 0.2 | |
| 9 | 9 | 0.1 | 0.0017 | 0.2 | 0.2 | |
| 10 | 10 | 0.1 | 0.0015 | 0.2 | 0.2 | |

* In carrying out these titrations, and also the tests, it is necessary to shake the racks after the addition of sensitized cells, at intervals of three or four minutes during the entire period of secondary incubation, so that the total action of the complement is exerted upon the hemolytic complex. The variation between the quantities of complement in the different tubes is so small that the difference in total hemolysis due to settling of the cells markedly affects the determination of the unit. This also applies to the test where reactions obtained with only one unit of complement are included in estimating the total fixation of complement and are, therefore, of diagnostic significance.

### *Standard Immune Serum*

The standard immune serum, which is used to test and control the activity of each antigen, is the serum of a horse immunized to the homologous strain of the tubercle bacillus (85). The serum is separated and bottled, and sterilized as directed in connection with patient's serum.

## STANDARDIZATION OF REAGENTS

### *General Directions*

In general, the procedure is the same as in the complement-fixation test for syphilis except that all reagents are diluted with buffered salt solution. In the titration of complement, follow the special technic prescribed in table 16.

### *Suspension of Sheep Red Blood Cells*

To see if the 5-per-cent suspension of sheep cells is of approximately standard density, dilute a 0.5-cc. quantity to 200 cc. with salt solution, in a volumetric flask, and determine its turbidity in a candle turbidimeter. If the turbidity lies between 220 and 230, consider the suspension satisfactory. If the turbidity does not lie between these values, make the necessary adjustments and determine that the turbidity of the adjusted suspension is satisfactory, before use.

### *Antisheep Amboceptor*

Follow the directions in the complement-fixation test for syphilis; standardization of reagents, but incubate the titrations for one hour instead of fifteen minutes in the water-bath at 37°C.

### *Complement*

Pool three or more guinea-pig sera which the preliminary tests, as performed for the complement-fixation test for syphilis, have shown to be of average quality. Determine the hemolytic value of the pooled complement by titrations in duplicate under the following conditions: complement alone, without preliminary incubation; complement alone, and plus the test dose of each antigen incubated for one and one-half hours at 37°C. before the addition of sensitized cells.

As a basis for the graded dilutions for titration, dilute all the pooled complement, 1:10, the actual quantity of the dilution being not less than 20 cc. Add four parts of 1:10 complement to six parts of salt solution in a 100-cc. bottle, the actual quantity of this 1:25 dilution being not less than 10 cc. Mix the 1:25 dilution thoroughly, remove one-tenth part of it to the first of a series of ten test tubes and replace with an equal quantity of salt solution. Mix the dilution, draw it up in the pipette which was used for removing the one-tenth portion and expel at least six times, then remove one-tenth part to the second test tube, replace with salt solution and mix as before. Repeat the operation described until ten dilutions have been prepared. When ready to perform the titrations, transfer 0.1 cc. from each of the ten dilutions of

complement into the corresponding small tubes which are used for titrations. For this purpose, use the upper tenth of an 0.2-cc. pipette, a separate pipette for each dilution, and pipette to the bottom of each tube.

TABLE 17

*Titration of complement after incubation with antigen**

| TUBE NUMBER | COMPLEMEMT | | | ANTIGEN† | BUFFERED SALT SOLUTION | FIXATION | SENSITIZED SHEEP CELLS | SECONDARY INCUBATION |
|---|---|---|---|---|---|---|---|---|
| | Dilution number | Amount pipetted | Actual amount of serum present | | | | | |
| | | cc. | cc. | cc. | cc. | | cc. | |
| 1 | 1 | 0.1 | 0.004 | 0.1 | 0.1 | Fix for one | 0.2 | Incubate for |
| 2 | 2 | 0.1 | 0.0036 | 0.1 | 0.1 | and one- | 0.2 | one hour in |
| 3 | 3 | 0.1 | 0.0032 | 0.1 | 0.1 | half hours | 0.2 | the water- |
| 4 | 4 | 0.1 | 0.0029 | 0.1 | 0.1 | in the water- | 0.2 | bath at 37°C. |
| 5 | 5 | 0.1 | 0.0026 | 0.1 | 0.1 | bath at 37° | 0.2 | |
| 6 | 6 | 0.1 | 0.0023 | 0.1 | 0.1 | C. | 0.2 | |
| 7 | 7 | 0.1 | 0.0021 | 0.1 | 0.1 | | 0.2 | |
| 8 | 8 | 0.1 | 0.0019 | 0.1 | 0.1 | | 0.2 | |
| 9 | 9 | 0.1 | 0.0017 | 0.1 | 0.1 | | 0.2 | |
| 10 | 10 | 0.1 | 0.0015 | 0.1 | 0.1 | | 0.2 | |

* In carrying out these titrations, and also the tests, it is necessary to shake the racks after the addition of sensitized cells, at intervals of three or four minutes during the entire period of secondary incubation, so that the total action of the complement is exerted upon the hemolytic complex. The variation between the quantities of complement in the different tubes is so small that the difference in total hemolysis due to settling of the cells markedly affects the determination of the unit. This also applies to the test where reactions obtained with only one unit of complement are included in estimating the total fixation of complement and are, therefore, of diagnostic significance.

† In the titration of complement incubated without antigen, substitute for 0.1 cc. of antigen, 0.1 cc. of salt solution, thus adding 0.2 cc. of salt solution to each tube.

Perform the titration of complement alone without preliminary incubation, as shown in table 16. Perform the titration of complement incubated with and without antigen as shown in table 17.[1]

The sensitized cells used in the complement titration should be a portion of the same sensitized cells prepared for that day's complement-fixation tests. The method of preparing sensitized cells is the same as that used in the complement-fixation test for syphilis.

[1] The titration of complement by the method used, in which each quantity is exactly one-tenth less than the immediately preceding quantity in the titration, gives a more accurate unit than the usual method of titration in which a progressive decrease in the difference between each quantity and the one immediately preceding occurs.

Definition: The unit of complement is arbitrarily regarded as the least quantity required to give complete hemolysis of 0.1 cc. of 5-per-cent sheep cells sensitized with two units of antisheep amboceptor after incubation for one hour in a water-bath at 37°C.

If the hemolytic unit is not the same in each pair of duplicate titrations, confirm the results by repetition. If the unit of the unincubated and the incubated complement is the same, repeat both sets of titrations. If the unit of the unincubated complement does not lie within the usual limits, 0.0019 to 0.0032 cc. of guinea-pig serum, repeat the titrations with new dilutions of complement. If the repeated titrations of the unincubated complement and the titrations of the incubated complement show the unit to be outside the usual limits, determine if there has been any error in the preparation of the amboceptor or the cells suspension.

If the complement unit in the presence of antigen is greater than 0.004 cc., the complement is unsatisfactory for tests with that antigen.

Dilute the complement for use in titration of amboceptor according to the titer of the unincubated complement, so that two units are contained in 0.1 cc.

Dilute the complement for use in titration of antigen and immune serum according to the titer of the complement incubated without antigen, so that two units are contained in 0.1 cc.

Dilute the complement for use in tests according to its titer in the presence of each antigen, so that one unit is contained in 0.04 cc.

### *Antigen*

Antigen is titrated to determine the lowest dilution which is not lytic or anticomplementary, and the highest dilution which is antigenic. Antibody titrations of the standard immune serum are then carried out with different dilutions of antigen within this range, to determine the dilution of greatest sensitivity. This dilution is selected as optimum for the test, providing it has not more than a slight effect upon the hemolytic activity of complement in the complement titration.

For purposes of comparison and control, different dilutions of antigen within the optimum range are used in complement-fixation tests of tuberculous patients' sera.

For purposes of control, corresponding dilutions of antigen are compared also in complement-fixation tests of sera from presumably nontuberculous cases, including sera giving no fixation as well as those giving complete fixation in the complement-fixation test for syphilis.

**Titration of Hemolytic Activity.**—In the preliminary titration of hemolytic activity, test antigen undiluted[2] excepting alcoholic extracts and those which are highly colored, such as tuberculins and culture filtrates. Dilute alcoholic antigen, 1:10, and dilute deeply colored antigens sufficiently to prevent the color interfering with the reading of the titration. As with antigens used in the complement-fixation test for syphilis, determine whether slow or rapid dispersion in salt solution gives better results and then adhere to the more favorable method.

TABLE 18

*Titration of anticomplementary properties of antigen*

| TUBE NUMBER | ANTIGEN | COMPLEMENT (2 UNITS) | BUFFERED SALT SOLUTION | FIXATION | SENSITIZED SHEEP CELLS | SECONDARY INCUBATION |
|---|---|---|---|---|---|---|
| | *cc.* | *cc.* | *drops* | | *cc.* | |
| 1 | 0.4 | 0.1 | None | Fix for one and one-half hours in the water-bath at 37°C. | 0.2 | Incubate for one hour in the water-bath at 37°C. |
| 2 | 0.2 | 0.1 | 1 | | 0.2 | |
| 3 | 0.1 | 0.1 | 2 | | 0.2 | |

TABLE 19

*Titration of antigenic properties of antigen*

| TUBE NUMBER | STANDARD IMMUNE SERUM | ANTIGEN | COMPLEMENT (2 UNITS) | BUFFERED SALT SOLUTION | FIXATION | SENSITIZED SHEEP CELLS | SECONDARY INCUBATION |
|---|---|---|---|---|---|---|---|
| | *cc.* | *cc.* | *cc.* | *drops* | | *cc.* | |
| 1 | 0.01 | 0.1 | 0.1 | 3 | Fix for one and one-half hours in the water-bath at 37°C. | 0.2 | Incubate for one hour in the water-bath at 37°C. |
| 2 | 0.01 | 0.05 | 0.1 | 4 | | 0.2 | |
| 3 | 0.01 | 0.025 | 0.1 | 4 | | 0.2 | |
| 4 | 0.01 | None | 0.1 | 4 | | 0.2 | |

Perform the titration of hemolytic activity as for the complement-fixation test for syphilis, but incubate it for one hour instead of for fifteen minutes in the water-bath at 37°C.

If the dilution of antigen tested proves hemolytic in 0.4 cc., test higher dilutions.

**Titration of Antigenic and Anticomplementary Properties.**—Beginning with the lowest dilution which is not hemolytic in 0.4 cc., titrate

[2] Before dilution, make aqueous extracts isotonic by adding one part of 8.5-per-cent sodium-chloride solution to nine parts of antigen.

the antigen for anticomplementary properties as shown in table 18, and for antigenic properties as shown in table 19. Place with the titrations for the secondary incubation a control of the sensitized cells, containing 0.2 cc. of sensitized cells, and 0.3 cc. of salt solution.

After the secondary incubation, centrifugalize the tubes which show more than partial inhibition of hemolysis, and record the degree of inhibition in all tubes.

At the time the titrations are read, the control of the sensitized cells should show no hemolysis. In the titration of anticomplementary properties, all three tubes should show complete hemolysis. If any amount of antigen shows the slightest inhibition of hemolysis, titrate higher dilutions. In the titration of antigenic properties, tube 1 should show complete fixation, i.e., complete inhibition of hemolysis, tubes 2 and 3 some degree of fixation, and tube 4, which is the serum control, should show complete hemolysis. If necessary, higher dilutions are tested to find the highest of which 0.1 cc. gives complete fixation.

TABLE 20

*Titration of antibody content of immune serum*

| TUBE NUMBER | IMMUNE SERUM (1:10) | ANTIGEN | COMPLEMENT (2 UNITS) | BUFFERED SALT SOLUTION | FIXATION | SENSITIZED SHEEP CELLS | SECONDARY INCUBATION |
|---|---|---|---|---|---|---|---|
| | *cc.* | *cc.* | *cc.* | *drops* | | *cc.* | |
| 1 | 0.1 | 0.1 | 0.1 | None | Fix for one and | 0.2 | Incubate for |
| 2 | 0.09 | 0.1 | 0.1 | None | one-half | 0.2 | one hour in |
| 3 | 0.08 | 0.1 | 0.1 | 1 | hours in the | 0.2 | the water- |
| 4 | 0.07 | 0.1 | 0.1 | 1 | water-bath | 0.2 | bath at 37°C. |
| 5 | 0.06 | 0.1 | 0.1 | 1 | at 37°C. | 0.2 | |
| 6 | 0.05 | 0.1 | 0.1 | 2 | | 0.2 | |
| 7 | 0.04 | 0.1 | 0.1 | 2 | | 0.2 | |
| 8 | 0.03 | 0.1 | 0.1 | 2 | | 0.2 | |
| 9 | 0.02 | 0.1 | 0.1 | 3 | | 0.2 | |
| 10 | 0.01 | 0.1 | 0.1 | 3 | | 0.2 | |
| 11 | 0.1 | None | 0.1 | 2 | | 0.2 | |

**Determination of Optimum Dilution of Antigen.**—Using different dilutions of antigen within the range which is antigenic, but not lytic or anticomplementary, perform antibody titrations of the standard immune serum as indicated in table 20. Select for use in tests the dilution which gives marked fixation with the least amount of the standard immune serum, providing it has no more than a slight effect upon the hemolytic activity of complement in the complement titration.

Compare the sensitivity of different dilutions of antigen within the optimum range in complement-fixation tests of tuberculous patients' sera as indicated in table 21.

For purposes of control, compare these different dilutions of antigen also in tests of presumably nontuberculous sera, including sera from

nonsyphilitic cases which give no fixation in the complement-fixation test for syphilis, and sera which give complete fixation in the test for syphilis.

TABLE 21

*Complement-fixation test with blood serum*

| TUBE NUMBER | PATIENT'S SERUM | STANDARD IMMUNE SERUM | ANTIGEN | COMPLEMENT* (0.04 CC. = 1 UNIT) | BUFFERED SALT SOLUTION | FIXATION | SENSITIZED CELLS | SECONDARY INCUBATION |
|---|---|---|---|---|---|---|---|---|
| **First row** | | | | | | | | |
| | *cc.* | *cc.* | *cc.* | *cc.* | *drops* | | *cc.* | |
| 1 | 0.05 | None | 0.1 | 0.08 | 1 | Fix for one and | 0.2 | Incubate for |
| 2 | 0.05 | None | 0.1 | 0.06 | 1 | one-half | 0.2 | one hour in |
| 3 | 0.05 | None | 0.1 | 0.04 | 1 | hours in the | 0.2 | the water- |
| 4 | None | 0.01 | 0.1 | 0.08 | 1 | water-bath | 0.2 | bath at 37°C. |
| 5 | None | None | 0.1 | 0.06 | 2 | at 37°C. | 0.2 | |
| 6 | None | None | 0.1 | 0.04 | 2 | | 0.2 | |
| **Second row** | | | | | | | | |
| 1 | 0.05 | None | None | 0.08 | 2 | Fix for one and | 0.2 | Incubate for |
| 2 | 0.05 | None | None | 0.06 | 2 | one-half | 0.2 | one hour in |
| 3 | 0.05 | None | None | 0.04 | 3 | hours in the water-bath at 37°C. | 0.2 | the water- bath at 37°C. |
| **Third row** | | | | | | | | |
| 1 | 0.02 | None | 0.1 | 0.08 | 1 | Fix for one and | 0.2 | Incubate for |
| 2 | 0.02 | None | 0.1 | 0.06 | 2 | one-half | 0.2 | one hour in |
| 3 | 0.02 | None | 0.1 | 0.04 | 2 | hours in the | 0.2 | the water- |
| 4 | 0.02 | None | None | 0.04 | 3 | water-bath at 37°C. | 0.2 | bath at 37°C. |
| **Fourth row** | | | | | | | | |
| 1 | 0.01 | None | 0.1 | 0.08 | 1 | Fix for one and | 0.2 | Incubate for |
| 2 | 0.01 | None | 0.1 | 0.06 | 2 | one-half | 0.2 | one hour in |
| 3 | 0.01 | None | 0.1 | 0.04 | 2 | hours in the | 0.2 | the water- |
| 4 | 0.01 | None | None | 0.04 | 3 | water-bath at 37°C. | 0.2 | bath at 37°C. |

* The complement is pipetted first to the bottom of the tubes, then the serum and antigen from the top.

**Comparative Trial of New Antigens.**—Before employing a new antigen routinely, test it in its optimim dilution parallel with the routine

antigens with at least fifty tuberculous sera, and over a period of at least four weeks.

**Controls of Antigen in Use.**—In addition to the controls included with the daily complement-fixation tests as indicated in table 21, titrate antigens once in every two weeks while in use.

If aqueous antigens are stored in the refrigerator for several days, they may prove anticomplementary unless heated for one-half hour at 54 to 56°C., before use. They are always heated after use, for one-half hour on three successive days at 56°C., to maintain their sterility.

### *Immune Serum*

Test the first trial bleedings of horses used for production of immune serum with homologous antigen. If standardized antigen is available, use the "optimum" dilution for testing immune serum. Otherwise, use one-fourth the largest amount of antigen which is neither lytic nor anticomplementary. Follow the method for testing patient's serum as given in table 21 but make the tests with two units of complement only. For control purposes, test trial bleedings also with the plain and the cholesterinized extracts used in the complement-fixation test for syphilis. Test parallel with the bleedings taken after immunization the "normal" bleedings from the same animal, i.e., the one taken before immunization.

When 0.01 cc. of the immune serum gives complete fixation with homologous antigen, determine its antibody content as indicated in table 20.

Select as a standard immune serum, one which gives complete fixation in 0.005 cc. or less with the homologous-strain tubercle antigen and no fixation in 0.02 cc. with the antigens used in the complement-fixation test for syphilis.

## THE COMPLEMENT-FIXATION TEST

### *Performing the Test*

Perform the test of patient's serum with a control test of the standard immune serum and controls of the anticomplementary properties of the antigen as illustrated in table 21.

### *Reading and Recording Results*

In the experimental study (85) of the complement-fixation reaction in tuberculosis, all readings were made with the cells in suspension. At present all readings are made by comparison with color standards prepared from the reagents in use as described in the complement-

fixation test for syphilis, and recorded in terms of percentage of inhibition of hemolysis. For reporting results of tests, a scale of values, varying somewhat from that used in the complement-fixation test for syphilis, has been tentatively adopted, as corresponding approximately to the values assigned to reactions in the experimental study. Further studies are being made of the color-standard values to determine whether they represent accurately the degree of reaction obtained.

| *Percentages of inhibition of hemolysis* | *Degree of fixation* |
|---|---|
| None (or less than 10 per cent)[3] | None, − |
| 10–30 per cent inclusive | Slight, ± |
| 35–65 per cent inclusive | Partial, + |
| 70–90 per cent inclusive | Marked, 2+ |
| Above 90 and less than 100 per cent | Nearly complete, 3+ |
| 100 per cent | Complete, 4+ |

If there is more than slight (±) fixation in the 0.05-cc. serum control with 1½ units of complement, or fixation in this control together with 40-per-cent or more inhibition in the 0.05-cc. serum control with 1 unit of complement and fixation in the 0.02-cc. serum control with 1 unit of complement, regard the serum as anticomplementary. Always retest an anticomplementary serum with another specimen of complement before reporting it as unsatisfactory. If the serum is but slightly anticomplementary, subtract the fixation in the serum controls from that in the tubes containing antigen, when computing the result.

It is necessary to give special consideration to the effect of anticomplementary properties in cases in which the serum reacts but slightly in the test. In such cases, if there is slight (±) fixation in both the 0.05-cc. serum control with 1½ units of complement and the 0.02-cc. serum control with 1 unit of complement and 25-per-cent inhibition of 0.05-cc. serum control with 1 unit of complement, the serum should be regarded as anticomplementary.

Any fixation in the antigen control is presumably due to technical inaccuracy since the complement unit was determined in the presence of the antigen. If there is fixation in two or more controls of the test dose of antigen, it would indicate an error in dilution of the complement and necessitate repetition of the test.

### *Reporting Results*

Compute the result to be reported as follows: Find the total fixation obtained in the nine tubes by adding together the reactions in all the tubes and subtracting the sum of the anticomplementary reactions in

[3] In all serum- and antigen-control tubes, record as ± the presence of even a trace of unhemolyzed cells, that is, less than 10-per-cent inhibition of hemolysis.

the corresponding serum-control tubes. On the basis of the total fixation obtained, report the result of the test as follows:

| | |
|---|---|
| Total fixation less than 4+ | = no cf. (no complement fixation) |
| Total fixation 4+ to $9\frac{1}{2}$+ inclusive | = ± (slight fixation) |
| Total fixation 10+ to $20\frac{1}{2}$+ inclusive | = + (partial fixation) |
| Total fixation 21+ to $32\frac{1}{2}$+ inclusive | = 2+ (marked fixation) |
| Total fixation 33+ to $35\frac{1}{2}$+ inclusive | = 3+ (nearly complete fixation) |
| Total fixation 36+ | = 4+ (complete fixation) |

*Letters to Precede or Accompany Reports*

When inadequate clinical data are given, send a letter requesting further information and indicate that the report is to be held until the data required are received. (This letter should be sent as soon as the specimen is received.)

When the clinician has given no definite diagnosis but has recorded the symptoms observed, send the report accompanied by a letter requesting the final diagnosis.

When a serum, which is not hemolyzed or contaminated, is to be reported anticomplementary, and it has given considerably greater fixation in the tubes containing antigen than in the controls, send a letter stating that the results are doubtful and requesting that another specimen be submitted.

*Interpretation of Reactions*

A study of the results obtained in tuberculous and nontuberculous cases has indicated that a degree of fixation classified as "slight" or better with a tubercle antigen may be interpreted as the specific reaction of an active process if confirmed by clinical manifestation. The significance of fixation with tubercle antigens is indeterminate, however, in cases in which the serum reacts also in the complement-fixation test for syphilis, on account of the possibility of cross fixation.

# SECTION IV

# THE ANATOMIC EXAMINATION OF TISSUES AND THE EXAMINATION OF ANIMALS FOR EVIDENCE OF RABIES

## CHAPTER 1

## THE ANATOMIC EXAMINATION OF TISSUES

### NUMBERING SPECIMENS

On receipt of specimens for anatomic examination, number the history blank as described under specimens for examination, opening and recording.

### EXAMINATION AND FIXATION OF GROSS SPECIMENS

Examine each specimen and write a concise description on the reverse side of the accompanying history blank. Select the parts necessary for microscopic examination and cut small blocks not larger than 2 by 2 by 0.3 centimeters. If the specimen has not been fixed, or if it shows evidence of incomplete fixation, place these small pieces in Zenker's fluid or 10-per-cent formalin, or in a fresh solution of the fixative in which the specimen was received; otherwise, place them in 90-per-cent alcohol for dehydration and embedding. When Zenker fixation is desired, place the small pieces of fresh tissue in Zenker's fluid and fix for from eight to twenty-four hours. Then wash thoroughly in running water for from twelve to twenty-four hours and preserve in 80-per-cent alcohol. When formalin fixation is desired, allow the small pieces to remain in 10-per-cent formalin for at least eight hours. Mark a tag with the miscellaneous number of the specimen, the year, and the number of pieces of tissue; draw a circle around the latter number and also, if other than formalin fixative is used, mark the tag to indicate the fixative used. Loop the string of the tag over the neck of the bottle which contains the blocks of tissue.

#### *Dehydrating and Embedding Tissue*

After the tissue has been properly fixed, dehydrate and clear it by immersing the pieces in the following series:

90-per-cent alcohol: 3 hours

95-per-cent alcohol: 3 to 4 hours
Absolute alcohol: 5 hours
Xylol: 8 to 16 hours

Transfer from xylol to melted paraffin in a 56- to 60°C.-incubator for from one to three hours.

In making these transfers, observe the following precautions:

Note the number of blocks indicated on the tag and be sure that none are left in the bottle.

Transfer the tag immediately after transferring the tissues.

Keep all bottles tightly stoppered.

Remove the tissues from the incubator, transfer them to a smooth, glazed surface, arrange two right-angled metal supports in the form of a rectangle around them, and fill the box so formed with melted paraffin, taking care that the tissues remain at the bottom of the box. Place the string of the tag in one corner of the paraffin so that when it congeals, the string will be securely fastened to the block. Allow the paraffin to congeal firmly, then remove the metal frames and cut away the surplus paraffin so that a small block containing the tissue is left. Trim the opposite surfaces parallel and close to the tissue. Place the blocks and the tag immediately in envelopes, $2\frac{1}{2}$ by $4\frac{1}{4}$ inches, which are used especially for this purpose. Mark in the upper, right-hand corner of each envelope the serial number and other information given on the tag. If more than one envelope is necessary to hold the blocks from one specimen, use two or more, but indicate on each, in addition to the above information, the number of envelopes used. Remelt the excess paraffin in the incubator and use it indefinitely.

### *Cutting Sections*

Mount a well-sharpened microtome knife in the holder of the microtome and clamp securely. Do not allow the edge of the blade to come in contact with hard objects. When bone or other hard material is to be cut, use a knife kept for the purpose or a safety-razor blade in a suitable holder.

Heat about 1 liter of water to steaming and pour it in a large flat dish. Place this on a hot plate near the microtome, and with the current turned off allow the water to cool to from 46 to 50°C. Thereafter, by regulating the hot plate, keep the water as near this temperature as possible.

Place several pieces of ice in a pan and add water to a depth of about one inch.

Open an envelope containing blocks to be sectioned. Compare the

number of blocks with the number indicated on the envelope and note the fixative. Fasten the blocks of one specimen only on the warmed object holders and keep them immersed in the iced water until they are cut. Clamp an object holder with a block in the microtome. Observe the following directions:

1. Adjust the knife holder so that the inner surface of the knife lies very nearly perpendicularly.
2. Adjust the surface of the block parallel to the line of the edge of the knife.
3. Adjust the knife holder so that the block does not quite come in contact with the edge of the knife; then lock the knife holder.
4. Set the advancing mechanism so that sections will be six microns in thickness.

Proceed to cut sections and float them on the warm water, stretching the "ribbon" to smooth out the wrinkles. Using a diamond-point pencil, mark slides at one end with the miscellaneous number, the year, and the abbreviation for the fixative used. Place a small drop of Mayer's glycerin-albumin mixture on a slide and rub it evenly over the surface. Float the sections on to the slide, drain off the excess of water and place the slide in a slide box. After all the sections have been cut and mounted, place the box containing them in the 56°C.-incubator for from one-half to one hour. Then remove the box and allow the slides to cool to room temperature.

### *Clearing Sections*

Transfer the slides to a glass staining dish, and treat as follows:

| | |
|---|---|
| Xylol: | 3 minutes |
| Absolute alcohol: | 2 minutes |
| 95-per-cent alcohol: | 1 minute |
| 70-per-cent alcohol: | 1 minute |

Wash in water.

If the sections are from Zenker-fixed tissues, immerse the slides for fifteen minutes in the Weigert modification of Gram's iodine solution. Following this, remove the excess of iodine by washing thoroughly in water and immersing in at least two changes of 80-per-cent alcohol until the sections are colorless; then wash in water. A fairly satisfactory mordant for formalin-fixed tissue, when the stain calls for Zenker fixation, is obtained by immersing the slides in Zenker's fluid, to which the acetic acid has not been added, for from twelve to sixteen hours, washing thoroughly in water, and then treating with iodine and alcohol, as with Zenker-fixed tissues.

After clearing, do not allow the sections to become dry; if it is not possible to begin the staining at once, keep them in water.

### *Staining Sections*

Stain sections from all specimens with hematoxylin and eosin.

Other stains are also used when requested by the pathologist. Although better results are often obtained by slight variations in the technic of staining, the directions given should be carefully followed.

In the dehydration of sections after staining with hematoxylin and eosin, do not leave the slides in alcohol longer than necessary, as some of the stains may be extracted. Keep the 90- and 95-per-cent alcohol slightly tinged with eosin as otherwise practically all the eosin is extracted from the sections. Leave the slides in xylol until the sections are clear.

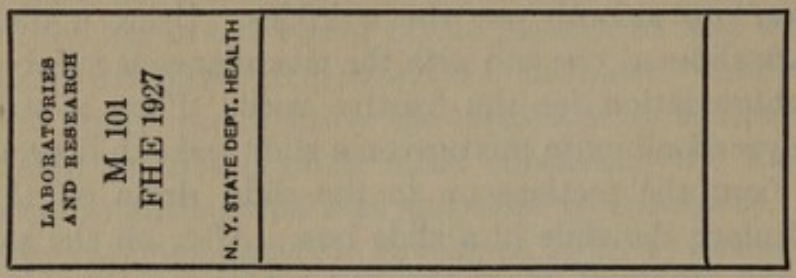


FIG. 27. SLIDE PREPARED FOR ANATOMIC EXAMINATION OF TISSUES

### *Mounting Sections*

Take one slide at a time from the xylol and remove the excess fluid with a piece of clean cloth. Place a drop of balsam on the section immediately, and then place a cover glass over the section. Remove the excess balsam from the edges of the cover glass with a cloth dipped in xylol.

### *Labeling Slides*

Use the labels especially designed for the purpose; these are thick enough to allow slides to be placed over one another without permitting the cover glass to come in contact with the second slide. Paste a label on one end of the slide and mark on it the same data that are on the slide and, in addition, indicate the stain used. Then paste a narrow strip, the thickness of the label, at the other end of the slide, see figure 27.

### *Microscopic Examination of Specimens*

As soon as the sections are prepared, place them in order in the tray and arrange the history blanks also in order for examination by the

pathologist who will record the result of the examination on the original history blank. After the examination is completed, file the slides.

### *Filing Gross Material, Blocks, and Slides*

Gross material which is not considered of value is discarded at the discretion of the pathologist.

**Procedure.**—Examine valuable material, add sufficient fixative, and seal the containers so that they are air-tight; then place them in a special box, list the miscellaneous numbers and tie the box securely. Paste labels on the box indicating its number and the year, and store it. Enter the miscellaneous numbers on index cards in order, also the number of the box, and the year in which the specimens were received.

Put in a permanent file, all paraffin blocks. Store the envelopes or jars and index them in a manner similar to that used for the gross material.

File all slides and keep them in special cabinets for at least six months. After this time, file permanently those on which the diagnosis is based, and file or discard the rest at the discretion of the pathologist. Keep an index of the sections in a manner similar to that used for gross material.

# CHAPTER 2

# THE EXAMINATION OF ANIMALS FOR EVIDENCE OF RABIES

## EXAMINATION OF BRAIN FOR NEGRI BODIES

### NUMBERING SPECIMENS

On receipt of specimens for examination for evidence of rabies, record the name of the messenger, and the time and date the specimen was received, on the history blank. Then number the history blank as described under specimens for examination, opening and recording. If all of the required data have not been given, attempt to obtain them from the physician or health officer in charge as soon as possible. If they are not available before the completion of the examination, send a form letter and the blank, "Information regarding specimens examined for evidence of rabies," with the first written report.

### REMOVAL OF THE BRAIN

Protect the hands with heavy, rubber gloves and the eyes with goggles. Spread paper on the work table and place the specimen on the paper. Hold the head firmly on the table and with a heavy scalpel or carving knife make a median, longitudinal incision through the skin, fascia, and muscles of the cranium to about the level of the eyes. Extend this incision laterally on both sides from a point slightly above the occipital protuberance, reflecting the soft tissue on both sides. Saw longitudinally through both parietal bones, about one inch lateral to the mid-line. Saw transversely through the skull just above the eyes. Insert bone forceps through the anterior bone incision and lift off the top of the skull. If necessary, broaden the opening through the skull by breaking away the sides with bone forceps. Remove the dura and expose the cerebral hemispheres. With a scalpel, or other suitable instrument, sever the nerves on the underside of the cerebrum, cut the tentorium and the spinal cord and remove the brain with as little laceration as possible. Place it in a paraffined-paper cup provided with a cover or in a sterile, Petri plate. After the brain has been removed, wrap the remains in the paper, remove at once to the furnace and supervise their incineration. Wash the table with 10-per-cent formalin

or 5-per-cent crude carbolic acid. Sponge the gloves in 10-per-cent formalin or 5-per-cent crude carbolic acid before removing from the hands, and then sterilize them with the instruments by boiling.

## EXAMINATION OF BRAIN

### *Macroscopic Examination*

Record on the back of the history blank the gross appearance of the brain, noting especially the state of preservation, the presence or absence of adhesions, presence or absence of exudate, and whether the blood vessels are injected.

### *Microscopic Examination*

**Selection of Material.**—Number three slides as for any other miscellaneous examination (See: specimens for miscellaneous examination, opening and recording), designating one for the cerebral cortex, one for the hippocampus, and one for the cerebellum.

Remove from the cerebral cortex, near the Rolandic fissure, a wedge-shaped piece of tissue about 2 mm. long, 2 mm. wide, and deep enough to include the entire thickness of the gray matter. Place this tissue at the numbered end of the corresponding slide.

Expose the hippocampus by making a vertical incision in the posterior third of a cerebral hemisphere, into the lateral ventricle and remove from it a cross section about 1 mm. in thickness. Place this tissue on the corresponding slide, tease away the outer white integument and discard it.

Cut the cerebellum across and remove a piece of tissue, parallel to the cut surface, about 1 mm. thick, 1 mm. wide, and extending from the periphery to the center. Place this tissue on the corresponding slide.

**Preparation of Films.**—With a clean slide or cover slip used as a spreader, press evenly and firmly upon the tissue until it covers almost the width of the slide. Then draw the spreader toward the opposite end of the slide, maintaining an even pressure. Discard the spreader by dropping it into 10-per-cent formalin or 5-per-cent crude, carbolic-acid solution. Place the films immediately in the picric-acid, methyl-alcohol fixative (See: stains and reagents) for about ten seconds and blot. Flood each film with modified Van Giesen's stain and gently heat it in a low flame to steaming. Wash the film in tap water and blot dry.

**Examination of Films.**—Examine each film first with the low-power

objective to locate large isolated nerve cells. Examine these cells with the oil-immersion lens for the presence of Negri bodies. In all cases, other than those in which typical Negri bodies are found, make additional films from the other half of the brain and examine not less than ten preparations for at least one hour.

All examinations should be confirmed by another examiner.

**Recording Findings.**—Record on the back of the history blank the microscopic findings, noting especially the condition of the large cells, the presence or absence of Negri bodies or suggestive structures, and the region where found.

It is desirable also to encircle interesting objects with an object marker.

### PRELIMINARY REPORT OF RESULTS

When Negri bodies are found, report immediately by telephone or telegraph to the health officer and to any physician concerned. Note on the history blank the date and the time this report was sent. When no Negri bodies are found or only suggestive but atypical bodies are present, report by telephone or telegraph and inform the health officer, or physician that paraffin sections are being prepared and that a more definite report will be available in a day or two. Base the preliminary report on a consideration of both sets of preparations.

### PREPARATION OF STAINED SECTIONS

Fix in Zenker's fluid small pieces of brain substance not over 1 by 1 by 0.2 centimeter in size from the three parts above mentioned, using ten parts of fluid to one of tissue; fix for exactly eight hours and wash them in running water for from eight to twenty-four hours. Preserve in 80-per-cent alcohol. Dehydrate and clear by immersing for one-half hour in 90-per-cent alcohol, one-half hour in 95-per-cent alcohol, one hour in absolute alcohol, one-half hour in xylol, one-half hour in xylol paraffin, and one-half hour in paraffin in the incubator. Embed in the usual manner and cut duplicate sections from each block. Stain one set, by Mallory's method, with eosin and methylene blue, and the other by Goodpasture's method with carbol anilin fuchsin. Give such specimens an anatomic serial number.

### ANIMAL INOCULATION

Inoculate animals with tissue from all brains other than those in which typical Negri bodies are found.

*Preservation of Material*

In all cases, remove pieces of gray matter from the cortex, hippocampus, and cerebellum and place them in sterile, neutral, C.P. glycerin, using three parts of glycerin to one of tissue. The material from each region should be about 1 by 1 by 0.5 centimeter in size. Store these in the cold room for three months unless used before the expiration of that period. Whenever the brain is heavily contaminated or decomposed, leave the material in glycerin for at least three days before using it for inoculation.

*Preparation of Material*

Withdraw, from the container, strips of the glycerinated tissue, using sterile technic. Remove the glycerin by rinsing in a few changes of sterile 0.85-per-cent salt solution and grind the strips in a sterile mortar with a small amount of pulverized glass. Then add from eight to ten parts of the salt solution and emulsify thoroughly; allow the emulsion to settle and use the supernatant fluid for inoculation. When it is necessary to carry this material to another part of the building for the animal inoculation, transfer it to a sterile, sputum jar.

*Technic*

Inoculate two guinea pigs weighing from 250 to 300 grams in each case. Anaesthetize the animals with ether. Wet the skin over the skull with alcohol, parting the hair in the mid-line while doing this. With a sterile scalpel, make a longitudinal incision about one-half inch long in the exposed skin. Cut through the subcutaneous tissue down to the bone. Spread the edges apart and with a small drill or trephine bore a hole through the skull one-quarter of an inch to one side of the mid-line. With a small syringe (22 to 26 gauge) attached to a needle three-quarters of an inch in length, inject from 0.2 to 0.3 cc. of the suspension into the brain. Withdraw the syringe and press the skin flaps together.

*Observation of Animals*

Place together only guinea pigs inoculated with material from the same specimen. Attach a tag to the cage, figure 28, and make a record of each inoculation on a special 4-by-6-inch card, see figure 29. Observe the animal daily for nine weeks unless it dies earlier.

If it dies within six days of the inoculation, death is probably due to some intercurrent infection and not to rabies.

In this instance, inoculate another animal from the glycerinated material. If the guinea pig dies after seven days, remove the brain and examine for Negri bodies. If no typical Negri bodies are found, complete the autopsy to determine, if possible, the cause of death. If

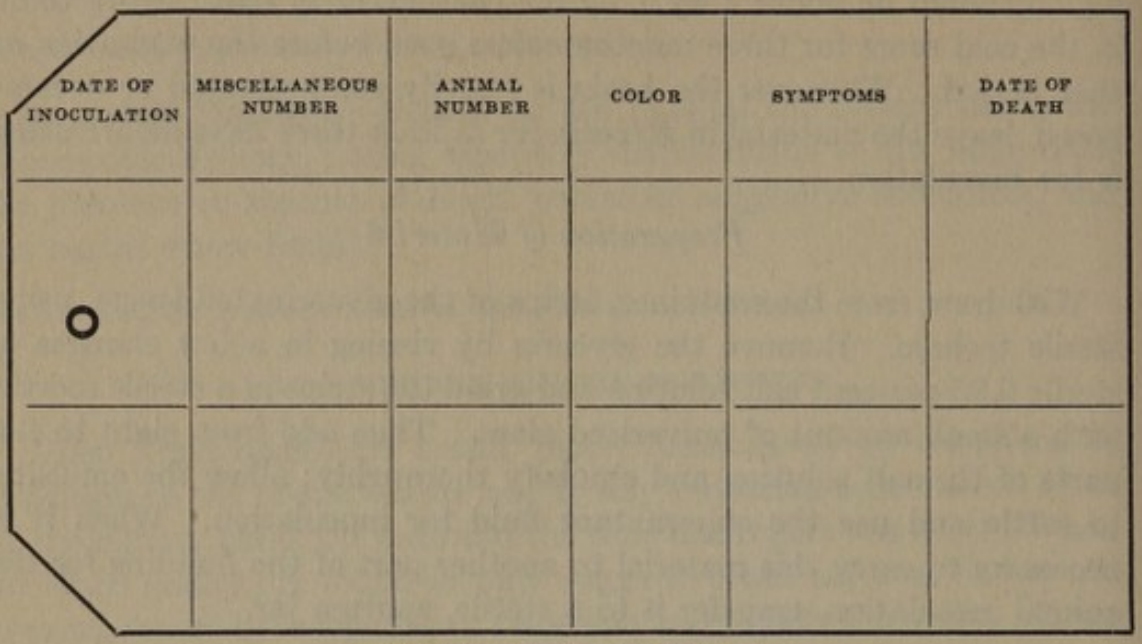


FIG. 28. ANIMAL CAGE TAG

RECORDS OF ANIMAL INOCULATIONS FOR RABIES

| SERIAL NUMBER OF DOG'S BRAIN | REPORT ON MISCELLANEOUS EXAMINATION OF DOG'S BRAIN | SERIAL NUMBER OF GUINEA PIG AUTOPSY | DATE OF GUINEA PIG INOCULATION | GUINEA PIG NUMBER | DATE OF DEATH | RESULT OF AUTOPSY | DATE FOR FINAL REPORT IF NEGATIVE | DATE REPORTED |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |
| | | | | | | | | |
| | | | | | | | | |
| | | | | | | | | |

FIG. 29. RECORD CARD

the animals are alive at the end of nine weeks, give them to the serum-diagnosis group to be killed, so that the blood will be available for use in that group.

For the care of these animals, see animals, experimental and test.

## REPORTING RESULTS

### *Preliminary Report*

1. "No Negri bodies were found in the microscopic examination of the (kind of animal) brain, received (date). Animals will be inoculated and a final report will be made within nine weeks," when no Negri bodies are found.

2. "Structures resembling Negri bodies but which were not perfectly typical in morphology were found in the microscopic examination of the (kind of animal) brain, received (date). Animals will be inoculated and a final report will be made within nine weeks. Persons bitten should be given vaccine treatments," when suggestive but not typical Negri bodies are found.

3. "The microscopic examination of the (kind of animal) brain was unsatisfactory due to the decomposition which had occurred in transit. Animals will be inoculated, and a final report will be made within nine weeks," when the brain is very soft and contaminated.

### *Final Report*

1. "Evidence of rabies was found in the microscopic examination of the (kind of animal) brain, received (date)," when intracellular Negri bodies are found.

2. "Animals inoculated with (kind of animal) brain, received (date) have shown definite evidence of rabies," when definite Negri bodies have been found in the guinea pigs' brains.

3. "Animals inoculated with the (kind of animal) brain, received (date) have not developed rabies up to nine weeks after inoculation," when the guinea pigs are alive nine weeks after inoculation.

# SECTION V

# THE EXAMINATION OF MISCELLANEOUS SPECIMENS

## CHAPTER I

## GENERAL INFORMATION AND INSTRUCTIONS

In this chapter are included directions for the general examination of discharges, body fluids, and miscellaneous specimens which comprise those submitted to be examined for evidence of the communicable diseases that occur infrequently in New York State, and specimens which must be collected from the patient at the time the examination is made.

### OPENING AND RECORDING SPECIMENS

When recording specimens for miscellaneous examination (See: specimens for examination, opening and recording), save each container until the nature of the desired examination has been ascertained. Discard in the furnace any container in which a specimen has been submitted for examination for pathogenic spore-bearing bacilli, such as anthrax, or tetanus.

When specimens are received on slides, write at one end with a diamond-point pencil "M," the serial number, and the year.

Whenever a miscellaneous specimen is to be examined for tubercle bacilli, diphtheria bacilli, or organisms of the enteric-disease group, cross-index the specimen in one of these other routine series, as indicated. The results of such examinations are incorporated in the general miscellaneous report. Should typhoid bacilli or diphtheria bacilli be isolated during the course of a routine miscellaneous examination, cross-index the specimen at this time.

When specimens are received from other laboratories for confirmatory examination, refer them to the bacteriologist in charge of the group who will assist in the work.

Whenever a request for an autogenous vaccine accompanies a specimen, refer the matter to the bacteriologist in charge of the group who will notify the physician who sent it that autogenous vaccines are not made in this laboratory as a routine procedure. Ascertain if he wishes the specimen sent elsewhere.

## GENERAL BACTERIOLOGICAL EXAMINATION

### MICROSCOPIC

Make four dried preparations of the specimen, marking the slides O.F. (Original Film) for later identification.

Stain one by Gram's method, and, if indicated, one by Ziehl-Neelsen's method for tubercle bacilli. The unstained slides are available for further study as indicated by the results of these examinations.

Record the results obtained.

### CULTURAL

#### *Aerobic*

**Plating Specimens.**—Inoculate a series of plates of: blood agar, if a general bacteriological examination is requested; blood-extract agar, if an examination for *B. influenzae* is requested.

#### *Anaerobic*

Employ anaerobic methods when the clinical history indicates the presence of anaerobic bacteria, or that the specimen is likely to contain bacteria that may develop more rapidly under anaerobic conditions. Cultures may be made in:

chopped-meat medium;
deep, dextrose agar;
the usual media, incubated in a hydrogen jar.

#### *Partially Anaerobic*

Certain cultures, when isolated directly from lesions, may develop more satisfactorily under conditions of partial anaerobiosis obtained in:

deep, dextrose-agar cultures;
partial $CO_2$ tension.

#### *Fishing Colonies*

After incubation, fish the colonies according to the nature of the organisms as indicated by the characteristics of the colony formation, by the morphology and Gram-staining reactions, or by both, to suitable subculture media, as follows:

Gram-positive cocci which, on blood agar, show an area of hemolysis or methemoglobin, suggesting streptococci or pneumococci—to blood-agar slants and to pneumococcus broth.

Gram-negative cocci which, on blood agar, present a glistening appearance, suggesting meningococci—to blood-agar slants or to serum-dextrose-agar slants.

Gram-positive cocci which are opaque on blood agar, suggesting staphylococci—to Loeffler's serum medium.

Gram-negative bacilli which are opaque on blood agar, with or without a zone of hemolysis, suggesting *B. coli*—to triple-sugar Andrade agar.

Gram-negative bacilli which on blood-extract agar form transparent, bluish-grey colonies with raised centers, suggesting *B. influenzae*—to coagulated-blood agar and dextrose-infusion agar. If growth appears on the first dextrose-agar culture, always make a second subculture from this first transfer, since the growth on the first culture may be due to the presence of blood carried over from the original medium.

Gram-negative bacilli which, on the differential media, suggest organisms of the enteric-disease group—to triple-sugar Andrade agar. Refer this work to the bacteriologist in charge of the cultural examinations for organisms of the enteric diseases.

If the original material contains an organism which spreads over the plates, making isolation of different organisms impossible, plate the material on medium containing tellurite which inhibits the growth of many species of spreading bacilli but affects very little the growth of Gram-positive cocci or molds.

### *Identification of Cultures Fished*

After incubation, make Gram-stained preparations of all the cultures. If the organisms cannot be identified, make further tests. Table 22 shows the characteristics by which some of the common pathogenic microörganisms may be identified.

## BLOOD CULTURES

When a blood culture is received in the standard Keidel outfit, open the tube in a flame. (Work in a draft-free room, if possible.) Transfer about 15 cc. of the culture with a bulb Pasteur pipette to a 6-by-$\frac{5}{8}$-inch test tube.

Make slide preparations of the culture and stain by Gram's method, and with methylene blue.

If Gram-negative bacilli are present, have an agglutination test made with typhoid immune serum. If the organisms are motile, and characteristic agglutination is obtained, the results may be reported.

TABLE 22

*The characteristics by which some of the common pathogenic microörganisms may be identified*

| NAME OF MICROÖRGANISM | STAINING REACTION | MORPHOLOGY | COLONY CHARACTERISTICS | CULTURAL REACTIONS | SEROLOGICAL TESTS |
|---|---|---|---|---|---|
| *Streptococcus hemolyticus* | Gram-positive | Cocci in chains | Blood agar, hemolysis | Insoluble in bile | |
| *Streptococcus viridans* | Gram-positive | Cocci in chains | Blood agar, methemoglobin | Insoluble in bile | |
| Pneumococcus | Gram-positive | Cocci in pairs or chains—usually lanceolate and encapsulated | Blood agar, methemoglobin | Soluble in bile | Specific agglutination in type serum except type IV |
| *B. influenzae* | Gram-negative | Bacilli—varying in size from short, coccoid to long thread-like bacilli | Blood-extract agar, bluish grey with raised center | No growth on dextrose, infusion agar | |
| Meningococcus | Gram-negative | Biscuit-shaped micrococci, generally in pairs | Blood agar, small and glistening | Ferments dextrose and maltose; not saccharose nor lactose | Specific agglutination in type serum |
| *B. coli* | Gram-negative | Bacilli, without spores | Endo's agar, red, with metallic luster | Acid and gas in triple-sugar Andrade agar, 10 per cent or more of gas in lactose broth; indol formed; nitrates reduced; gelatin not liquefied; acid produced in litmus milk | |
| Organisms of the enteric-disease group | Gram-negative | Bacilli, without spores | See: Cultural examination of organisms of the enteric-diseases | | |
| *Staphylococcus albus* | Gram-positive | Cocci, in micrococcus grouping | Blood agar, white, opaque | Loeffler's serum medium, white growth | |
| *Staphylococcus aureus* | Gram-positive | Cocci, in micrococcus grouping | Blood agar, yellowish, opaque | Loeffler's serum medium, orange-yellow growth | |

Make confirmatory tests, as described under typhoid bacillus, isolation of from blood.

If organisms other than Gram-negative bacilli are present, streak blood-agar plates.

If no organisms are seen, inoculate the following media.

Inoculate two 50-cc. amounts of dextrose-beef-infusion broth with from 3 cc. to 5 cc. of the blood culture.

Inoculate two beef-infusion-agar pour plates, with about 2 cc. of the culture.

Streak two blood-agar plates.

Inoculate two deep tubes of dextrose-beef-infusion agar.

Incubate one set of these preparations aerobically and the other under partial $CO_2$ tension. If, after incubation, any growth appears on the plates, fish colonies to identify the organisms.

If no growth appears in any of the media after twenty-four hours' incubation, reincubate the subcultures and observe daily for one week. If no growth appears after forty-eight hours, send a preliminary report. If no growth appears by the end of the week, and the broth culture is cloudy, make a morphologic examination. If growth is found, make the further examinations that are necessary. If no growth is then found, send the final report.

When blood for cultural examination is received in any outfit other than the standard Keidel tube, inoculate enrichment broth at once, and then, after incubation, proceed as described above.

### ANIMAL INOCULATION

Inoculate a guinea pig in the following instances: when no acid-fast bacilli are found in the stained preparations and the history of the case indicates that the lesion may be due to tuberculosis; when acid-fast bacilli are found in specimens of urine, feces, or in any case where there is the slightest doubt as to the identity of the organisms seen.

#### *Preparation of Specimen*

When the specimen is fluid and not badly contaminated, centrifugalize part of it directly, to concentrate the organisms. When the specimen is contaminated with a large number of other organisms, as in feces or sputum, digest part of it before centrifugalizing, according to Petroff's method (57). To from 3 to 5 cc. of the specimen add an equal amount of 3-per-cent NaOH and incubate the mixture at from 35 to 37°C. for one hour. At the end of this time, neutralize the emulsion

with an equivalent solution of HCl using litmus paper or phenolphthalein as an indicator. Centrifugalize the digested material for one-half hour, and then pour the supernatant liquid into a jar of disinfectant.

### *Inoculation of Animals*

Suspend the sediment obtained by either method of preparing the specimen in from 1 to 2 cc. of sterile 0.85-per-cent salt solution and inoculate the suspension subcutaneously in the groin of a guinea pig.

Make a record of each inoculation on a special card, 4 by 6 inches, see figure 30.

### *Inspecting and Killing Animals*

Inspect the animals carefully at least once a week, renumbering any of which have lost their identification tags, and isolating in special cages

RECORD OF ANIMAL INOCULATION

| ANIMAL NUMBER | WEIGHT | COLOR | MISCELLANEOUS NUMBER | MATERIAL | DATE INOCULATED | DATE KILLED | DATE OF AUTOPSY | DATE REPORTED |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | | |
| | | | | | | | | |
| | | | | | | | | |

FIG. 30. RECORD CARD

any that show enlarged glands, skin lesions, or any evidence of illness. In the case of discharging glands, make stained preparations from the discharge by the Ziehl-Neelsen method. If tubercle bacilli are found, proceed with the autopsy at once. Kill and autopsy all animals which have enlarged glands, at the end of five weeks.

Kill all other animals which have not died within eight weeks and observe the following procedure:

Obtain from the file the miscellaneous history blank bearing the original record of the inoculation and check the animal as to tag number, material inoculated, color, and sex. Give the examinations made at autopsy a new miscellaneous serial number and cross-index it with that of the original history blank. Then record the weight of the animal and give it to the serum-diagnosis group to be killed so that the blood will be available for use in that group. If later autopsy findings should indi-

cate the presence of tuberculosis or other lesions, notify the serum-diagnosis group immediately, so that the blood can be discarded.

### *Autopsies*

**Killed Guinea Pigs.**—Special autopsy procedure: Examine carefully the inguinal and axillary glands and remove any that show nodules, abscesses, or any enlargement. Then proceed with the autopsy, examining the spleen, liver, kidneys, adrenals, mesenteric lymph nodes, and finally the lungs for tuberculous lesions. If no abnormalities are noted, report without making any further tests. If any definite or suggestive lesions are noted, remove a portion of each abnormal organ and make stained preparations by the Ziehl-Neelsen method. If no tubercle bacilli are found in these, make cultures to eliminate the possibility of a secondary infection which produces lesions of pseudotuberculosis, and place a portion of each organ affected in 10-per-cent formalin for anatomic examination. Whenever there is any doubt concerning the examination of the gross material, have portions of the liver, spleen, lungs, and lymph nodes examined anatomically. Enter the date of the autopsy and the date of reporting the result of the autopsy on the record cards.

**Guinea Pigs Found Dead.**—Whenever a test animal dies, follow the procedure given above. If a guinea pig dies a month or more after inoculation, make preparations from the regional glands so that if tubercle bacilli are found a definite report can be made.

If no evidence of tuberculosis is noted, make direct slide preparations for Gram's stain, cultures from all abnormal organs, and from the heart's blood. Place portions of the abnormal organs in 10-per-cent formalin to be used for anatomic examination, if the microscopic and cultural examinations are indefinite.

Record the results of the autopsy on the record slip.

If the autopsy findings are markedly at variance with results of the preliminary examination, refer the matter to the bacteriologist in charge of the group so that inquiries may be made with regard to the outcome of the illness. Also make a search for inaccuracies which might have occurred.

## REPORTING RESULTS

Report the results of miscellaneous bacteriological examinations according to the following general form:

*Microscopic Examination*

"———were found with Gram's stain."
"———were found with Ziehl-Neelsen's stain."

In the case of feces or specimens from the urogenital tract, report "Acid-fast bacilli were found," or "No acid-fast bacilli were found." For all other specimens, report "Tubercle bacilli were found" or "No tubercle bacilli were found."

*Cultural Examination*

"———were found in pure culture," or
"———was the predominating organism found."
"———was also present."

*Blood Cultures*

"———was isolated from the blood culture submitted in (kind of outfit)," when an organism has been isolated and identified.

Preliminary report after forty-eight hours' incubation: "No growth of bacteria was obtained in the blood culture submitted in (kind of outfit)."

Final report after seven days' incubation: "No growth of bacteria was obtained in the blood culture submitted in (kind of outfit)."

"Specimen of blood submitted in (kind of outfit) was unsatisfactory for examination, due to the presence of contaminating organisms."

*Animal Inoculation*

"A guinea pig has been inoculated with some of the specimen. The result of the animal inoculation will be reported in about eight weeks," when a report of the presence of tubercle bacilli or acid-fast bacilli is made; or

"A guinea pig has been inoculated with some of the specimen to determine the presence of tubercle bacilli. The result of the animal inoculation will be reported in about eight weeks," when no acid-fast bacilli are found.

*Autopsies*

"The autopsy of guinea pig (tag number), inoculated on (date), shows definite evidence of tuberculosis," when the animal is killed or dies, and lesions of tuberculosis are found.

"The autopsy of guinea pig (tag number), inoculated on (date),

shows no evidence of tuberculosis," when the animal is killed eight weeks after inoculation and no lesions of tuberculosis are found.

"Guinea pig (tag number), inoculated on (date), died (date) of intercurrent infection. If another specmen is submitted, the animal inoculation will be repeated," when the animal dies within six weeks after inoculation and no evidence of tuberculosis is found. If the inciting organism is not isolated, state that it "died apparently of intercurrent infection."

"Guinea pig (tag number), inoculated on (date), died (date) of intercurrent infection. No evidence of tuberculosis was found at autopsy," when the animal dies a few days before the date set for killing and no tuberculous lesions are found at autopsy. If the inciting organism is not isolated, state that it "died apparently of intercurrent infection."

Record on the lower margin of the history blank the total number of examinations made, noting the number of chemical, bacteriological, microscopic examinations, and animal tests.

## CHAPTER 2

# THE EXAMINATION OF SPINAL AND OTHER BODY FLUIDS

## SPINAL FLUIDS

Examine spinal fluids as soon as received.

### *Recording Appearance*

Record the appearance as follows:

| | |
|---|---|
| Opacity | clear<br>slightly cloudy<br>cloudy |
| Color | whatever is noted |
| Presence of blood | tinged with hemoglobin<br>very small amount<br>moderate amount<br>large amount |
| Presence of coagulum | |

### *Preparation of Specimens*

**For Cell Count.**—Shake the specimen vigorously. Pour all but about 1 cc. of the fluid into a sterile, centrifuge tube and use the material left in the original tube for the count.

**For Microscopic Examination.**—If a coagulum is present, remove it to be stained by the Ziehl-Neelsen method. Centrifugalize the specimen for one-half hour. Then pour the supernatant fluid into a sterile, test tube and prepare films from the sediment, using a fine capillary pipette to obtain the material.

**For Cultural Examination.**—Plate some of the sediment obtained.

**For Chemical Examination.**—Test the supernatant fluid.

**For Animal Inoculation.**—Suspend what remains of the sediment in from 1 to 2 cc. of the supernatant fluid or in 0.85-per-cent salt solution.

**For Complement-Fixation Test.**—Give at least 2 cc. of the supernatant fluid to the serum-diagnosis group.

### *Cell Count*

If red blood cells are present, a direct count is usually of little value and the result should be reported as unsatisfactory unless the ratio of red to white blood cells differs markedly from that found in the blood.

**Direct Count.**—Make a cell count on all satisfactory specimens received from suspected cases of encephalitis, poliomyelitis, meningitis, and whenever it is requested.

Before making the count, shake the specimen vigorously. Draw glacial acetic acid up to the mark "11" of a white-cell pipette and expel it. Then fill the pipette with the fluid, shake well, and transfer a drop to the counting chamber. Count all the cells in the nine large squares of the ruled areas and multiply by 10/9 to obtain the number of cells found per cubic millimeter. Calculate the average from a count of two drops before making the report. If the specimen contains so many cells that it is impossible to make an accurate count in this manner, dilute the fluid as for a leucocyte count.

**Differential Count.**—Prepare a film from the sediment, stain it with Loeffler's alkaline methylene blue and record the relative number of lymphocytes or mononuclear and polymorphonuclear leucocytes.

### *Microscopic Examination of Stained Preparations*

Prepare films from the sediment obtained after centrifugalization. As a routine, stain two by the Ziehl-Neelsen method and one by Gram's method, if bacteria or a number of pus cells were found in the preparation stained with methylene blue. For recording results, follow directions given under methods of reporting laboratory examinations.

Make a very careful and prolonged examination of the stained preparations for tubercle bacilli, as these bacilli are usually present in very small numbers. If no acid-fast bacilli are found, examine for at least fifteen minutes, using a mechanical stage.

Examine the preparations stained by Gram's method. If biscuit-shaped Gram-negative intracellular micrococci are found, consider them meningococci.

Such organisms found in an extracellular position are probably meningococci. If large numbers of polymorphonuclears are present and no bacteria can be found, especially in specimens received from a distance, the probability of a meningitis due to meningococci should be considered since these cocci autolize readily.

In such cases, examine a number of preparations.

If streptococci, pneumococci, or influenza bacilli are found in specimens of spinal fluid, they are usually present in large numbers.

Confirm all microscopic findings by cultural or serological tests.

*Cultural Examination*

Follow the procedure described under cultural examination of miscellaneous specimens.

*Chemical Examination*

**Test for Globulin (Noguchi's Butyric Acid Test)** (86).—Test for an increase in globulin all spinal fluids which are satisfactory for this examination. To from 0.1 to 0.2 cc. of spinal fluid, add 0.5 cc. of 10-percent butyric acid and heat to boiling. Add slowly 0.1 cc. of normal NaOH, again heat the fluid to the boiling point, and allow it to cool. A white flocculent precipitate shows the presence of increased globulin. Control the test by carrying through the procedures without the addition of spinal fluid. In the presence of red blood cells or a slight tinge of hemoglobin, the results are of questionable value. Record the results after the test has been left at room temperature for one hour.

Record results as follows:

"Marked increase in globulin," when a large amount of flocculent precipitate is present.

"Moderate increase in globulin," when a moderate amount of granular precipitate is present.

"Slight increase in globulin," when a heavy cloud and a slight granular precipitate are present.

"No increase in globulin," when a slight cloud only is present.

**Test for Sugar (Fehling's).**—Test for sugar, specimens being examined for evidence of meningitis. Mix and boil equal parts of Fehling's solutions I and II. Add the same amount of spinal fluid and heat again to the boiling point. Allow the tubes to stand at least one-half hour before making the final reading. Reduction of the solution as shown by the appearance of a copper-red precipitate at the bottom of the tube indicates the presence of sugar.

**The Quantitative Determination of Sugar.**—Meningitis, due to tubercle bacilli, meningococci or pyogenic cocci, usually decreases the sugar content of the fluid while in the case of syphilitic meningitis the percentage of sugar may not be essentially altered. If it is necessary

to make a quantitative determination of the sugar content in a spinal fluid, the procedure outlined by Folin and Wu (87) may be followed.

*Animal Inoculation*

Inoculate a guinea pig when no organisms of pathogenic significance can be found in a specimen from a case with symptoms of meningitis or that has been diagnosed as meningitis.

*Agglutination Tests for Meningococci*

**Macroscopic Slide Agglutination.**—Make a macroscopic slide-agglutination test when colonies suggestive of meningococci are found on the plates. Prepare with 0.85-per-cent salt solution 1:10 dilutions of normal and antimeningococcus horse serum for this purpose, and place a loopful of each on a glass slide. Emulsify some of the colony in the drop containing normal serum and then transfer some of this suspension to the one containing antimeningococcus horse serum. Watch for clumping of the organisms in the antimeningococcus serum. Record the results. Test not more than four colonies on one slide and then stain the preparation by Gram's method so that a permanent record of the test will be available. If the organisms are Gram-negative and are agglutinated in the antimeningococcus serum only, consider them to be meningococci.

**Macroscopic Tube Agglutination.**—Make a heavy suspension of a density of the barium sulfate standard No. 3, using the culture grown on blood-agar slants, or serum, dextrose-agar slants, in 0.85-per-cent salt solution. Then make a 1:25 dilution of normal horse serum, and dilutions of the polyvalent antimeningococcus horse serum and monovalent antimeningococcus rabbit sera from animals immunized against groups I, II, and III, according to the titer of the serum. Put 0.3 cc. of each of the serum dilutions in agglutination tubes and add 0.3 cc. of the suspension of the organisms. As an additional control, use 0.3 cc. of the culture suspension and 0.3 cc. of 0.85-per-cent salt solution. Shake the tubes thoroughly and incubate for twenty-four hours at 55°C.

*Interpretation of Results*

If the organisms tested are meningococci, they should be strongly agglutinated by the polyvalent horse serum, and by the monovalent rabbit serum of the homologous type. They should not be agglutinated in the salt solution, and should be only very slightly agglutinated in normal horse serum, diluted 1:50.

There are nonpathogenic Gram-negative cocci found in the nasopharynx that are strongly agglutinated by the normal horse serum, diluted 1:50.

## TABLE 23

*Characteristics of spinal fluids found under various meningeal conditions**

| MENINGEAL CONDITION | PRESSURE | APPEARANCE | CYTOLOGY | BACTERIOLOGY | GLOBULIN | FEHLING'S SOLUTION | ANIMAL INOCULATION |
|---|---|---|---|---|---|---|---|
| Normal | Normal | Clear | 5 to 10 cells per cubic millimeter | Sterile | No increase | Reduced | |
| Meningismus | Increased | Clear | Very few cells | Sterile | No increase | Reduced | |
| Poliomyelitis | Increased | Clear, sometimes slight fibrin web | 30 or more cells per cubic millimeter. Early polynucleosis—late lymphocytosis up to 98 per cent | Sterile | Slight increase | Reduced | |
| Encephalitis lethargica | Somewhat increased | Clear | Slightly increased, rarely over 100, 10 to 20 usually, partly mononuclear | Sterile | No increase to slight increase | Reduced | |
| Tuberculous meningitis | Increased | Clear, fibrin web | 100 to 300 cells per cubic millimeter—lymphocytosis up to 98 per cent | Tubercle bacilli | Slight increase to moderate increase | Not reduced in 25 per cent of cases | Tuberculosis in four to eight weeks |
| Epidemic cerebrospinal meningitis | Increased | Cloudy | Few hundred to several thousand cells per cubic millimeter—polynucleosis up to 98 per cent | Meningococci | Slight increase to marked increase | Reduced or not reduced according to severity and stage | |
| Meningitis of undetermined origin | Increased | Cloudy | Marked increase in number of cells—polynucleosis up to 98 per cent | Infecting organism | Slight increase to marked increase | Reduced or not reduced according to severity and stage | |
| Syphilis of central nervous system | Variable, increased at times | Usually clear | Cells increased, usually a lymphocytosis | Spirochetes may be found | Slight increase to marked increase | Reduced | |
| Tumor | Usually increased | Usually clear, may have yellow tint | Variable, slight increase at times | Sterile | No increase to moderate increase | Reduced | |
| Multiple sclerosis | Normal | Clear | Variable, slight increase at times | Sterile | No increase to slight increase | Reduced | |
| Cerebral or spinal hemorrhage | Usually increased | Usually bloody immediately after hemorrhage. Later often clear with a yellow tint | Very few cells or red and white blood cells present in large numbers | Sterile | Slight increase to marked increase | Reduced | |

* Amplification of table given in Park and Williams, Pathogenic Microörganisms (88).

If the results of the agglutination tests are not satisfactory, repeat the tests, using a wider range of dilutions of the serum that gave unsatisfactory results.

If after these repeated tests, a definite result is not obtained, refer the culture to the serum-production group for further study.

## REPORTING RESULTS

In making the report of the complete examination, record the results under the following headings:

### *Appearance*

Describe the opacity and color and if blood is present, describe the amount.

### *Cell Count*

Report the number of cells per cubic millimeter and the ratio of lymphocytes or mononuclears to polymorphonuclears.

### *Chemical Examination*

When a specimen is received to be examined for evidence of encephalitis, send a report of the globulin test and cell count by telegram. Report marked, definite, slight, or no increase in globulin, and that Fehling's solution was or was not reduced, or that the specimen was unsatisfactory for the reason specified.

### *Bacteriological Examination*

Whenever a preliminary report based on morphology is made, state that the result of the cultural examination will follow.

**Preliminary Report.**—Report: "Organisms having the morphology of ——— were found," or "No bacteria were found," or "The examination was unsatisfactory (for the reason specified)."

When organisms having the morphology of meningococci are found, telephone or telegraph the results, whether or not an early report is requested.

When large numbers of polymorphonuclear cells are found and no bacteria can be demonstrated, telegraph these results and suggest that the use of antimeningococcus serum should be considered.

**Cultural.**—Report as described under methods of reporting laboratory examinations.

**Animal Inoculation.**—Report as described under methods of reporting laboratory examinations.

## PLEURAL, ASCITIC, AND OTHER FLUIDS

Apart from any academic question of whether there is any real fundamental distinction or difference, body fluids in pathologic conditions are classified for practical purposes as transudates and exudates. Transudates are considered noninflammatory in origin, contain a few cells and a small percentage of albumin. Exudates are inflammatory in origin, contain more cells and a higher percentage of albumin than transudates and also tend to coagulate on standing.

Usual differential characteristics:

| | *Transudates* | *Exudates* |
|---|---|---|
| Specific gravity: | Lower than 1.018 | Higher than 1.018 |
| Appearance: | Clear, straw color | Cloudy, purulent |
| Albumin: | Less than 2½ per cent | 2½ per cent or more |
| Microscopic examination: | No bacteria, few red blood cells | Bacteria, pus cells |

Exudates incited by tubercle bacilli may show some of the characteristics of transudates. A reaction in such cases may appear as follows:

| | |
|---|---|
| Specific gravity | 1.018 |
| Appearance | Clear |
| Albumin | 2½ per cent |
| Microscopic examination | Tubercle bacilli<br>Lymphocytes |

### DIFFERENTIATION BETWEEN TRANSUDATES AND EXUDATES

#### *Specific Gravity*

Determine specific gravity by means of a hydrometer.

#### *Test for Protein (Rivalta; Smoke Test)*

Add two drops of glacial acetic acid to 200 cc. of water and into this solution allow a few drops of the body fluid to fall.

If the fluid contains a large amount of the protein characteristic of exudates, the drops in sinking to the bottom of the container produce a turbidity which resembles a ring of smoke. If a small amount of the protein is present, the cloudiness will be slight and will develop slowly. If the protein is absent, no turbidity will appear.

### *Test for Albumin*

Use Esbach's method for determining the quantity of albumin.

### *Microscopic Examination*

Centrifugalize the specimen for one-half hour. Then follow the procedure outlined under microscopic examination of miscellaneous specimens. When the clinical diagnosis indicates the possibility of a malignant condition, make two additional preparations and stain by Wright's method. Refer these to the pathologist for examination.

### *Cultural Examination*

Follow the procedure outlined under cultural examination of miscellaneous specimens.

### *Animal Inoculation*

When the history of the case indicates that the infection is acute, omit the animal inoculation. Refer such specimens to the bacteriologist in charge of the group. Otherwise, inoculate from 1 to 2 cc. of the sediment suspended in supernatant fluid, if available, or in salt solution, subcutaneously into the groin of a guinea pig.

## REPORTING RESULTS

Write a complete report of the examinations. (See: methods of reporting laboratory examinations.)

# CHAPTER 3

# THE EXAMINATION OF INTESTINAL AND OTHER DISCHARGES

## EXAMINATION OF INTESTINAL DISCHARGES

### CHEMICAL EXAMINATION OF FECES FOR BLOOD

This examination is of little value except when the patient has been on a meat-free diet for several days prior to the time of collecting the specimen, and has not had bismuth or iron medication.

**Preparation of the Specimen.**—Suspend a small fragment of feces in 2 cc. of water, and boil.

**Benzidin Test.**—Mix equal quanitites of hydrogen peroxide and a saturated solution of benzidin in glacial acetic acid.

Add a few drops of the feces suspension to this mixture. A blue color denotes the presence of blood.

If desired, a small amount of feces solution may be put on blotting paper and a few drops of the benzidin reagent added. A blue color will appear in the blotting paper, on the edge of the feces emulsion, if blood is present.

Controls: Always control these tests by substituting water for the specimen under examination, when a reaction is obtained, and by adding a drop of blood to the reagents, when the reaction is not obtained.

### MICROSCOPIC EXAMINATION

#### *Unstained Material for Ova*

**Moist Preparation.**—Place a small particle of feces on a slide, dilute it with 0.85-per-cent salt solution, if necessary, and place a cover slip over it. Among the structures recognizable under the microscope are remnants of food, epithelial cells, leucocytes, red blood cells, crystals, bacteria, and the ova of animal parasites.

**Saturated Brine Method** (89).—Thoroughly emulsify a small amount of the specimen in saturated salt solution and allow it to stand for about fifteen minutes. Ova, if present, will usually be found in the surface layer of the fluid. Examine several moist preparations.

### *Material for Protozoa*

Whenever an examination for amoebae or other protozoa is requested, and the specimen is submitted immediately after collection, examine it, using a warm-stage apparatus.

Since an examination for vegetative protozoa is not entirely satisfactory if the specimen is cold, suggest in such cases that specimens be examined in a nearby laboratory.

Examine all such specimens for cysts. Make at least two moist preparations of the material, of such density that ordinary newspaper print is just visible through them. Add a large drop of aqueous eosin solution and examine. Living protozoa remain unstained on a pink background.

If protozoa are found, make a further examination in strong iodine solution (90) (iodine protozoan stain). This kills and stains all protozoa.

If unable to identify the protozoan parasites in the iodine preparation, make several preparations and stain them with iron hematoxylin, as follows:

1. Prepare thin films of the stool on slides; do not allow them to dry.
2. Place these preparations, film down, in warm Schaudinn's fluid for from two to five minutes. Heat this solution only to the point at which steam rises.
3. Harden by passing through graded alcohols:

*a.* 50-per-cent alcohol: 5 minutes
*b.* 70-per-cent alcohol: 5 minutes
*c.* 95-per-cent alcohol: 15 minutes
*d.* 70-per-cent alcohol: 5 minutes
*e.* 50-per-cent alcohol: 5 minutes

4. Mordant in 2-per-cent aqueous solution of iron alum (ammonium ferric sulfate) for from thirty minutes to two hours.
5. Wash in distilled water for five minutes.
6. Stain in 0.5-per-cent aqueous-hematoxylin solution for from two to sixteen hours or longer.
7. Wash in distilled water for five minutes.
8. Decolorize in 2-per-cent aqueous solution of iron alum. Wash carefully.
9. Wash in distilled water for five minutes.
10. Dehydrate by passing through:

50-per-cent alcohol: 5 minutes
70-per-cent alcohol: 5 minutes

11. Counterstain with 1-per-cent alcoholic eosin for from five to ten seconds.

12. Continue dehydration by using:

95-per-cent alcohol: 15 minutes
Absolute alcohol: 10 minutes

13. Clear in xylol: 15 minutes.

14. Mount in balsam and examine.

When no protozoa are found after this examination, advise the physician to submit a specimen in 10-per-cent formalin. Prepare moist preparations and examine them by the iron-hematoxylin method beginning with step 3 c.

### CULTURAL EXAMINATION

In case no evidence of animal parasites or protozoa is found in specimens of feces submitted for these examinations, give them to the bacteriologist in charge of the isolation of organisms of the enteric-disease group for routine examination.

**For B. welchii.**—Inoculate two sterile tubes of milk with about 0.5 cc. of the material, heat at 80°C. for fifteen minutes, then cool without shaking. Make subcultures from these tubes on dextrose beef-infusion agar and blood-agar plates. Incubate the tubes of milk and the plates in an anaerobic jar for from twenty-four to forty-eight hours.

If the tubes of milk show "stormy" fermentation and have an odor of butyric acid, make Gram-stained preparations and examine the motility of the organisms. If Gram-positive, nonmotile bacilli are found, *B. welchii* is indicated.

If further cultural tests and animal inoculations are desired, the procedure outlined by Hall (91) may be consulted.

### REPORTING RESULTS

Report results as follows:

"Blood was demonstrated by the benzidin test."

"No blood was demonstrated by the benzidin test."

"Ova of———were found."

"(Species of protozoa) were found."

"No evidence of parasitic infection was found."

"*B. welchii* was indicated."

"*B. welchii* was isolated."

"The presence of *B. welchii* was not indicated."

## EXAMINATION OF PURULENT DISCHARGES

### MICROSCOPIC EXAMINATION

Follow the procedure outlined for general bacteriological examinations. (See: microscopic examination.)

### BACTERIOLOGICAL EXAMINATION

If the material is received on a swab or in a test tube, suspend some of it in broth and make a cultural examination of the suspension. (See: cultural examination.)

The microscopic examination will indicate the amount of dilution necessary.

### REPORTING RESULTS

Report the results as outlined under methods of reporting laboratory examinations.

## EXAMINATION OF MATERIAL FROM THE NASOPHARYNX RECEIVED ON SWABS, FOR MENINGOCOCCI

The examination must be made immediately after collection.

### INOCULATION OF PLATES

Inoculate blood-agar plates which have been warmed to 37°C. Deposit all the material on one small area of the medium and spread it in fan-like radiations. (See: plating methods.) Incubate the plates immediately.

### MICROSCOPIC EXAMINATION

Make direct preparations from the swab and stain them by Gram's method. Examine and note the presence of Gram-negative micrococci which may be meningococci.

### CULTURAL EXAMINATION

Follow the procedure for general bacteriological examinations (See: cultural examination), and make an agglutination test for meningococci, when indicated.

### REPORTING RESULTS

Report the results as outlined under methods of reporting laboratory examinations.

## EXAMINATION OF SLIDE PREPARATIONS FOR VINCENT'S ANGINA

If only one slide is received, stain with aqueous gentian violet or dilute carbolfuchsin. If two are received, stain one by Gram's method and the other with aqueous gentian violet, or dilute carbolfuchsin and examine them for spirochetes and fusiform bacilli associated with Vincent's angina.

These organisms are often found near the teeth, especially when pyorrhea is present, and in any necrotic tissue in the mouth or nasopharynx.

## EXAMINATION OF DISCHARGES FOR GONOCOCCI

### MICROSCOPIC EXAMINATION

Make slide preparations of the discharge, stain them by Gram's method and examine for morphologically typical gonococci. (See: exudates.)

TABLE 24

*Fermentation reactions of Gram-negative micrococci*

| | DEXTROSE | MALTOSE | SACCHAROSE |
|---|---|---|---|
| Gonococcus | Acid | No acid | No acid |
| Meningococcus | Acid | Acid | No acid |
| *Micrococcus catarrhalis* | No acid | No acid | No acid |
| *Micrococcus flavus* | Acid | Acid | No acid |
| *Micrococcus pharyngis* | Acid | Acid | Acid |

### CULTURAL EXAMINATION

(This examination is not attempted unless cultures can be made immediately after the material is collected from the patient.)

Plate the material on beef testicular blood agar and, after incubation, fish typical colonies to slants of the same medium. If this medium is not available, use blood agar. Gonococcus colonies on this medium are characteristically small, finely granular and have scalloped edges. The granulations are very marked in the center of the colony, but the periphery is so colorless that it is sometimes difficult to distinguish. After incubation, make slide preparations from the cultures fished and stain them by Gram's method. Gonococci grown on artificial medium may vary in size and shape and may stain unevenly. They may be distinguished from other Gram-negative micrococci by (1) the scantiness of their growth on plain agar and (2) their fermentation reactions in sugar-serum-agar media. Table 24 gives the fermentation reactions of Gram-negative micrococci which morphologically resemble gonococci.

#### REPORTING RESULTS

Report results as follows:
"Gonococci were isolated."
"No gonococci were isolated."

## EXAMINATION OF DISCHARGES FOR TREPONEMA PALLIDUM

#### COLLECTION OF MATERIAL

Wear rubber gloves. To remove contaminating organisms, wash the suspected lesion with sterile 0.85-per-cent salt solution, using sterile gauze or cotton to remove the surface exudate. Lightly curette the surface of the suspected chancre or lesion, wipe off the blood, and then squeeze the surrounding tissues until a drop of serum, containing a few or no red blood cells, collects.

### *Moist Preparation*

Using a sterile capillary pipette, transfer a drop of this serum to a clean glass slide and place a cover slip over it. Examine over a dark field immediately.

If *Treponema pallidum* is not found after examining several preparations over the dark field, and if glands, near the lesion, are palpable, it is desirable to examine fluid aspirated from them.

Draw about 0.5 cc. of sterile 0.85-per-cent salt solution into a small sterile syringe to which is attached a 20- to 22-gauge needle 1 inch long. After the skin has been sterilized, hold the gland firmly and pierce it with the needle. Force the salt solution into it, moving the point of the needle about slightly while doing this, then aspirate as much fluid as possible and examine it over the dark field.

### *Dried Preparation (92)*

Stain slide preparations submitted for an examination for *Treponema pallidum* by Fontana's method.

#### REPORTING RESULTS

### *Moist Preparation*

Report results as follows:
"Morphologically typical *Treponema pallidum* was found. A specimen of blood should be submitted for the complement-fixation test for syphilis."

"No morphologically typical *Treponema pallidum* was found. A specimen of blood should be submitted for the complement-fixation test for syphilis."

*Dried Preparation*

"*Treponema pallidum* was found. A specimen of blood should be submitted for the complement-fixation test for syphilis."

"No *Treponema pallidum* was found. As the examination of dried preparations is less satisfactory than the direct examination over a dark field, the patient should, if possible, be sent to a nearby laboratory where this examination may be made. A specimen of blood for the complement-fixation test for syphilis should be submitted."

## CHAPTER 4

# THE EXAMINATION OF BLOOD FOR MALARIAL PARASITES

Staining of blood film: Stain blood films to be examined for malarial parasites by Wright's method. If this examination is not made at frequent intervals, make a stained preparation of normal blood before staining the specimen, as a control for the stain and the technic. Prepare and examine only one of the specimen slides at a time, so that if the first preparation is not satisfactory there will be another for study.

### STAINED PREPARATIONS

Always use a mechanical stage when examining blood films and whenever a parasite is seen, record on the history blank the readings of the vernier, the number of the microscope and the type of stage used, or encircle the area on the slide with the object marker.

### REPORTING RESULTS

Report results as follows:

"Malarial parasites were found."

"No malarial parasites were found. Specimens should be submitted for confirmatory examination."

"No malarial parasites were found. Since quinine has been administered, the probability of finding the parasites is lessened. Specimens taken previous to the administration of quinine, and just before the expected chill, are most satisfactory for examination."

# CHAPTER 5

## THE EXAMINATION OF SPECIMENS FOR EVIDENCE OF HIGHLY PATHOGENIC MICROÖRGANISMS AND FOR PARASITIC FUNGI

In handling specimens to be examined for *B. anthracis*, *B. mallei*, *B. pestis*, *Sp. cholerae*, *B. melitensis*, and *B. tularense*, special precautions must be observed.

### EXAMINATION FOR B. ANTHRACIS

#### SPECIMENS FROM CASES OF ANTHRAX

##### *Microscopic Examination*

Make slide preparations from the specimen and stain by Gram's method. When large Gram-positive bacilli that resemble *B. anthracis* in morphology are found, make a preliminary report of the findings.

##### *Cultural Examination*

Inoculate beef-infusion broth, agar slants, and agar pour plates with the culture. After incubation, examine the cultures for large Gram-positive nonmotile bacilli which produce characteristic "Medusa head" colonies on plain agar and "cotton fluff" sediment in broth.

##### *Animal Inoculation*

Inoculate guinea pigs and mice subcutaneously with some of the material from the lesion. If the animals die in from one to four days, and if aerobic nonmotile bacilli, having the typical colony formation and morphology of *B. anthracis*, are found in cultures and in stained preparations from the spleen, liver, or blood, it is proof that *B. anthracis* is present.

It may be desirable to check these results by inoculating a guinea pig with the cultures recovered from the animals.

In case the animals do not die and organisms resembling anthrax bacilli are found in the cultures, use them for inoculating other animals.

More of the original material may also be used for repeating the animal inoculation.

### SPECIMENS OF HAIR, BRISTLES, ETC.

#### *Preparation of Specimen*

Prepare an emulsion of part of the material submitted by cutting it in fine pieces and grinding them in a mortar with a small amount of 0.85-per-cent salt solution. When a shaving brush is under investigation, examine a piece of the stub, in addition to the hair. Heat a portion of the emulsion at 80°C. for thirty minutes and make a separate examination of both the heated and the unheated portions.

#### *Microscopic Examination*

Prepare films, stain them by Gram's method and record the presence of organisms morphologically resembling *B. anthracis*.

#### *Cultural Examination*

Make a cultural examination as described under specimens from cases of anthrax.

#### *Animal Inoculation*

Inoculate guinea pigs and mice as described under specimens from cases of anthrax.

### REPORTING RESULTS

Report results as follows:

"*B. anthracis* was found," when the cultural and morphologic findings are confirmed by proof of the pathogenicity of the organisms.

"No *B. anthracis* was found," when no organisms morphologically or culturally typical of *B. anthracis* are found.

## EXAMINATION FOR B. MALLEI

#### *Microscopic Examination*

Prepare films from the lesion and stain by Gram's method and also with Loeffler's alkaline methylene blue. Note the presence of granular, Gram-negative, straight, or slightly curved bacilli. In all cases, confirm results of morphologic examinations by cultural, serologic, and animal tests.

#### *Cultural Examination*

For isolating the organism, streak the suspected material on glycerin-agar, glycerin-potato agar, or serum agar plates. When a pure cul-

ture is obtained, inoculate several tubes of potato medium, as well as of milk, gelatin, and peptone water containing dextrose, mannite, saccharose, and lactose.

*B. mallei* grows well on most of the ordinary culture media. The appearance of the growth on potato is characteristic and is an important factor in the identification of the organism. After incubation for forty-eight hours at 37°C., a yellowish, thick viscous film appears which, during the next few days, becomes brown. The potato in the region of the growth turns dark.

### *Agglutination Test*

When an organism with the morphology and cultural characteristics of *B. mallei* has been isolated, an agglutination test, although of questionable value, may be attempted. *B. mallei* is not, as a rule, easily agglutinated when freshly isolated from the animal body, and it is difficult also to obtain a serum with a high agglutinating titer.

For the test, heat several 48-hour glycerin-agar, or glycerin-potato-agar slant cultures at 60°C. for two hours and then suspend the growth in 0.85-per-cent salt solution to which 0.1 per cent of phenol has been added. Make as even an emulsion as possible, pass it through soft filter paper, and dilute the filtrate with the phenolized salt solution until it is faintly turbid. Set up the agglutination tests, using 0.3 cc. of the suspension of organisms and 0.3 cc. of the following serum dilutions: 1:50, 1:100, 1:200, 1:300, 1:400, 1:500, 1:600, 1:800, and 1:1000. Incubate the test for from twenty-four to thirty hours at from 35 to 37°C., and record the reactions, basing the reading on the presence of a sediment consisting of agglutinated bacteria.

Always control the reaction by testing the organism in normal horse serum and by testing a culture known to be *B. mallei* in both the agglutinating serum and in the normal horse serum.

When serum agglutination tests are made with either human or horse serum, employ the technic already described, using a culture known to be *B. mallei*.

Since normal horse serum often agglutinates *B. mallei* equally as well as the serum of a horse suffering with glanders, this test is of little value. Very little is known of the agglutination reaction of human serum in this disease.

### *Animal Inoculation*

When a pure culture is obtained, inoculate a male guinea pig intraperitoneally.

If the organism is *B. mallei*, a characteristic enlargement of the testicles (Straus reaction) will become apparent in two or three days and the animal will die in from one to two weeks. Grayish nodules are usually found in the spleen and other organs. The test is not absolutely specific, since other organisms besides *B. mallei* have been shown to produce an analogous orchitis.

If the material contains organisms other than *B. mallei*, inoculate a guinea pig subcutaneously and after the death of this animal, inoculate a second male guinea pig intraperitoneally with a portion of the lymph gland from the first.

### REPORTING RESULTS

Report results as follows:
"*B. mallei* was isolated."
"No *B. mallei* was isolated."

### EXAMINATION FOR B. PESTIS

Consider the possibility of *B. tularense* in material sent from inflamed and suppurating glands. This organism induces lesions similar to those induced by *B. pestis*, but can be cultivated on special enrichment medium only.

### BUBONIC TYPE OF PLAGUE

#### *Microscopic Examination*

Prepare films from the bubo and stain by Gram's method and also with methylene blue. Gram-negative bacilli which have a tendency to stain more intensely at the poles suggest *B. pestis*.

#### *Cultural Examination*

Inoculate both blood-agar plates from pH 7.8 to 8.2 and 3-per-cent sodium-chloride agar plates. After incubation, examine them for characteristic colonies; on blood agar, colonies of *B. pestis* can be pushed along the surface of the medium; on salt agar after staining, involution forms will be found.

If these characteristics are noted, make a preliminary report.

Inoculate broth and gelatin media with a pure culture of the organisms isolated. Examine the broth for stalactite formation which is characteristic. Examine the gelatin for liquefaction which will not occur if the organisms are *B. pestis*.

### *Animal Inoculation*

Use two guinea pigs. Inoculate one subcutaneously, and rub some of the material on a dry, shaved area on the abdomen of the other.

If the material inoculated contains *B. pestis*, the animals will usually die in from three to five days after inoculation and the bacilli will be found in large numbers in the lymphatic glands, liver, spleen, and blood.

## PNEUMONIC TYPE OF PLAGUE

Examine specimens of sputum in the same manner as described for material from a bubo.

## SEPTICEMIC TYPE OF PLAGUE

Make blood cultures. If growth is obtained, test the cultures as described for material from the bubo.

## REPORTING RESULTS

Report results as follows:

### *Preliminary Report*

"A preliminary examination indicates the presence of *B. pestis;* the results of confirmatory tests will be reported later."

### *Final Report*

"*B. pestis* was isolated," if the results of the animal tests confirm the cultural and morphologic findings.

"No *B. pestis* was isolated."

## EXAMINATION FOR SP. CHOLERAE

Control all cultural examination of feces for *Sp. cholerae* by an examination for organisms of the enteric-disease group, especially for *B. dysenteriae.*

In cases of suspected Asiatic cholera it is advisable also to examine blood films for the spirochetes of relapsing fever.

### *Microscopic Examination*

Prepare a film of the feces and stain by Gram's method; also with methylene blue. If Gram-negative, comma-shaped spirilla are present, examine a moist preparation. If numerous morphologically typical, actively motile spirilla are found, make a prelminary report.

*Cultural Examination*

*Sp. cholerae* die rapidly in 30-per-cent glycerin. For this reason, if specimens are received in outfits which contain glycerin, those designed for submitting specimens of feces to be examined for organisms of the enteric-disease group, the examination for *Sp. cholerae* should be considered unsatisfactory, unless the organisms are isolated.

**Enrichment.**—Inoculate a tube of freshly prepared Dunham's peptone medium with a loopful of feces. After from six to twelve hours' incubation, prepare films from the surface growth and stain by Gram's method. If numerous Gram-negative spirilla are present, make microscopic agglutination tests, using standard dilutions of immune serum and a preparation with normal serum as a control. If definite agglutination occurs, make a preliminary report.

When spirilla are not found, inoculate a second tube of Dunham's peptone medium from the surface growth of the first, and examine after from six to twelve hours' incubation.

*Confirmatory Tests*

If the feces contains a large number of cholera-like spirilla, make a heavy suspension and inoculate plates of Dieudonné's medium, or Krumwiede's alkaline egg medium, directly.

If enrichment was necessary, inoculate the plates from the surface growth in the Dunham's peptone medium. Use the first or second tube, depending on the character of the growth. After incubation attempt to isolate the organism in pure culture. When a pure culture has been secured, identify it by: (1) morphology in Gram-stained preparations; (2) characteristic, rapid liquefaction of gelatin; (3) nitrate-indol reaction in Dunham's peptone medium.

REPORTING RESULTS

Report results as follows:
"*Sp. cholerae* was isolated."
"No *Sp. cholerae* was isolated."
"Result of examination for *Sp. cholerae* was unsatisfactory."

## EXAMINATION FOR B. MELITENSIS AND B. ABORTUS

### SPECIMENS FROM SUSPECTED CASES OF UNDULANT FEVER

*Blood (for Cultures)*

Follow the procedure described under blood cultures inoculating in addition liver-infusion agar. Incubate duplicate cultures in a jar in

which 10 per cent of the air has been replaced by $CO_2$. (See: cultural methods.)

The colonies are small, colorless, and transparent. When a slow-growing, small, Gram-negative cocco-bacillus which ferments none of the carbohydrates is isolated, *B. melitensis* is indicated.

Confirm the findings by the agglutination test.

**Agglutination Test.**—Suspend the growth from an agar slant in a small amount of 0.85-per-cent salt solution, and adjust to a density comparable to a barium sulfate standard No. 3. Heat the suspension in a water-bath at 60°C. for one hour and set up an agglutination test, using 0.3 cc. of the bacterial suspension and 0.3 cc. of specific dilutions of a melitensis agglutinating serum. Always control the reaction by testing the serum with a standard suspension of *B. melitensis*. Also test the patient's serum, if it is available, with the culture isolated. Record the reactions after incubation for two hours at from 35 to 37°C., and again after they have been allowed to stand overnight in the cold room.

**Animal Inoculation.**—After sufficient material for the cultural examination has been removed, inoculate two light-colored guinea pigs (250 to 300 grams) intraperitoneally with the remainder of the specimen. If the animals are still alive eight weeks after inoculation, etherize them and perform autopsies. Remove the liver and spleen. Make stained preparations and cultures from each and preserve a portion in formalin for pathologic examination. Make stained preparations and cultures from the heart's blood. In culturing autopsy material, use liver-infusion agar as well as deep tubes of dextrose-beef-infusion agar and incubate duplicate cultures in a jar containing 10-per-cent $CO_2$.

### *Urine*

Follow the procedure described under urine, bacteriological examination, for the cultural examination of urine and in addition inoculate plates of liver-infusion agar and incubate duplicate cultures in 10-per-cent $CO_2$.

### REPORTING RESULTS

Report results as follows:

"*B. melitensis* was isolated."

"No *B. melitensis* was isolated; (other organisms) were isolated."

"No growth of bacteria was obtained."

*Blood (for Agglutination Test)*

Separate the serum by centrifugalization.

**Cultural Examination.**—Follow the procedure for the cultural examination of blood clots and also the special procedures described above for the identification of *B. melitensis.*

**Agglutination Test.**—Inactivate the serum by heating at 55°C. for thirty minutes and make the following dilutions with 0.85-per-cent salt solution: 1:5, 1:10, 1:20, 1:40, 1:80, 1:160. To 0.3 cc. of each dilution add 0.3 cc. of a suspension of *B. melitensis,* in salt solution, killed either by heating at 60°C., for one hour or by the addition of 0.1-per-cent formalin (the density comparable to a barium sulfate standard No. 3). Always control the reaction by testing the suspension in a serum known to agglutinate *B. melitensis.* Also test the patient's serum with *B. typhosus* and *B. paratyphosus* A, and B. Record the reactions after the tests have been incubated for two hours at 37°C. and again after they have been allowed to stand overnight in the cold room.

Definite agglutination in the 1:80-dilution (final) is considered diagnostic.

REPORTING RESULTS

Report results as follows:

"Definite agglutination of *B. melitensis* was obtained."

"No agglutination of *B. melitensis* was obtained."

SPECIMENS FROM ANIMALS SUSPECTED OF BEING THE SOURCE OF HUMAN CASES OF UNDULANT FEVER

*Blood (for Cultures and Agglutination Tests)*

Follow the procedure for the examination of blood from suspected cases of undulant fever.

*Milk*

**Cultural Examination.**—Centrifugalize the milk, remove the cream and with this and with the sediment, inoculate culture media, as follows: blood-agar plates, liver-infusion-agar plates, and deep tubes of dextrose-beef-infusion agar. Incubate duplicate cultures in 10-per-cent $CO_2$. Follow the special procedures described for the isolation and identification of *B. melitensis.*

**Animal Inoculation.**—Inoculate two light-colored guinea pigs (250 to 300 grams) intraperitoneally with 5 cc. each of cream and two others

with 5 cc. each of sediment suspended in whole milk. Unless the guinea pigs die of acute infection, which may occur within a few days after inoculation, chloroform them after six or eight weeks. Autopsy the animals.

## EXAMINATION FOR B. TULARENSE (93)

### SPECIMENS FROM CASES OF TULAREMIA

#### *Pus or Discharge from Glands*

**Microscopic Examination.**—Prepare films from the specimen and stain by Gram's method. Note any organisms that may be present.

*B. tularense* is stained with difficulty in body fluids or tissue.

**Cultural Examination.**—Because of the difficulty with which *B. tularense* is cultivated on artificial culture media, no attempt is made to obtain cultures directly from the specimens. Inoculate blood-agar plates and make agar-pour-plate cultures to determine the presence of other organisms.

**Animal Inoculation.**—Use two guinea pigs. Inoculate one subcutaneously, and on the other rub some of the material on the skin of the abdomen which has been abraded by shaving with a dull razor.

If the material inoculated contains *B. tularense*, the animals will usually die in from five to ten days after inoculation. The lymph glands will be enlarged and filled with caseous material. Numerous white, caseous nodules will be found in the liver and spleen.

If desired, an attempt may be made to obtain from the lesions, cultures of *B. tularense* on egg-yolk medium and cystine agar on which *B. tularense* grows very slowly. The cultures should, therefore, be incubated for at least three weeks, unless growth appears sooner.

#### *Clotted Blood*

Separate the serum by centrifugalization. Prepare the following dilutions of the serum in 0.85-per-cent salt solution: 1:5, 1:10, 1:20, 1:40, 1:80, 1:160. Make agglutination tests, using 0.3 cc. of the serum dilutions and 0.3 cc. of a suspension of *B. tularense* in 0.85-per-cent salt solution killed by the addition of 0.1-per-cent formalin, the density of the suspension being comparable to a barium sulfate standard No. 4. Always control the reaction by testing the suspension of *B. tularense* in a serum known to agglutinate it. Also test the patient's serum with cultures of *B. typhosus*, *B. paratyphosus* A, and B, and *B. melitensis*. Incubate the tests for two hours at from 35 to 37°C. and record the

reactions. If a definite reaction is obtained, report at once. Allow the tests to stand in the cold room overnight and record the reactions on the following day.

Definite agglutination in the 1:80-dilution is considered diagnostic.

### RABBITS OR OTHER RODENTS

Perform a careful autopsy, removing any enlarged glands or organs showing the white, caseous nodules suggestive of tularemia.

**Microscopic Examination.**—Stain preparations from abnormal tissues with Gram's stain, Loeffler's methylene blue, and with the Ziehl-Neelsen stains.

**Cultural Examination.**—Inoculate blood agar with the heart's blood and with material from any lesion which may warrant it, to determine if organisms other than *B. tularense* may be present.

**Animal Inoculation.**—Inoculate two guinea pigs with any tissue showing the changes characteristic of tularemia.

### REPORTING RESULTS

Report results as follows:

"Guinea pigs inoculated with the specimen showed evidence of tularemia."

"Guinea pigs inoculated with the specimen showed no evidence of tularemia."

"Definite agglutination of *B. tularense* was obtained."

"No agglutination of *B. tularense* was obtained."

"The (species of animal) showed definite evidence of tularemia."

"The (species of animal) showed no evidence of tularemia."

## EXAMINATION FOR PARASITIC FUNGI

**Microscopic Examination.**—Examine moist preparations for sulfur granules, hyphae, etc. In the case of actinomycosis, crush the sulfur-like granules in the pus under a cover glass and examine the unstained material.

In the case of the parasitic diseases of the skin, the parasites may be demonstrated by softening a hair or a fragment of crust with a few drops of 10-per-cent sodium hydroxide.

Examine films stained by Gram's method, methylene blue, or gentian-violet stain.

**Cultural Examination.**—Inoculate Sabouraud's agar and tellurite agar. Seal the cultures with adhesive plaster or place them in an air-

tight jar to prevent drying. As the organisms grow slowly, incubate for from one to two weeks.

If cultures of higher bacteria, yeasts, or molds, are secured, it is usually necessary to make a complete cultural study, as well as animal inoculations, in order to identify them. White rats are especially susceptible to some of the pathogenic fungi.

# CHAPTER 6

## THE EXAMINATION OF SPECIMENS IN CASES OF FOOD POISONING WITH B. BOTULINUS AND B. ENTERITIDIS

In many instances, requests for the examination of food stuffs are based on insufficient evidence. Time is so often wasted by examining these specimens, that considerable care and discrimination must be exercised in deciding what should be done. Refer every specimen of this kind to the bacteriologist in charge of the department who will request from the physician information concerning the history of the case. For description of symptoms in cases of food poisoning, see reports by Burke (94), and Dickson (95).

Examine specimens from suspected cases of food poisoning for evidence of either *B. botulinus* or *B. enteritidis*, depending on the clinical symptoms. If the symptoms are indefinite, examine for both organisms. Refer specimens also to the chemical department in every case in which the symptoms suggest chemical poisoning, or are indefinite.

While waiting for further information when the symptoms of food poisoning are indefinite, make a preliminary examination to determine the presence of toxic substances. Use aseptic precautions throughout this and the following examinations. Inject one guinea pig intraperitoneally, and feed another with some of the specimen. (Kittens may also be used.) If both animals survive, further tests may be unnecessary, but if one or both of the animals die and signs of poisoning with *B. botulinus* or of infection with *B. enteritidis* are noted, make a complete examination as follows:

### EXAMINATION FOR B. BOTULINUS

#### SPECIMENS OF FOOD

##### *Microscopic Examination*

Make slide preparations from all specimens and stain by Gram's method. Note the different kinds of organisms present, especially the relative numbers of large Gram-positive spore-bearing bacilli. If the specimen is solid, make the stained preparation from a suspension in 0.85-per-cent salt solution.

### *Toxicity Tests*

Preparation of specimen: If the specimen is liquid, centrifugalize part of it for from one-half to one hour at high speed and test the supernatant fluid.

If the specimen is solid, suspend from 2 to 5 grams of it in from 20 to 30 cc. of 0.85-per-cent salt solution by grinding in a mortar, and extract for from one to two hours at room temperature. Centrifugalize the extract and test the supernatant fluid.

Make slide preparations of the supernatant fluid in either case and, if large numbers of contaminating organisms are present, pass the material through a filter candle.

For control, boil a portion of the material for thirty minutes.

### *Animal Inoculation*

Inject intraperitoneally 0.5 cc. of the heated material into each of of two mice and 1 cc. into each of two guinea pigs which weigh not over 300 grams. Inject the same quantity of the unheated material into each of three mice and three guinea pigs, one of each receiving the toxin alone; another, the toxin and from 100 to 300 units of type A botulinus antitoxin; and another, the toxin and from 100 to 300 units of type B antitoxin. Allow the toxin-antitoxin mixture to stand for from one-half to three hours before the animals are inoculated.

*Animal Feeding.*—If desired, portions of the specimens may also be fed directly to a normal guinea pig and to guinea pigs protected against both types of botulinus toxin. Immunize the animals just before feeding.

**Results of Animal Inoculation.**—Observe all inoculated animals at frequent intervals for symptoms of botulinus poisoning.

With guinea pigs that do not succumb to infection in from four to eighteen hours, the following are the most characteristic symptoms that may be observed: striking emaciation, extreme weakness, flabby abdominal muscles, and paralysis of the throat muscles with increased secretion of mucus from nose and mouth. If the specimen contains botulinus toxin only, the normal animals which received the unheated material should die in from four to seventy-two hours, while those which received the heated material should remain alive. The type of toxin is determined by the protection obtained with one of the antitoxins. Death of animals receiving heated material indicates the presence of other toxic substances.

If botulinus toxin causes the death of the guinea pigs, the following findings should be noted at autopsy:

Site of inoculation: usually no macroscopic changes; occasionally slight infiltration or oedema, with or without hemorrhages, may be noted.

Abdominal organs: There is usually congestion with dark-colored liver and spleen. In acute intoxications, these lesions may be absent.

Bladder: frequently distended.

Lungs: hyperemic; may show large or small petechiae.

Always culture the heart's blood at autopsy to determine the possibility of infection from other organisms. Identify any organisms isolated.

### *Cultural Tests for B. botulinus*

Always confirm the toxicity tests by cultural examination.

**Preparation of the Specimen.**—Heat part of the original specimen at 80°C. for thirty minutes, or at 70°C. for from one to two hours, to destroy the vegetative forms. If the specimen is very acid, neutralize it before heating.

**Enrichment Cultures.**—For enrichment, inoculate modified van Ermengem's broth and chopped-meat medium with from 2 to 3 cc. of heated and unheated suspensions of the sediment from the centrifugalized specimen. Incubate for from five to ten days at from 35 to 37°C.

Make dried preparations, stain by Gram's method and note the presence of Gram-positive spore-bearing bacilli morphologically resembling *B. botulinus*. Test the toxicity of the cultures which show typical bacilli. Pool the growth if necessary.

For the toxicity tests, centrifugalize the enrichment broth cultures for one hour or filter through a filter candle. Inoculate three guinea pigs subcutaneously with from 1 to 3 cc. of supernatant fluid or filtrate, one of the animals receiving toxin alone, another toxin and type-A antitoxin, and another toxin and type-B antitoxin.

### *Methods of Isolation (96, 97)*

**Hydrogen-Jar Method.**—Inoculate a series of blood-agar plates from dilutions of the original material as for aerobic growth, as described under plating methods, surface inoculation (1). Include a plate inoculated with a culture known to be *B. botulinus* as a control.

Incubate the plates in the hydrogen jar for anaerobic growth at from 35 to 37°C. for from forty-eight to seventy-two hours. Then fish typical or suggestive colonies to dextrose-infusion semisolid agar, or chopped-meat medium. Colonies of *B. botulinus* on blood agar are small, transparent to gray, hemolytic, moist, and flat. They vary in shape from round to irregular.

Incubate the fishings for from twenty-four to forty-eight hours. Then make slide preparations from each and transfer each one that shows growth of morphologically typical bacilli, to enrichment broth. Incubate the broth cultures for from five to ten days at from 35 to 37°C. and determine their toxicity.

If the organisms cannot be isolated from the original material, and

the material has been found toxic, inoculate plates from the sediment of the first enrichment culture.

**Deep-Agar Culture.**—If the apparatus for the hydrogen-jar method is not available, inoculate in duplicate, deep tubes of 1-per-cent dextrose-beef-infusion agar, from pH 6.8 to 7.2. Incubate the cultures for from two to four days, one set at from 35 to 37°C. and the duplicate at from 25 to 28°C. At the end of this period examine the tubes for evidence of growth and gas production. Fish typical disk-shaped colonies to enrichment-broth medium, and proceed as already described.

#### SPECIMENS OF FOOD IN CANS

Open aseptically all cans of food suspected of containing *B. botulinus*. Scrub the container with soap and hot water, and allow it to stand in 10-per-cent cresol compound for a few minutes. Then flame the lid and open with a sterile can opener. Test the contents as described under specimens of food.

#### SPECIMENS OF FECES

Dilute a large amount of the specimen with salt solution and heat for thirty minutes at 80°C. or for one hour at 70°C. Inoculate culture media and test as described under specimens of food.

#### REPORTING RESULTS

Refer the results of both the animal and cultural tests to the bacteriologist in charge of the group who will report the findings in a special letter.

### EXAMINATION FOR B. ENTERITIDIS

#### SPECIMENS OF FOOD

##### *Microscopic Examination*

Make slide preparations and stain by Gram's method. Record the kinds of organisms present, noting chiefly the relative number of Gram-negative bacilli.

##### *Animal Feeding*

If the specimen is solid, suspend part of it by grinding in a mortar with 0.85-per-cent salt solution. Feed 2 cc. of this suspension, or of the material itself, if liquid, to each of two guinea pigs. Observe the animals for evidence of illness. If death occurs, attempt to determine the cause.

*Cultural Examination*

Plate some of the specimen or suspension on blood agar and on the differential plating media used in the examination of specimens from cases of enteric diseases. After incubation, fish suggestive colonies to triple-sugar Andrade agar. A reaction showing acid and gas in the butt is suggestive of *B. enteritidis*. Make agglutination tests with immune sera for the various organisms of the enteric-disease group, and also with *B. enteritidis* immune serum.

SPECIMENS OF FECES

Make a cultural examination of specimens of feces in the same manner as when examining specimens of food.

SPECIMENS OF BLOOD

If specimens of blood are received, make cultural examinations and agglutination tests with organisms of the enteric-disease group, and also with any organisms resembling *B. enteritidis* which may have been isolated from the food or feces.

REPORTING RESULTS

Refer the results of both the animal and cultural tests to the bacteriologist in charge of the group who will report the findings in a letter.

## CHAPTER 7

# THE EXAMINATION FOR EVIDENCE OF INFECTION WITH LEPTOSPIRA ICTEROHAEMORRHAGIAE AND THE WEIL-FELIX AGGLUTINATION TEST FOR TYPHUS FEVER

Specimens from cases of jaundice may be examined for *Leptospira icterohaemorrhagiae* (98).

### URINE

Centrifugalize 30 cc. of urine at high speed for one-half hour and remove the supernatant fluid.

#### *Chemical Examination*

Perform the routine chemical tests on the supernatant fluid. (See: urine, chemical examination.)

#### *Microscopic Examination*

Make the usual microscopic examination of the sediment. (See: urine, microscopic examination.) Also examine over dark-field illumination for leptospira.

#### *Cultural Examination*

If the specimen was collected with a catheter, inoculate three tubes of the rabbit-serum medium of Noguchi (See: leptospira medium), placing a loopful of the sediment in each.

#### *Animal Inoculation*

Suspend the remainder of the sediment in 5 cc. of the supernatant fluid and inoculate two light-colored, male guinea pigs weighing from 250 to 300 grams, intraperitoneally with 2.5 cc. each.

### CLOTTED BLOOD

Centrifugalize at low speed for ten minutes and remove the serum.

#### *Serologic Tests*

**Test for the Pfeiffer Phenomenon.**—Test the serum for the Pfeiffer phenomenon with *Leptospira icterohaemorrhagiae* as follows: Select an active culture of *Leptospira icterohaemorrhagiae* which has been grown

in the rabbit-serum medium. To 0.5 cc. of the upper layer of culture, which contains the heaviest growth, add 0.5 cc. of the patient's serum and 1.5 cc. of 0.85-per-cent salt solution and inoculate a guinea pig intraperitoneally with the mixture. At intervals of fifteen minutes and thirty minutes after the injection, withdraw a small amount of fluid from the peritoneal cavity with a capillary pipette and examine it over the dark-field illumination.

If the serum contains no antibodies for *Leptospira icterohaemorrhagiae*, active organisms should be seen in the fluid at both examinations.

If the serum contains such antibodies, some agglutination and lysis of the organisms is usually evident at the end of fifteen minutes and, if lysis is complete, no leptospirae should be seen at the end of thirty minutes.

When this result is obtained, control it by performing a test on a guinea pig inoculated with corresponding amounts of leptospira culture combined with normal human serum.

In case a virulent culture is used for the Pfeiffer reaction, keep the animals under observation for at least two weeks to see if the unknown serum exerts any protective effect. In case an avirulent culture is used, no further observation of the animals is necessary.

**Agglutination Test.**—The agglutination test may also be made according to the following procedure: If a luxuriant culture is available for this test, use equal parts of culture and undiluted serum, as well as serum dilutions of 1:5, 1:10, 1:25, and 1:50. Mix the culture and serum dilutions in 11-by-75-millimeter tubes and incubate for two hours at 37°C. Examine moist preparations over dark-field illumination for evidence of agglutination.

If only a moderately rich culture is available, combine four parts of culture with one of serum, thus obtaining final dilutions of 1:5, 1:10, 1:25, etc.

### *Animal Inoculation*

In case a specimen of clotted blood is submitted during the first week of the disease, grind the clot in a mortar with 0.85-per-cent salt solution and inoculate two light-colored, male guinea pigs weighing from 250 to 300 grams, intraperitoneally with this emulsion.

## CITRATED BLOOD

### *Microscopic Examination*

Examine over dark-field illumination for leptospirae.

### *Cultural Examination*

Inoculate a few tubes of rabbit-serum medium with 0.5 cc. each.

### *Animal Inoculation*

Inoculate two light-colored, male guinea pigs weighing from 250 to 300 grams, with 2 or 3 cc. of the specimen intraperitoneally.

## WILD RATS

Since wild rats are known to be carriers of *Leptospira icterohaemorrhagiae,* these animals are occasionally submitted for examination from the locality in which cases of suspected infectious jaundice have occurred.

### *Autopsy*

If a wild rat is received alive, anaesthetize it and bleed to death from the heart. Perform an autopsy and remove as much urine as possible with a sterile syringe. Remove the kidneys aseptically. Macerate them in a mortar with ground glass, and suspend in about 5 cc. of salt solution, then proceed as follows:

### *Microscopic Examination*

Examine the urine and the kidney suspension over dark-field illumination for leptospirae.

### *Cultural Examination*

Inoculate tubes of culture media with the urine and the kidney suspension.

### *Animal Inoculation*

Inoculate one light-colored, male guinea pig weighing from 250 to 300 grams, intraperitoneally with the urine and two with the kidney emulsion.

## CARE AND OBSERVATION OF CULTURES

Incubate the cultures at about 25°C. Examine each culture over dark-field illumination weekly for four weeks, or until active leptospirae are seen. As soon as organisms are found in moderately large numbers, transfer about 0.1 cc. of the top layer of the culture to two or three tubes of fresh medium. If, at this time, the guinea pigs inoculated with the original material have shown no signs of leptospira infection, inoculate others with the remainder of the culture. Discard culture tubes which show no growth at the end of a month's incubation.

### OBSERVATION AND AUTOPSY OF GUINEA PIGS

Examine the animals daily for indication of jaundice in the visible portion of the skin and mucous membranes, particularly the sclerae. Take the temperature daily and when a marked rise is noted, anaesthetize the animal and take 2 or 3 cc. of blood from the heart. Add a few crystals of sodium citrate, or equal parts of 2-per-cent sodium citrate in 0.85-per-cent salt solution, and shake to prevent clotting. Examine over dark-field illumination and, if leptospirae are found, chloroform the guinea pig and perform an autopsy. If no leptospirae are found in the heart's blood but the animal appears jaundiced one week or ten days after inoculation, chloroform it and make an autopsy.

If infected with *Leptospira icterohaemorrhagiae*, all the tissues will appear intensely jaundiced and numerous petechial hemorrhages will be found in the subcutaneous tissues.

Hemorrhages are evident in practically all the tissues, but are especially prominent in the tissues of the inguinal region and in the lungs where they have been called "butterfly hemorrhages."

Remove the urine with a sterile syringe. Macerate the kidneys in 0.85-per-cent salt solution, avoiding contamination as far as possible. Examine the urine and the kidney emulsion over dark-field illumination and, if leptospirae are found, either or both may be used for seeding new cultures and inoculating other guinea pigs, if a culture of the organism is desired. If no leptospirae are found, place pieces of the lungs, liver, kidneys, and testicles in 15-per-cent formalin so that they will be available for sectioning.

### REPORTING RESULTS

Report the results obtained, in a letter.

## WEIL-FELIX AGGLUTINATION TEST FOR TYPHUS FEVER

### *Macroscopic Tube Agglutination Test*

When a request is received for this test, obtain a 24-hour agar-slant culture of *B. proteus* X 19 from the bacterial collection. Prepare a suspension by washing off the growth with 0.85-per-cent salt solution and dilute it until its density corresponds to that of barium sulfate standard No. 2. Dilute the patient's serum 1:10, 1:20, 1:40, 1:50, and 1:100, and to 0.3 cc. of each dilution add 0.3 cc. of the suspension of *B. proteus* X 19. For controls, add 0.3 cc. of the suspension to 0.3 cc. of each of a 1:50-and 1:100-dilution of serum from a case of typhus

fever, or serum from a rabbit immunized against *B. proteus* X 19, and 0.3 cc. of the suspension to an equal amount of 0.85-per-cent salt solution. Incubate the tests in a water-bath at 45°C. for two hours.

The reaction is usually apparent at the end of this time, but the final readings should be made after the tests have been allowed to stand in the cold room overnight. Agglutination in the 1:100-dilution is considered diagnostic.

Reports have appeared in the literature, indicating that sera from typhus cases when heated to from 56 to 58°C. generally lose their ability to agglutinate cultures of *B. proteus* X 19, and may be thus differentiated from sera from cases of proteus infection or those from animals inoculated with cultures of *B. proteus*. Such sera are relatively thermostabile.

Whenever agglutination is obtained with *B. proteus* X 19, therefore, determine the thermolability (99) of the serum and report these results, together with those of the agglutination test.

### REPORTING RESULTS

Report results as follows:

"Definite agglutination was obtained by the Weil-Felix test for typhus fever. The serum, when heated at ——°C. for one hour loses (or does not lose) its power to agglutinate the culture of *B. proteus* X 19."

"No agglutination was obtained by the Weil-Felix test for typhus fever."

# CHAPTER 8

# THE EXAMINATION OF URINE, BLOOD, AND STOMACH CONTENTS

## URINE

Routine urine analyses, cytological examination and hemoglobin determination of blood, as well as the examination of stomach contents are made in connection with the physical examinations of the department staff and special investigations. Occasionally specimens are submitted by physicians from localities where facilities for the work are not available.

If the specimen shows evidence of decomposition, it is unsatisfactory for these tests.

### PHYSICAL AND QUALITATIVE CHEMICAL EXAMINATION

The following tests are included in the routine analyses.

#### *Appearance*

Record the color and turbidity of the specimen.

#### *Reaction*

Record the reaction, as determined by means of litmus paper.

#### *Specific Gravity*

Record the specific gravity as determined by a urinometer, which must float freely in the fluid. Remove any bubbles or debris from the surface of the urine with a piece of dry filter paper. Always read from the bottom of the meniscus.

#### *Albumin*

Filter the specimen until it is clear before attempting any of the following tests. Use Purdy's test as a routine.

**Purdy's Heat Test.**—To from 15 to 20 cc. of urine in a test tube add approximately 3 cc. of a saturated solution of sodium chloride and eight drops of a 50-per-cent solution of acetic acid. Mix by inverting the tube and cautiously boil the upper portion of the fluid. A white

cloud in the heated area denotes the presence of albumin. Bence-Jones' protein produces a white cloud which disappears upon boiling and reappears upon cooling.

**Heat and Nitric-Acid Test.**—After boiling about 5 cc. of urine in a test tube, add from one to three drops of concentrated nitric acid. The presence of albumin is indicated by a white cloud or flocculent precipitate. An excess of nitric acid is to be avoided since it may cause the resolution of the precipitate. Use this test when it is desired to remove albumin before testing for sugar.

**Heller's Ring Test.**—Pour 2 cc. of nitric acid into a test tube, tilt the tube, and cautiously layer the same amount of urine over the acid by allowing it to run slowly down the side of the tube. A white ring at the zone of contact of the two fluids indicates the presence of albumin.

Whenever the tests show more than a faint trace of albumin, estimate the amount by Esbach's method.

**Recording Results.**—Record the amount of albumin obtained as follows:

Large amount.
Moderate amount.
Heavy trace.
Trace.
Faint trace.
Very faint trace.
None.

*Sugar*

To determine the presence of sugar, use Benedict's solution.

**Benedict's Test.**—If the specimen has been preserved with chloroform, boil it, or if albumin in more than a trace has been found, boil, acidify, and filter before attempting this test.

To 5 cc. of Benedict's solution (qualitative) in a test tube, add eight drops of the urine to be tested. Heat the mixture for five minutes in a bath of boiling water, and then allow it to cool slowly. The formation of a brick-red, yellow, or green precipitate demonstrates the presence of sugar. The reading is not made until the mixture is cool since precipitates due to very small amounts of sugar may not become visible until then.

When sugar is present, examine the specimen also for both acetone and diacetic acid.

**Recording Results.**—Record the results as follows:

Present.
Trace.
None.

### *Bile*

Shake the urine in a bottle or test tube. If bile pigments are present, they will impart a yellowish or brownish color to the foam. In case tests are to be made for bile acids and bile salts, follow the procedure outlined by Hawk (100).

**Recording Results.**—Record the results as follows:

Present.

None.

### *Indican*[1]

**Obermayer's Test.**—To 3 cc. of urine, slightly warmed, add 3 cc. of Obermayer's reagent and 1 cc. of chloroform. Shake this mixture vigorously for a few seconds and allow it to stand for about ten minutes. A blue coloring of the chloroform indicates the presence of indican, the depth of color depending upon the degree of indicanuria.

**Recording Results.**—Record the results as follows:

Large amount.

Moderate amount.

Small amount.

None.

### *Blood*[1]

Tests for the detection of blood in the urine are of value only when the possibility of extraneous blood (e.g. menstrual) is eliminated. The following tests may be used.

**Benzidin Test.**—Mix equal quantities of urine and the benzidin reagent. A blue color appears in the presence of blood.

**Recording Results.**—Record the results as follows:

Present.

None.

### *Acetone*[1]

**Lange's Test.**—To 5 cc. of urine in a test tube, add five drops of glacial acetic acid and five drops of a saturated aqueous solution of sodium nitroprusside. Tilt the tube and carefully layer with ammonium hydroxide. A purple color at the zone of contact denotes the presence of acetone.

**Rothera's Test.**—To from 5 to 10 cc. of urine in a test tube, add 1 gram of ammonium sulfate and two or three drops of a fresh concentrated solution of sodium nitroprusside. Tilt the tube and carefully

[1] This test is made only upon request.

layer with ammonium hydroxide. A permanganate-colored ring indicates the presence of acetone.

**Recording Results.**—Record the results as follows:

Present.

None.

### *Diacetic Acid*[1]

**Gerhardt's Test.**—To from 5 to 10 cc. of the urine in a test tube add, drop by drop, a 10-per-cent solution of ferric chloride until the precipitation of the phosphates is complete. Then filter and add a small quantity of the ferric-chloride solution to the filtrate. If diacetic acid is present, a Bordeaux red color is formed which disappears upon boiling. Several minutes' boiling is required; simply bringing the fluid to the boiling point will not suffice. The test may also be performed by the ring method, when the coloration is more definite.

[1] If desired, a few drops of a 35- to 40-per-cent solution of ferric chloride may be used in place of the 10-per-cent solution. This will eliminate the necessity for filtering.

**Recording Results.**—Record the results as follows:

Present.

None.

## QUANTITATIVE CHEMICAL EXAMINATION

Make quantitative tests only if requested and when a sample of a 24-hour specimen has been submitted, except in the case of albumin.

### *Albumin*

**Esbach's Method.**—Filter the urine and, if alkaline, add sufficient acetic acid to give an acid reaction on litmus paper. If the qualitative test has indicated a large amount of albumin, dilute the urine with water. Pour the urine into an Esbach's tube to the "U" mark and bring the volume to the "R" mark by addition of Esbach's reagent. Place a rubber stopper in the tube, mix the contents by inverting the tube slowly several times, and then place it in the cold room. After twenty-four hours, the height of the precipitate, as determined by graduations on the tube, indicates the amount of albumin in grams per liter. Divide by ten to obtain the percentage, and, if the urine was diluted, multiply by the dilution factor.

### *Sugar*

**Benedict's Macro Test.**—Place 25 cc. of Benedict's solution for quantitative estimation of sugar in a porcelain evaporating dish with from

10 to 20 grams of sodium-carbonate crystals, or one-half this weight of the anhydrous salt, and a few small pieces of pumice stone. Bring the mixture to the boiling point and keep it boiling vigorously throughout the titration. Add the urine rapidly from a graduated burette. If the sugar content is high (more than a slight trace), dilute the urine 1:10. A white precipitate will form and the color of the solution will fade. When the blue color becomes pale, add the urine more slowly and then drop by drop until the end-point is reached, i.e. the disappearance of the blue color. Read the amount of urine used from the burette and calculate the percentage as follows:

Let X equal the quantity of undiluted urine from the burette.

$$\frac{0.05}{X} \times 100 = \text{percentage of glucose present.}$$

(0.05 gram of glucose is required to reduce 25 cc. of the reagent.)

**Fermentation Test for Glucose.**—Sometimes it is desirable to determine if the sugar present is glucose. For this purpose, sterilize the urine in an Arnold sterilizer for twenty minutes. If the specimen contains more than 1 per cent of sugar, dilute with a known amount of water so that the portion used contains less than this amount. Emulsify in the urine approximately half a gram of fresh yeast cake, free the mixture from any air bubbles, and transfer the whole to a Smith fermentation tube. Fill the cylindrical portion, allowing no air bubbles to remain, and half fill the bulbous portion. Incubate the tube twenty-four hours or overnight so that fermentation will be complete. The presence of gas ($CO_2$) denotes the fermentation of glucose. Lactose will reduce Benedict's solution but it is generally believed not to be fermentable by yeast.[2]

Always control the test by testing normal, sterilized urine with the yeast, since yeast itself sometimes gives off gas. Also test a known glucose solution to determine if the yeast is active.

If an Einhorn saccharimeter is available, use it for this test (103). The percentage of glucose present can be obtained from the graduations on this apparatus. Always calculate for the dilution when this tube is used.

[2] While this fact is commonly accepted and is stated in many manuals of methods, Castellani, A. and Taylor, F. E., published a paper in the Journal of the American Medical Association, 1926, 86, 523 (101), in which they assert that bakers' yeast will, as a rule, ferment a very large number of sugars, including maltose, galactose, saccharose, and lactose. Instead of a yeast method, they advocate the use of a mycologic method first described by them in 1917 in the British Medical Journal, 2, 855 (102).

### *Chlorides*

When a quantitative estimate of chlorides is requested, use the Volhard-Arnold method (104). Obtain the standardized solution necessary for the test from the chemical department.

### *Urea*

When a quantitative estimation of urea is requested, use Marshall's urease method (105).

### *Functional Test—Phenolsulfonephthalein*

When a phenolsulfonephthalein-functional test is requested, use the methods described by Todd (106), or Rowntree and Geraghty (107).

## MICROSCOPIC EXAMINATION

### *Unstained Sediment*

Centrifugalize the urine from three to five minutes at low speed. Pour off the supernatant fluid, transfer a small amount of the sediment to a glass slide by means of a capillary pipette and place a cover slip over it. Examine several such preparations, making them separately from the upper and lower portions of sediment when it is abundant. Make a careful search for pus cells, red blood cells, tissue cells, casts, and crystals, and note, in general, the number of bacteria. To obtain the best results in the examination of urinary sediment, use a low-power objective and subdued illumination.

**Recording Results.**—Record the relative numbers of the different elements noted as follows:

Large number.
Many.
Moderate number.
Few.
Very few.
None.

### *Stained Sediment*

When a request is made for an examination for tubercle bacilli or other organisms, centrifugalize the urine for one-half hour at high speed and prepare films from the sediment; stain two by the Ziehl-Neelsen method for acid-fast organisms. Except in special instances, Gram-stained preparations are made only on catheterized urines when a bac-

teriological examination is requested. Search very carefully for acid-fast bacilli. The finding of these organisms in a catheterized specimen is indicative of tuberculous infection.

Examine the preparations stained by Gram's method and note the relative number of different kinds of bacteria.

**Recording Results.**—For recording results, follow directions under methods of recording.

*Bacteriological Examination*

Unless aseptic precautions have been observed during collection, the specimens are generally unsatisfactory for these tests.

Plate a small amount of the sediment on blood agar and identify the different types of organisms present (See: table 22).

When special requests are made for determining the presence of *B. coli*, plate the sediment on Endo's medium and if the colonies are characteristic of *B. coli*, identify the organisms further by methods given in table 22. Record the organisms identified by morphologic, serologic, and cultural tests.

## ANIMAL INOCULATION

In all cases when there is an indication of tuberculosis of the genito-urinary organs, inoculate a guinea pig with some of the sediment whether or not acid-fast bacilli are found in the stained preparation.

## REPORTING RESULTS

In reporting the results of the routine examinations, use the following form:

*Physical and Chemical Examination*

Appearance.....
Color.....
Specific gravity.....
Reaction.....
Albumin.....
Sugar.....
Bile.....

*Microscopic Examination*

Leucocytes (pus cells).....
Red blood cells.....
Casts.....

Epithelial cells.....
Bacteria.....
Crystals.....
Amorphous deposits.....
Add the results of other tests that have been made.

## BLOOD

Cell counts and hemoglobin determinations are made in connection with the routine physical examinations of the department staff and when special investigations are undertaken.

### STAINED PREPARATIONS

**Preparation of Blood Film.**—Use slides which have been cleaned and kept in alcohol. Burn off the alcohol before using. Place a small drop of blood, either from the patient's ear or finger tip, on the slide near one end. Place a second slide upon the first at an angle to it and against the edge of the drop. Then spread the drop by drawing the second slide across the surface of the first in a continuous sweep. A thin film will be left in its path. Allow to dry without the use of heat.

**Differential Leucocyte Count.**—Staining of blood films: Stain the blood films submitted for differential count with Wright's stain, using a normal blood control as described under malaria. When two slides are submitted, stain one at a time as indicated under malaria. The second may be stained by Goodpasture's method if the history or examination indicates the presence of leukemia.

Examine the stained preparation carefully, using a mechanical stage. Count at least three hundred leucocytes, five hundred if feasible, classify each, and calculate the percentage of each variety. Select representative portions of the film for counting, as the leucocytes are usually unevenly distributed on the slide.

Note any abnormal leucocytes or erythrocytes. If nucleated erythrocytes are present, record the ratio of the number seen to the number of leucocytes counted.

### REPORTING RESULTS

Stamp the following classification form on the back of the history blank and enter the results of the examination accordingly.

*Differential Count*

Lymphocytes (large and small).....
Large mononuclears and transitionals.....

Polymorphonuclears { eosinophiles ..... neutrophiles ..... basophiles .....
Myelocytes .....
Normoblasts .....
Megaloblasts .....
.....
.....
.....cells counted

PREPARATIONS STAINED BY GOODPASTURE'S METHOD

**Staining of Blood Films.**—If there is evidence of leukemia, stain blood films by Goodpasture's method. If two slides are submitted, stain only one with this stain and the other with Wright's stain.

**Examination.**—Blood stained by Goodpasture's method presents the following picture:

*Leucocytes.*—The nuclei of the cells are clear red and the cytoplasm is pink. The granular elements differ as follows:

Polymorphonuclears: Neutrophilic granules, deep blue, and sharply defined. Eosinophilic granules, dark blue about the periphery with lighter center. Basophilic granules do not stain.

Myelocytes: Granules are stained as above. The cells are differentiated by morphology of nucleus.

Transitional cells: Contain fairly numerous and sharply defined granules.

Lymphocytes and most mononuclear cells: No granules.

Mononuclear cells of doubtful classification: Certain mononuclear cells of doubtful classification show a moderate number of blue granules. If stained by Wright's method, these cells would probably be classified as lymphocytes.

*Erythrocytes.*—A smooth buff color.

*Platelets.*—Pink.

**Reporting Results.**—If definite results are obtained, record:

"Myelocytes were found (Goodpasture's stain)."

"No myelocytes were found (Goodpasture's stain)."

If the results are questionable, send the report in a letter.

ENUMERATION OF ERYTHROCYTES AND LEUCOCYTES

When counts are not made at frequent intervals, control each by an examination of blood from a normal person. When making direct counts of erythrocytes and leucocytes, examine at least two separate preparations for each.

**Erythrocyte Count.**—Make a 1:200 dilution of freshly drawn blood with Hayem's solution in a pipette provided for the purpose and mix, shaking by hand for three minutes or in a shaking machine for one minute. Fill a Levy counting chamber and count the cells in eighty small squares. This number multiplied by ten thousand gives the number of erythrocytes per cubic millimeter.

**Leucocyte Count.**—Make a 1:20 dilution of freshly drawn blood with a 1-per-cent solution of glacial-acetic acid, using a pipette designed for the purpose, and shake by hand for two minutes or in a shaking machine for one minute. Fill a Levy counting chamber and count the cells in at least five of the 1-mm. squares. The average multiplied by two hundred gives the number of leucocytes per cubic millimeter.

### HEMOGLOBIN DETERMINATION

As a preliminary procedure, the Tallquist method may be used and if any evidence of abnormality is noted, use either the Dare or Sahli method.

**Dare's Method** (108).—Allow undiluted freshly drawn blood to flow into the capillary chamber formed by two small pieces of glass and place it in the hemoglobinometer. When observed in this instrument, the specimen appears as a stationary red disk, side by side with a similar disk which is part of a revolving color scale. Compare the two in a darkened room by the light of the candle or electric bulb attached to the instrument, revolving the scale until the colored disks match. Read the percentage of hemoglobin directly from the scale on the instrument.

It is well to restandardize each instrument by making several determinations on specimens of normal blood.

**Sahli's Method.**—Fill the graduated tube of the Sahli hemoglobinometer to the mark 10 with N/10 HCl and add to it, by means of a pipette provided for the purpose, 0.02 cc. of freshly drawn blood. Allow this to stand for at least one minute while a change to acid hematin is produced. Then dilute the fluid drop by drop with distilled water, until the color matches that of the standard tube. Read the percentage of hemoglobin in the blood directly from the graduated tube.

Both pipette and tube should be standardized before they are put into use, and at intervals thereafter, by testing several specimens of normal blood from both men and women. The normal for the instrument used may then be recorded with each report.

**Reporting Results.**—Report results as follows:

"Hemoglobin———per cent (Sahli). The normal for the instrument used is———," or

"Hemoglobin———per cent (Dare)."

### *Color Index*

The color index is the ratio of the amount of hemoglobin in a single erythrocyte to the normal amount. It is determined by dividing the percentage of hemoglobin by the percentage of erythrocytes. To obtain the percentage of erythrocytes, multiply the first two figures of the total erythrocyte count by 2, if the count is one million or more.

## COAGULATION TIME

Allow three large drops of fresh blood to fall freely on a glass slide and note the time. At half-minute intervals, draw a needle through one or another of them and as soon as a clot is dragged along by the needle, record the time again. The interval between the time the blood was drawn and the time the clot was formed is the coagulation time. For normal blood this interval is from two to eight minutes, usually about four and one-half.

**Reporting Results.**—Report results as follows:

"Coagulation time———minutes."

## STOMACH CONTENTS (109)

### CHEMICAL EXAMINATION

When gastric contents are submitted for examination, perform the following tests when requested: If the quantity of material is small, dilute it with distilled water before testing. Give the tests precedence in the order listed. Filter the material through coarse filter paper before any of the tests are made.

### *Blood*

**Benzidin test.**—Perform a benzidin test.

### *Lactic Acid*

**Kelling's Test.**—To from 15 to 20 cc. of water, add from one to two drops of a saturated solution of ferric chloride and mix thoroughly. Pour one-half the mixture into each of two clear test tubes. To one tube, add a small amount of the filtrate from the gastric contents and

to the other tube a similar volume of water. Lactic acid is indicated by the immediate development of a distinct yellow color in the tube containing the gastric contents.

**Reporting Results.**—Report results as follows:

"Lactic acid was present (Kelling's test)."

"No lactic acid was indicated (Kelling's test)."

### *Total Acidity*

If a request is received for the determination of the acidity of stomach contents, send a letter stating that this test, to be of value, should be performed in a local laboratory.

## MICROSCOPIC EXAMINATION

Examine the sediment on a cover-slip preparation for red blood cells, pus cells, and Boas-Oppler bacilli and report the findings.

# METHODS USED IN THE LABORATORIES FOR SANITARY AND ANALYTICAL CHEMISTRY

LEONARD M. WACHTER, *Chemist in Charge*

## INTRODUCTION

The department examines water and sewage, whenever the results of the examination are likely to be directly applicable to the prevention of disease and the protection of public health. Bacteriological milk counts are made in coöperation with the Division of Sanitation in connection with its supervision of local milk pasteurization plants.

Prior to the year 1914 the laboratory made water examinations in large numbers without requiring that the samples be accompanied by accurate data concerning the sanitary conditions existing at the source from which the samples were taken. Obviously, such results were of but limited practical value. Since 1914 the water laboratory does not report on any sample unless adequate data concerning the sanitary conditions of the source of the water are furnished. This gives a sound basis for the interpretation of the results of water examinations of samples from either public or private sources. The policy eliminated useless work and opened up the opportunity for citizens to have samples from their private supplies examined where public health problems were involved and the source of which was duly inspected by the local health officer or other qualified officials.

Sanitary inspections have not been required by some state laboratories. The absence of such information undoubtedly was a serious handicap in the past to the United States Public Health Service in the certification of waters used on interstate railroads, boats and other carriers, as presumably such certifications would have to be based on the results of the laboratory examinations. However, the regulations concerning the certification of water supplies promulgated by the Secretary of the Treasury on May 3, 1921, Section 19, paragraph B, state "as determined through a survey of the sanitary conditions under which the supply is produced and the results of bacteriological and chemical analysis of samples of the water."

The general analytical work carried on by this department covers a broad scope. It involves problems referred to the laboratory by local health officers, by the Divisions of Administration, Communicable Diseases, and Sanitation and also coöperative work with other departments of the laboratory. Some of the activities approximate research in character and for such, standard methods are not now available. The formulation of standard methods for much of this work is a line of endeavor that it is hoped to accomplish in the future.

The staff consists of two chemists, one assistant chemist, one technical assistant and one laboratory helper.

In general the methods used for water, sewage and milk examinations are those of the "Standard Methods of Water Analysis" and the "Standard Methods of Milk Analysis" of the American Public Health Association. Only outlines of such methods are given. These outlines serve to indicate which of several procedures are used where several alternate methods are given in the American Public Health Association methods. They also record the slight deviations that have been found best adapted to our work.

The policies that define the relations of the sanitary and analytical laboratories to outside agencies as regards the work that will be undertaken, for whom it is done, and the distribution of reports are stated in detail. The methods for receiving samples, for checking them and the accompanying data and the procedures for reporting are described.

*Administration of the Department*

The department is administered by the chemist in charge. The bacteriological and the chemical examinations are made by two closely coöperating groups of workers located in adjacent rooms. As the Division of Sanitation is responsible for the inspection and supervision of public water supplies, close coöperation is maintained with the members of it. Through frequent conferences, these laboratories also coöperate with the district state health officers and with the local health officers.

## SECTION I

## THE EXAMINATION OF WATER

Samples of water are examined by the laboratory at the request of the directors of the divisions of the State Department of Health, the district state health officers, and the local health officers. Private individuals are referred to their local health officer. If in his judgment examinations are necessary or desirable, they will be made by the laboratory when the health officer obtains the necessary containers from the laboratory, collects the samples, and furnishes the facts concerning the sanitary conditions existing at the source from which the samples are collected. No examinations are made unless the samples are taken in the containers provided by the laboratory, and unless the required data are furnished concerning the sanitary conditions of the source of the water.

### RECEIVING AND RECORDING SAMPLES AND THE ACCOMPANYING DATA

When samples of water are received at the laboratory, the large samples are delivered to the chemical laboratory, and the cases containing the small samples are delivered to the bacteriological water laboratory. Samples received on holidays, Sundays, and after laboratory hours are placed in the cold room by the person receiving them.

**Procedure.**—Before beginning the examinations, record on the daily record card the shipper's name, the place of shipment given on the shipping tag or label, and the identification number on the boxes.

Open the boxes, also the envelopes. Compare the data on the sanitary-inspection record cards and on the tops of the individual sample bottles. Mark each sample bottle with a serial number and a day number; the series of numbers being distinct for the chemical and the bacteriological samples. Place this serial number on the proper laboratory working card, also the name of the municipality, the name of the sender, the source of the sample, and any identification marks that may be recorded on the descriptive card accompanying the sample or on the top of the sample bottle. Compare the identification data on the descriptive cards that accompany the samples and on the laboratory working cards. Enter the samples in the accession book, giving the bacteriological sample the first accession number, and writing the letter "B" after it

to indicate that it is a bacteriological sample. If a sample for chemical examination was collected from the same point, give it the succeeding accession number followed by the letter "C." Enter the accession numbers on the sanitary-record cards and also on the bacteriological- and chemical-laboratory working cards.

## PHYSICAL AND SANITARY CHEMICAL EXAMINATION

Comparatively rapid changes in the amounts of certain compounds take place in water samples. This is particularly true of nitrogen compounds.

**Procedure.**—Make determinations in the following sequence: free ammonia, albuminoid ammonia, nitrites, nitrates, and oxygen consumed. Make the other determinations later if necessary. Express results in parts per million.

In general, the details of technic employed are those described in "Standard Methods for the Examination of Water and Sewage of the American Public Health Association," Fifth Edition.

### *Physical Examinations*

(1) **Color.**—Determine by the platinum-cobalt standard in 50-cc. Nessler tubes. If the water is turbid, pass it through a Berkefeld filter.

(2) **Turbidity.**—Determine turbidities to 100 by turbidity standards. Determine turbidities over 100 by a standard candle turbidimeter. Use the platinum-wire method (U. S. Geological Survey turbidity rod) in the field.

(3) **Odor (Cold).**—Determine in a partially filled bottle after shaking.

(4) **Odor (Hot).**—Heat the water nearly to the boiling point in a partly filled, tall beaker or an Erlenmeyer flask covered with a watch glass. Allow to cool for not more than five minutes and determine the odor.

### *Chemical Examinations*

(1) **Total Solids.**—Evaporate a measured quantity of water in a weighed, platinum dish. Heat the residue at 180°C. for one hour; cool in a desiccator and weigh. Compute for 1 liter of water.

(2) **Loss on Ignition.**—Ignite the dish plus the total solid residue to dull redness, in a radiator, cool in a desiccator and weigh.

Loss in weight computed for 1 liter of water is the loss on ignition.

(3) **Mineral Residue.**—Total solids minus loss on ignition gives the mineral residue.

(4) **Nitrogen as Free or Saline Ammonia.**—Add 500 cc. of water to a distilling flask. If the water is acid to methyl-orange indicator, add sufficient sodium carbonate to make it slightly alkaline. Distill off in Nessler tubes three portions of 50 cc. each. Add 1 cc. of Nessler reagent. If the third tube contains more than a small amount of ammonia, distill off one or more additional 50-cc. portions. Compare the Nesslerized tubes with ammonium-chloride standards, or permanent standards adjusted to ammonium-chloride standards.

(5) **Nitrogen as Albuminoid Ammonia (Total).**—Cool the residue in the flask from the distillation of the free ammonia, add 50 cc. of alkaline, potassium-permanganate solution, connect the flask with the condenser and distill 5 portions of 50 cc. of each. Nesslerize and compare with the standards.

(6) **Nitrogen as Albuminoid Ammonia (Dissolved).**—When necessary to determine also the dissolved albuminoid ammonia, filter the water through ammonia-free filter paper and proceed as above.

(7) **Nitrogen as Nitrites.**—Treat colored and turbid waters with aluminum hydrate and filter before testing. Add 1 cc. of sulfanilic-acid solution and 1 cc. of *a*-naphthlamine acetate to 100 cc. of the water in a Nessler jar. Mix them and allow the mixture to stand at least ten minutes. Compare with standard nitrite solution treated with the same reagents in similar jars.

(8) **Nitrogen as Nitrates.**—Treat colored or turbid water with aluminum hydrate, and filter. Evaporate to dryness 50-cc. or smaller volumes, depending on the nitrate content, in a porcelain dish on a water-bath. Moisten residue with 2 cc. of phenol-disulfonic acid. Rub with a glass rod. Dilute with distilled water and make distinctly alkaline with potassium hydrate. Wash into a 100-cc. Nessler jar and dilute to 100 cc. Compare with standard solutions of potassium nitrate treated as above.

If the water contains more than 30 parts per million of chlorine, nearly neutralize the alkalinity with N/50 normal sulfuric acid, treat with silver sulfate free from nitrates to remove all but 0.1 mg. of chlorine. Treat with aluminum hydrate, filter, wash with a small amount of hot water. Evaporate to dryness, moisten with 2 cc. of phenol-disulfonic acid and proceed as above.

(9) **Organic Nitrogen.**—Boil off the free ammonia from 500 cc. of sample. To the residue, add 5 cc. of nitrogen-free, concentrated, sulfuric acid. Mix by shaking. Boil until copious fumes of sulfuric acid are given off and the liquid is colorless. Add 5 grams anhydrous-sodium sulfate if a higher temperature is required to obtain complete

digestion. Cool, dilute with ammonia-free water. Make alkaline with a 10-per-cent ammonia-free sodium-hydrate solution. Distill the ammonia and Nesslerize.

(10) **Oxygen Consumed.**—Place 100-cc. or, when necessary, smaller volumes of the water sample, diluted to 100 cc. with distilled water free from organic matter, in a flask, add 10 cc. of dilute sulfuric acid (1:3) and 10 cc. of standard potassium permanganate. Submerge the flask in boiling water for thirty minutes. Remove from the bath, add 10 cc. of standard ammonium oxalate; then titrate the excess of ammonium oxalate with the permanganate solution.

(11) **Dissolved Oxygen.**—Dissolved oxygen is determined by the Winkler method modified as recommended by the "Standard Methods of the American Public Health Association," (1925).

(12) **Chlorides.**—Clarify turbid and colored samples with aluminum hydrate; samples of water containing small amounts of chlorides to be concentrated by evaporation. Evaporate 250 cc. to a volume of 50 cc. in a 6-in. evaporating dish. Add 1 cc. of potassium-chromate indicator and titrate with standard silver-nitrate solution: each cubic centimeter is equivalent to 0.0005 gram of chlorides.

(13) **Total Hardness.**—Determine by the soap method. Standardize alcoholic-soap solution against calcium chloride made from Iceland spar treated with hydrochloric acid and evaporated several times to remove excess acid.

(14) **Chlorine.**—Mix 1 cc. of orthotolidine reagent with 100 cc. of the sample in a 100-cc. Nessler jar and allow the mixture to stand at leave five minutes. In cold weather, warm the sample to 20°C.

For quantitative results compare with permanent standards. Small amounts of chlorine give a yellow color and larger amounts an orange color.

(15) **Alkalinity.**—Add 1 cc. of erythrosine indicator and 5 cc. of neutral chloroform to 100 cc. of the sample in a 250-cc. clear glass-stoppered bottle. Titrate with N/50 sulfuric acid. Add small amounts at a time. Shake the bottle after each addition.

The end-point is reached, when chloroform becomes colorless. Methyl orange may be used as indicator for waters that have not been treated with iron sulfate or aluminum sulfate.

## BACTERIOLOGICAL EXAMINATION OF WATER

Samples of water are collected in sterile glass-stoppered bottles, the tops and necks of which are covered with tinfoil and a cloth secured by a string. Samples should be collected only by some one capable of using sterile technic. A record of the sanitary conditions of the

source of the water must accompany the samples. Samples of water for bacteriological examination should be forwarded to the laboratory in shipping cases that make proper provision for keeping the samples cold by the use of ice. Samples must be kept cold until examined. The sample bottles should be shaken twenty-five times before removing portions for examination.

### *Qualitative Examination*

**Test for the Presence of Members of the B. coli Group.**—To determine the presence of bacilli of the *B. coli* group, inoculate three fermentation tubes containing lactose broth with each volume of the water that is tested; 10 cc., 1 cc., and 0.1 cc. being the volumes of potable water usually tested. When examining grossly polluted water, test smaller volumes, in order to determine the volume that will give a negative test.

If after from eighteen to twenty-four hours' incubation, gas is produced in one or more of the fermentation tubes inoculated with any given volume of water, make a streak subculture on an Endo or eosin-methylene-blue plate from one of the fermentation tubes producing gas. Make streak plates,[1] if gas is absent after from eighteen to twenty-four hours, but is present after forty-eight hours' incubation. Make similar streak plates from one culture of any other volume of water that produced gas.

To make the streak plate cultures, transfer 1 loopful of the culture from the fermentation tube to a test tube containing approximately 10 cc. of sterile water. Place a loopful of this diluted culture on an Endo or eosin-methylene-blue plate and streak the surface with a sterile, bent glass rod. Incubate from eighteen to twenty-four hours. If characteristic or strongly suggestive *B. coli*-like colonies have developed, pick colonies from the plate and transfer part of each colony to an Andrade lactose-agar slant and also to a lactose-broth fermentation tube. If no typical or suggestive colonies appear upon the plate within twenty-four hours, reincubate the plate twenty-four hours longer and pick colonies

[1] Three-per-cent Endo's agar base: Sodium-sulfite solution. Ten-per-cent standardized solution. Keep under a layer of mineral oil. Draw off with a siphon.

Basic fuchsin solution. Ninety-five-per-cent alcohol saturated with the dye.

Melt 3-per-cent agar base. To each 100 cc. of base add one gram of pure lactose or 5 cc. of 20-per-cent lactose previously sterilized in test tubes. Add, after mixing, 2.25 cc. of 10-per-cent medium sulfite solution and 0.75 cc. of saturated, basic, fuchsin solution.

considered most likely to be of the *B. coli* group, whether typical or otherwise, and transfer to an Andrade lactose-agar slant and to a lactose-broth fermentation tube. Incubate the slant and fermentation tubes for forty-eight hours.

If gas is produced in a lactose-broth fermentation tube inoculated from a colony on an Endo or eosin-methylene agar plate, make a Gram-stain preparation from the Andrade lactose-agar-slant culture inoculated from the same colony.

**Results of Tests.**—Report *B. coli* group as present in the volume of water inoculated in the original fermentation tube, when the microscopic examination demonstrates that the slant culture consists of non-spore-bearing bacilli and gas is produced in the confirmatory fermentation tube.

Report *B. coli* group as absent from the volume of water inoculated in the original fermentation tubes, if gas is not produced after forty-eight hours' incubation.

Report *B. coli* group as absent from the volume of water inoculated in the original fermentation tubes, if gas is not produced in the confirmatory fermentation tube or if the microscopic examination indicates the presence of spores.

### *Quantitative Examination*

**Colony Count on Plates.**—If the water is known to contain 300 or a smaller number of bacteria per cubic centimeter, plate 1 cc. If the water contains a larger number per cubic centimeter, dilute the sample with sterile water, 1 cc. of the sample to 9 cc., or 1 cc. to 99 cc. respectively, or similar further dilutions if necessary.

The plating media are standard beef-extract gelatin and standard beef-extract agar, the reaction to be between pH 6.8 and 7.2.

**Procedure.**—Count gelatin plates after forty-eight hours' incubation at 20°C.

Count agar plates after twenty-four hours' incubation at 37°C.

Except when water contains less than 30 bacteria per cubic centimeter, count those plates that contain between 30 and 300 colonies.

Take the average of at least two plates for computing the number of bacteria per cubic centimeter in the water.

Make all counts over a standard ruled counter with a standard lens magnifying 3.5 ×.

## REPORTING RESULTS

Reports are furnished only when the laboratory has received a satisfactory record of the sanitary conditions of the source of the water to be examined.

**Procedure.**—When health officers or others fail to send the information with the samples, write a letter asking that the information be furnished.

When all the individual tests have been completed, the information recorded on the descriptive card, the chemical working card, and the bacteriological working card which refer to the same sample, are brought together. The person in charge of the water laboratories writes the interpretations and the conclusions on the back of the bacteriological working card.

Base the interpretations upon the results of the laboratory examinations and the data, obtained by inspection of the sources of the water, submitted by health officers, district state health officers, or members of the department staff.

The reporting group makes typewritten reports from the data on the working cards. These reports and the cards are compared with members of the water group.

The laboratory reports directly on private supplies. One copy of the report is sent to the local health officer, one to the district state health officer, and one to the Division of Administration.

Report the results of the laboratory examination of samples collected from public supplies to the director of the Division of Sanitation.

These reports give only the actual laboratory results without an interpretation. Two copies of these reports are sent to the Division of Sanitation accompanied by the sanitary-survey record cards. These cards are later returned to the laboratory. One copy of the report is sent to the Division of Administration. The Division of Sanitation incorporates the laboratory report in its report to the commissioner of health.

**Recording Results in Accession Book.**—Record the results of the bacteriological examinations in an accession book. Clip together and send to the office for filing the chemical and bacteriological laboratory working cards and the record of the sanitary survey.

## SECTION II

# THE EXAMINATION OF SEWAGE, SEWAGE EFFLUENTS, AND INDUSTRIAL WASTES

## CHEMICAL EXAMINATION

### *Collection of Samples*

Collect samples in two separate portions in glass-stoppered bottles. Preserve one portion to be used for the determination of the compounds of nitrogen, except nitrites, by adding 1 cc. of dilute sulfuric acid (1 part concentrated acid, sp. gr. 1.84, to 3 parts of water) to each 100 cc. of the sample. Preserve the second portion to be used for the determination of chlorides, total solids, loss on ignition, nitrite nitrogen, and fats by adding 1 cc. of dilute formaldehyde (1 part 40-per-cent formalin to 3 parts water) to each 100 cc. of the sample.

At least two liters are required as final samples to be sent to the laboratory. Except in special cases, samples are composites and are made up of portions taken at definite intervals. Portions should be taken at least every four hours if the composite is to represent a single day. Ordinarily, the aliquot portions of a sample are of equal volumes, 100 cc. or more, depending upon the number taken to make up the final sample. Samples must be accompanied by a card giving the name of the municipality, the date and the time when each portion was taken, the source of the sample and details concerning the source, and methods of operation at the time of sampling, if the sample is taken from a sewage-treatment plant.

### *Methods for Chemical Examination*

(1) **Total Solids.**—Proceed as directed for water, except dry the residue at 103°C.

(2) **Suspended Solids.**—In a Gooch crucible prepare a mat about 4 mm. thick with asbestos that has been shredded, thoroughly ignited, treated with concentrated hydrochloric acid overnight, and washed with distilled water until acid-free. Dry the crucible and prepared mat at 103°C., cool, and weigh. Filter 1000 cc., or an aliquot part of a liter, through the crucible (residue shall not exceed 30 mg.), dry at 103°C. for one hour, cool in a desiccator and weigh.

The increase in the weight of the crucible in milligrams equals the suspended matter in parts per million of one liter of the sewage filtered.

(3) **Ammonia Nitrogen.**—

Reagents:

Copper sulfate, 100 grams per liter.

Lead acetate, 100 grams per liter.

Sodium hydroxide, 500 grams diluted to 1 liter with distilled water.

**Procedure.**—To 100 cc. of the sample, add 1 cc. of copper-sulfate solution; mix thoroughly, add 1 cc. of sodium-hydroxide solution, mix, and allow precipitate to settle. If sample contains hydrogen sulfide, add 1 cc. of lead-acetate solution and 1 cc. of sodium-hydroxide solution. Dilute a portion of the supernatant liquid in a Nessler jar to the 50-cc. mark. Nesslerize and compare with standards. (See: water analysis.)

(4) **Albuminoid Nitrogen.**—The ratio between this nitrogen and total nitrogen in sewage is irregular. At times the data are desired to make comparisons between sewage and the stream into which it flows. When the determination is required, see water analysis.

(5) **Organic Nitrogen.**—

Reagents:

Copper sulfate solution.

Sulfuric acid, concentrated, low in nitrogen.

Potassium sulfate, powdered.

Sodium hydroxide, 500 grams in 1 liter.

Sulfuric acid, 0.05 N.

Sodium hydroxide, 0.05 N.

Methyl red, 1 gram in 100 cc. of 95-per-cent alcohol.

**Procedure.**—Add 10 cc. of nitrogen-free sulfuric acid, 1 cc. of copper-sulfate solution and 5 grams of potassium sulfate to 100 cc., or a less volume, of the sewage in a 500-cc. Kjeldahl flask. Digest in hood for thirty minutes after the liquid becomes colorless. Cool somewhat, dilute to about 250 cc. with ammonia-free water. Make alkaline with caustic-soda solution, using phenolphthalein as indicator. Connect the flask immediately to condenser and distill into Nessler tubes. Nesslerize and compare with standards. Make a blank determination, using the same amount of reagents and deduct the nitrogen content of the reagents.

Determine the free ammonia content in a separate portion of sewage by direct Nesslerization.

The organic nitrogen is equal to the difference between the nitrogen found as ammonia after digestion and the free-ammonia nitrogen.

(6) **Nitrate Nitrogen.**—

Reagents:

Sodium hydroxide. Dissolve 250 grams in 1.25 liters of distilled water. Add strips of aluminum foil and allow to reduce overnight. Concentrate to 1 liter.

Aluminum foil. Strips of about 0.33 mm. thick, weighing about 0.5 gram.

**Procedure.**—Concentrate to 20 cc., by boiling 100 cc. of the sample in a 300-cc. casserole after 2 cc. of the sodium-hydroxide solution has been added. Pour the concentrated sample into a 100-cc. Nessler jar or tube of similar dimensions. Rinse the casserole several times with nitrogen-free water and pour the rinsings into the Nessler jar; the total volume in the jar being about 75 cc. Add a strip of aluminum foil to the jar. Close the jar with a rubber stopper containing a bent, glass tube about 5 mm. in diameter.

One arm of the tube is flush with the bottom of the stopper, and the longer arm dips below the surface of distilled water contained in another test tube, which serves as a trap. The action is continued usually overnight and never less than four hours.

TABLE 25

*Relative stability numbers**

| TIME REQUIRED FOR DECOLORIZATION AT 20°C. | RELATIVE STABILITY S | TIME REQUIRED FOR DECOLORIZATION AT 20°C. | RELATIVE STABILITY S |
|---|---|---|---|
| *days* | *percentage* | *days* | *percentage* |
| 0.5 | 11 | 8.0 | 84 |
| 1.0 | 21 | 9.0 | 87 |
| 1.5 | 30 | 10.0 | 90 |
| 2.0 | 37 | 11.0 | 92 |
| 2.5 | 44 | 12.0 | 94 |
| 3.0 | 50 | 13.0 | 95 |
| 4.0 | 60 | 14.0 | 96 |
| 5.0 | 68 | 16.0 | 97 |
| 6.0 | 75 | 18.0 | 98 |
| 7.0 | 80 | 20.0 | 99 |

* From Standard Methods for the Examination of Water and Sewage, Amer. Pub. Health Assoc., 1923, 76.

Pour the contents of the tube into a distilling flask and dilute to 250 cc. with ammonia-free water. Distill, and collect the distillates, and Nesslerize. If the sample is high in nitrates, mix the distillates in a 200-cc. flask filled to capacity with ammonia-free water. Nesslerize an aliquot part of this, compare with a standard, and multiply the result by the necessary factor to give the nitrogen content in parts per million of one liter of the sample.

(7) **Oxygen Consumed.**—See: water.

(8) **Dissolved Oxygen.**—See: water.

(9) **Relative Stability of Effluents.**—

Reagents:

Methylene blue. Dissolve 0.5 gram of the double zinc salt, or commercial variety, in water.

**Procedure.**—Use a glass-stoppered bottle of 150-cc. capacity. In collecting the sample, use precautions similar to those for dissolved oxygen samples (See: water analysis), if the amount of dissolved oxygen is low. Add 0.4 cc., not more, of the methylene-blue solution, insert stopper in completely filled bottle, and incubate ten days at 20°C. Observe samples twice daily and record the time when the color disappears.

Table 25 gives the relative stability numbers.

## BACTERIOLOGICAL EXAMINATION

Proceed as for water but make higher dilutions.

## SECTION III

## THE EXAMINATION OF ICE

### RECEIPT OF SAMPLES

A representative piece of ice weighing about fifty pounds (more if shipped to the laboratory from a distance in warm weather), must come to the laboratory packed in sawdust.

If it is natural ice, the sample must be accompanied by a record of a sanitary survey of the source of the water from which the ice was cut, giving detailed information concerning any sources of pollution located near the ice field, or upstream from it, that might affect the quality of the ice.

### PREPARATION AT THE LABORATORY

At the laboratory, remove the sawdust from the outside of the cake of ice and rinse the surface with sterile water. Remove the outside surfaces by chipping with an axe that has been sterilized by flaming, and split the ice at right angles to the original horizontal plane. Remove chips from this newly fractured surface with the sterile axe and place them in a 6-ounce, sterile, wide-mouth bottle, using a sterile metal spoon.

If the cake contains snow ice and clear ice in distinct layers, a sample of each may be chipped from the cake and placed in separate bottles.

### EXAMINATION

Allow the ice in the bottles to melt completely at room temperature and proceed as in the examination of a sample of water.

## SECTION IV

## THE BACTERIOLOGICAL EXAMINATION OF MILK AND CREAM

### SAMPLING AND PACKING

All the samples sent to the laboratory must be accompanied by a record card on which are given the identification marks on the original container and the name and address of the dairy, bottling plant, creamery, producer, or distributor from whom the milk was obtained, date and time of milking and of packing the sample; also the grade of milk or cream, as raw or pasteurized. This information and any other details concerning the quality of the milk must be recorded on the card.

**Sampling.**—Obtain representative samples of milk. When possible, invert the original container several times to mix the milk. Stir milk in larger containers with a sterile rod or pipette. Use a different sterile mixer for each container that is sampled.

Milk may be stirred with a dipper or other stirrer that is already in the container from which the sample is to be taken.

Obtain samples from cans or tanks by passing slowly, vertically downward through the surface of the milk to the bottom of the can or tank, a sterile aluminum tube or straight-walled pipette, leaving the top of the tube open while passing it into the milk.

These aluminum pipettes should be long enough to reach to the bottom of the usual milk can and approximately 6 mm. in diameter.

Place all of the milk withdrawn in the pipette in a sterile bottle or glass tube with metal cap and cork gasket.

**Packing.**—Enter the number or other identification on the bottle, on the record card. Place the sample in a cylindrical, metal, watertight box with a seamless cover. Place a tight-fitting rubber band over the joint between the box and the cover. Pack this box containing the milk samples, in ice in the milk can or other suitable container.

There must be sufficient ice to last during the time consumed in transit. If all the ice has melted when the samples reach the laboratory, the examinations are not made. The results would not be of value and might be misleading. When samples of bottled milk or cream are obtained from but a short distance, the original bottles may be brought to the laboratory, surrounded by ice, or the bottles may be thoroughly shaken and a representative sample withdrawn with a sterile pipette, placed in a small, sterile bottle or tube, and packed as previously described for samples from milk cans or tanks.

## LABORATORY RECORDS

As soon as the sample is received, give it the laboratory accession number, which is entered on the descriptive card accompanying the sample to the laboratory and also on a laboratory working card. Enter on this card the name of the place, the source of the sample, and the distinguishing marks or number on the sample bottle, also the date and time of arrival at the laboratory. Enter also the date and time of plating and counting, the dilutions plated, the counts obtained on the plates, the computations, and the counts per cubic centimeter.

Following the sample number in the laboratory accession book, record the details concerning the sample and, when the examination is completed, the results.

## OFFICIAL PLATE COUNT[1]

**Dilutions.**—Before the milk is withdrawn for plating, shake the sample twenty-five times. Withdraw 1 cc. of the milk from the sample bottle with a graduated pipette and mix with 99 cc. of sterile water. After shaking twenty-five times, make a further dilution, or dilutions if necessary, by adding 1 cc. of the previous dilutions to another 99-cc. volume of sterile water or to 9 cc. of sterile water. Shake each dilution twenty-five times before removing any with a pipette.

**Plating.**—Keep the samples on ice until ready to plate. If the approximate bacteriological content of the milk is not known, plate the milk in dilutions of 1:100, 1:1000, and 1:10,000. If the milk is known to be of good quality, omit the 1:1000- and the 1:10,000-dilutions. Omit the 1:100-dilution when examining milk known to have a high bacteriological content.

Use standard, straight-walled, sterile pipettes, graduated to deliver 1 cc., and glass-covered Petri plates 10 cm. in diameter and 15 mm. deep, for plating.

With a wax pencil, mark on the cover the number of the sample and the dilution used in the plate. Make duplicate plates.

Place 1 cc. of the diluted milk in the center of the dish and afterward 10 cc. of standard beef-extract agar that has been previously melted and cooled to 43°C. Thoroughly mix the milk and agar and allow the plates to harden. Invert the plates and then incubate for forty-eight hours at 37°C.

[1] Standard Methods of Milk Analysis of the American Public Health Association and the Association of Official Agricultural Chemists. Fourth editions are used for details of technical procedure (110).

**Counting.**—Count the plates over a standard ruled counter with a standard 3.5 X engraver's lens such as a Bausch and Lomb No. 146.

Count by preference the plates having between 30 and 300 colonies. If it is necessary to count a plate having a large number of colonies, count a representative fractional area of the plate and multiply this number by the necessary factor to obtain the number of colonies on the plate. Then multiply by the factor of dilution. Keep a record of the exact counts obtained from the plates.

**Reporting.**—Report, as official plate count or colonies per cubic centimeter. In reporting use only the two significant left-hand digits.

### DIRECT MICROSCOPIC COUNT

It is possible to determine by this method, in a comparatively short time, whether a sample of raw milk is decidedly above or below a definite grade. The method is, therefore, of great value in controlling the quality of raw milk from the individual producer, as brought to the milk station or pasteurizing plant, or even the individual can of raw milk. It may be used for the control of all grades of raw milk and raw cream and for grading "Grade B Raw" milk and cream. It is not entirely satisfactory for accurately grading "Certified" and "Grade A Raw" milk. It will, however, detect marked deviations from these grades. The direct microscopic-count method must not be used for determining the number of living bacteria in pasteurized milk or pasteurized cream.

When the direct microscopic method is used, duplicate tests should be made from time to time with samples of milk from the sources usually tested, the direct microscopic method being used, and the standard agar-plate method, for the purpose of checking the results. Records of these duplicate tests should be preserved.

**Apparatus.**—

Standard special capillary pipettes graduated to deliver 0.01 cc.

Glass microscopic slides, the usual 1-by-3-inch slides or 2-by-4.5-inch slides.

Glass guide plate, ruled with areas 1 cm. square.

Stiff inoculating needle.

Microscope (mechanical stage).

Objective, oil immersion, 1.9 mm. ($\frac{1}{12}$ inch).

Eyepieces.

Eyepiece micrometer marked with a circle 8 mm. in diameter and divided into quadrants.

**Reagents.**—

Methylene-blue stain (Loeffler's).

Xylol.

Ethyl alcohol (90 per cent).

**Slide Preparations.**—Mark the microscopic slides with the sample numbers. Thoroughly shake the milk sample. Place a clean glass slide, over the glass guide plate, or paper ruled in square centimeters. With a calibrated pipette place 0.01 cc. of the milk or cream on the slide, and with a stiff needle spread the milk evenly over an area of 1 square centimeter using the marking on the ruled glass plate, or paper, as a guide. Place the slide upon a level surface in a warm place.

The milk should dry within ten minutes but must not be excessively heated or the film will separate from the glass.

After drying, dip the slide in xylol for from one to two minutes to dissolve the fats, drain and completely dry. Immerse the slides in 90-per-cent ethyl alcohol for one minute or more and then transfer to a freshly made and filtered solution of Loeffler's methylene blue prepared as follows: saturated alcoholic solution of methylene blue, 30 cc.; caustic potash in a 0.01-per-cent solution, 100 cc. Stain the slides from five seconds to one minute or longer, depending on the depth of color desired. After withdrawal from the stain, rinse the slides with water to remove the excess of stain and decolorize with alcohol.

The slide must be observed while decolorizing. The decolorizing may require from a few seconds to a minute. A properly decolorized film will have, in general, a faint blue background. After decolorizing, the slides are dried and may then be examined or preserved for future reference.

**Standardizing the Microscope.**—To standardize the microscope, place a stage micrometer on the stage of the microscope, and by experiment find the combination of eyepiece and the tube length that will cover a field 0.205 mm. in diameter.

When the field covered has a diameter of 0.205 mm., it will cover almost exactly the area covered by 1/300,000 of a cubic centimeter of milk dried on the smear when prepared as directed. A higher magnification may be obtained by a different adjustment of tube length and the use of a higher-power ocular. For this purpose, an eyepiece micrometer graduated with a circle 8 mm. in diameter may be used. The microscope must be adjusted so that this circle covers a field on the slide exactly 0.146 mm. in diameter. When so adjusted, each field will cover approximately 1/600,000 of a cubic centimeter of milk when dried on the slide.

**Counting.**—Count all the bacteria observed in at least thirty fields, when examining milk approximately "Grade B Raw" in quality.

The microscope adjustment covering a field 0.205 mm. in diameter may be used. If thirty fields 0.205 mm. in diameter are observed, the number of bacteria found, multiplied by 10,000 will give the estimated count of bacteria per cubic centimeter.

When examining milk of the higher grades, where the bacteria are few in number, use the microscope, adjusted so that it covers a field 0.146 mm. in diameter, and count sixty fields.

The number of bacteria found in the sixty fields 0.146 mm. in diameter, multiplied by 10,000, will give the estimated count per cubic centimeter.

**Preserving the Slide Preparations.**—Clean the slide preparations with xylol, dry, and preserve them for three months.

**Reporting Results.**—Record the number of fields examined and the exact counts obtained from the slide, on the laboratory cards. In the final report, give the two significant left-hand digits only.

The official agar-plate count being considered standard, and which records the number of colonies per cubic centimeter, will not be directly equivalent to the direct microscopic count, which records the individual bacteria. It should be assumed that under usual conditions the direct microscopic count of bacteria per cubic centimeter will average four times the colony count obtained by the official plate method.

# METHODS USED IN THE ANTITOXIN, SERUM, AND VACCINE LABORATORIES

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# INTRODUCTION

The standard methods of the antitoxin, serum, and vaccine laboratories deal largely with processes that cannot be standardized. Until the obscure reactions of infection and immunity are more clearly understood, work based on their practical application must be largely empirical. The technical details are, therefore, constantly under revision and an entire procedure may at any time be discarded. The routine methods can give at most, only a cross section of a particular stage in the development of each problem. Moreover, the balance between precise directions, and information or explanation, necessarily varies with the data available for each process. This state of flux makes impossible uniformity of presentation. At the same time, it makes all the more essential, a detailed statement of present procedure as a point of departure for future progress.

The methods have not been formulated for publication. Nor have they been focused for any particular grade of worker. Few if any of the procedures are original. Many are based directly on the experience of others, to whom hearty acknowledgment is made. They have developed as the joint effort of the staff of the department as a whole, to meet their particular requirements. Directly or indirectly each member of the staff has had a part in the preparation. Thus the methods are intended for the instruction and use of the younger workers and technicians and also as an aid and a reference volume for the more experienced workers. Their aim is to insure uniform procedure in technical detail and, by placing responsibility, to relieve those in authority from the irksome task of such constant close supervision as is imperative if methods are less precisely formulated. The more competent workers are thus released for administrative duties or for related study and research.

Figure 31 gives graphically the work of the antitoxin, serum, and vaccine laboratories and the relation of the various groups and subgroups included in it. The activities are divided among a number of small units each of which is in charge of a competent assistant bacteriologist or chemist or veterinarian, under whom are the assistants necessary to carry on the work. There are several subgroups in charge of highly trained technical workers supervised, in an advisory capacity, by an assistant bacteriologist. At the farm a worker is directly in

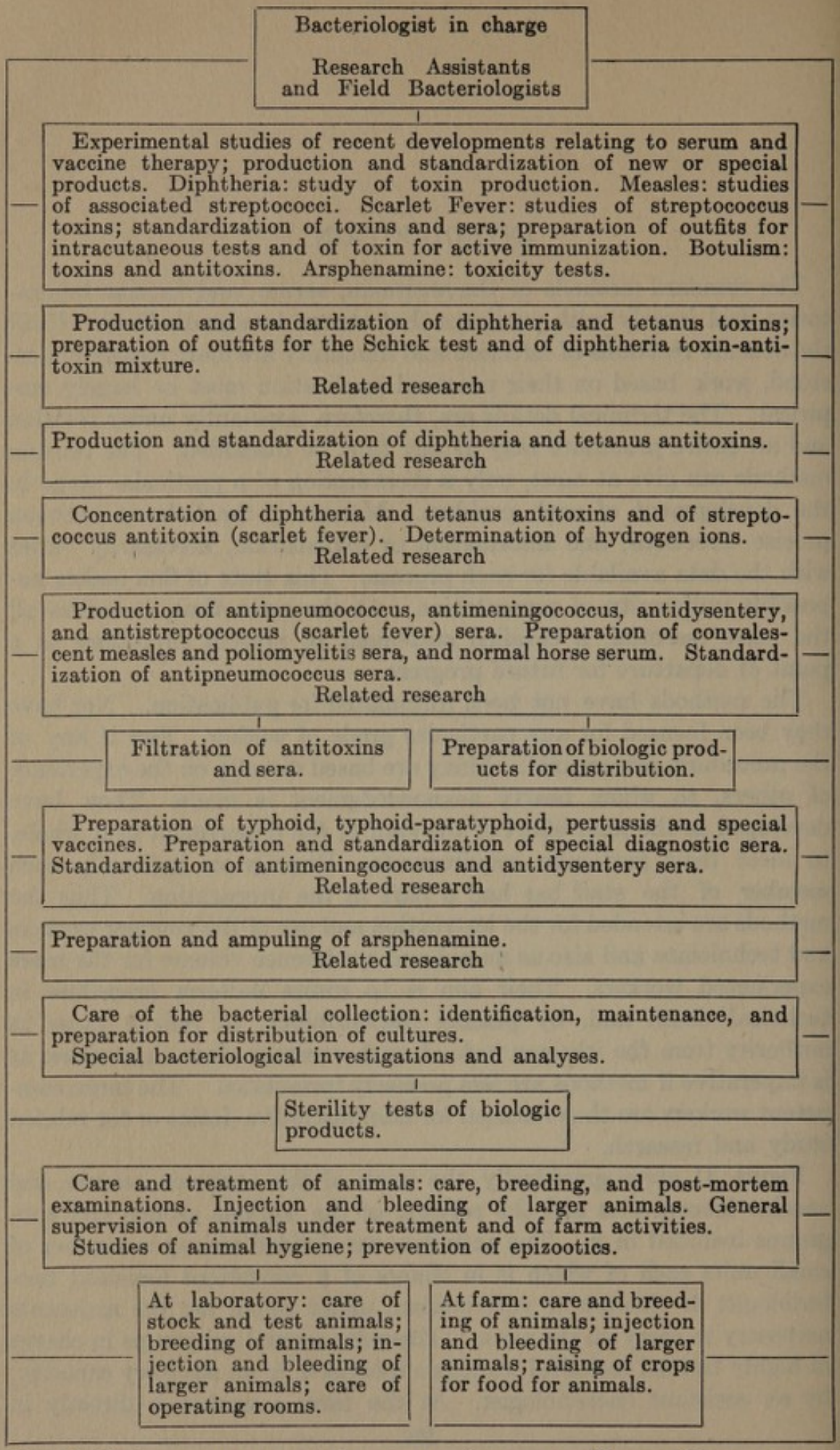


FIG. 31. ORGANIZATION OF THE ANTITOXIN, SERUM, AND VACCINE LABORATORIES

charge, and under him there are six or eight technical assistants and laborers. The division and arrangement of the groups is, to some extent, one of practical convenience and expediency and has been adopted to meet special conditions. Thus, the care of the bacterial collection and the work covering the care and treatment of the animals, and farm activities, which are also general service functions, have been included in the antitoxin, serum, and vaccine laboratories, owing to their very close connection with this department. Similarly, the secretary of the department is in charge of the office shipping group under the executive clerk, the bacteriologist in charge acting in an advisory capacity.

Only a few of the large number of record forms in use are shown in the text. At the end of each chapter, however, is appended a brief description of the permanent records used. Statistical, general, and research reports covering the work of the different groups are submitted each month. Space is left at the end of each general report for suggested changes in routine procedures and for a record of changes adopted during the month. Before final inclusion in the standard procedures, such changes must be tested in actual practice and be approved by the bacteriologist in charge and by the director.

New workers on entering the department prepare weekly reports of work they have seen and learned. It has been found that instead of repressing initiative the formulated methods give an incentive as well as a concise and firm basis to those who are capable of progress along lines of related research.

Since the form and arrangement of the methods have been based solely on the convenience of the workers, instructions of purely local bearing, information, and explanation, have been inserted, without consideration for the reader, when and where they would be most useful. Inconsistencies and omissions will doubtless become all too evident in the collected methods, and in many procedures, through lack of space, it has been impossible to explain why certain things are done in certain ways.

The methods do, however, represent the accumulated practical experience of a number of years, during which the laboratory has prepared and distributed biologic products of a high average grade.

## SECTION I

## CHAPTER 1

# PRODUCTION AND STANDARDIZATION OF DIPHTHERIA TOXIN

The diphtheria bacillus secretes an extracellular poison or exotoxin, which is obtained in quantity *in vitro* by cultivation of the bacillus in suitable fluid medium.[1] Diphtheria toxin thus produced, after being standardized by potency tests on guinea pigs, is used in the production of antitoxic serum in horses, in standardization tests of unconcentrated and concentrated sera, as the diagnostic agent in the Schick test, and in the preparation of toxin-antitoxin mixtures for active immunization of persons against diphtheria.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

### *Standard Strain*

The standard strain used in the production of toxin is:

*B. diphtheriae:* Park-Williams, No. 8. Collection No. 110. Received in February, 1915 from the New York City Research Laboratory, where it was isolated in 1894.

**Maintenance of Strain.**—Two cultures of the strain are maintained independently. The cultures, grown in 0.2-per-cent dextrose-veal-infusion broth (125-by-13-mm. tubes containing 2 cc. of medium), are incubated at 34°C. for twenty-four hours and then transferred in a draft-free room to fresh medium. Before a new batch of medium is used for the daily transfers, two successive transfers are made to ascertain whether the medium is favorable for growth. Care should be

[1] The procedures, formulated empirically as the result of practical experience, while complicated, have been found to yield high-grade toxin if carefully followed. Recent studies made in this laboratory of toxin production in synthetic media, attempting to determine the essential conditions and to simplify the procedure, indicate an interaction between the protein constituents of peptone and certain inorganic salts (Ca, Mg, and the phosphates) as necessary for toxin production. While progress has been made, the results have not reached the practical stage of replacing the empirical procedures.

taken not to shake the tubes while transferring, or while carrying the container to and from the incubator.

Remove the 24-hour cultures from the incubator and observe whether the appearance is typical (pellicle with clear sparkling broth below). Transfer a loop of the pellicle from one of the cultures to the surface of the fresh medium, then slant the tube in a small, flat, wire tray so that as large a surface of the broth as possible is exposed to the oxygen in the air. Repeat the procedure with the second culture. Place the tray containing the inoculated tubes in the incubator. Retain the cultures from which transfers are made, for one week in the draft-free room.

**Identification and Purity Tests.**—Observe the cultures carefully when making the daily transfers. Once a week, or oftener if anything unusual is noted, examine the cultures microscopically, staining with methylene blue and by Gram's method. Also, make plate cultures by placing a loop of pellicle on one of two 0.2-per-cent dextrose-beef-infusion agar plates (poured and incubated for two days in advance), and proceed as directed under "General Bacteriological Technic; Cultural Examination," p. 7. Examine the inoculated plates after twenty-four and forty-eight hours' incubation. If suspicious colonies are noted, make stained preparations and examine. Should a culture be contaminated, go back to a previous seeding. Resort to fishings only if the strain would otherwise be lost.

Every three months control the cultures further by fermentation tests. Inoculate tubes containing dextrose, dextrin, and saccharose serum waters, and incubate together with one uninoculated tube of each sugar. Make readings at the end of twenty-four and forty-eight hours' incubation and record the reactions as ++ (acid and coagulation), + (acid), ± (very slight acid production), and − (no change). If dextrose or dextrin is not fermented, or if saccharose is fermented, make and examine slide preparations.

## PRODUCTION OF TOXIN

For toxin production, cultures of the standard strain are grown for seven days in 2-liter Erlenmeyer flasks containing 700 cc. of diphtheria-toxin broth; the preservative is then added, and the toxin-broth culture passed through paper pulp and a filter candle to free it from organisms. Twenty-eight flasks are usually inoculated at one time.

Requisition for media: Place orders for the necessary media at least ten days before the flasks are to be inoculated. Order dextrose, which is added to the

flasks at the time of inoculation, in Pyrex tubes (150 by 16 mm.) containing 7 cc. of a 20-per-cent solution which, added to the 700 cc. of broth in each flask, gives approximately a 0.2-per-cent solution. Order for transfers, broth containing dextrose in 2-cc. volumes in tubes (125 by 13 mm.); for the broth for the preliminary seed cultures, broth from the same lot as that used in the flasks to be inoculated, dispensed in Pyrex tubes (125 by 13 mm.).

### *Preliminary Seed Cultures*

Two days before the flasks are inoculated, make three or four extra transfers from the 24-hour stock culture selected for toxin production. The next day make microscopic preparations from these tubes and stain them with methylene blue and by Gram's method. Reject any cultures showing atypical forms. From the remainder, inoculate the required number of tubes for seed cultures, that is, two or three more than the number of flasks to be inoculated. Slant the tubes and incubate them for twenty-four hours.

### *Inoculation and Incubation of Flasks*

Three days before inoculation bring the flasks containing freshly prepared medium to the draft-free room used for diphtheria toxin and leave them there to permit reabsorption of oxygen after sterilization. Examine the seed tubes before taking them from the incubator room, and discard any that do not have the characteristic appearance. To inoculate the flasks, place one culture tube and one tube containing dextrose solution, side by side in the right hand, with the mouths on a level. Flame and remove the plugs, and flame the mouths, while an assistant removes the paper cap and plug from one of the flasks, and, after flaming the opening, brings the flame to a horizontal position above it. Then, quickly pour the contents of both tubes into the flask. The assistant immediately flames the neck of the flask, and replaces the plug and the paper cap. Place the empty tubes at once in a pail to be sterilized in the autoclave, holding the plugs under the necks to catch any drops. Immediately after inoculating each flask, enter the lot number and date on the tag. Incubate the inoculated flasks for seven days at an even temperature (34°C.). If the incubator is opened frequently, protect the flasks from the light with a dark cloth. Do not disturb them during the period of incubation.

### *Addition of Preservative*

On the seventh day, remove the flasks to the toxin-filtration room, examining each carefully as it is taken from the shelf of the incubator.

If the broth beneath the pellicle is not perfectly clear and sparkling, or if the appearance of the pellicle is unusual, make slide preparations from both pellicle and underlying broth; stain the broth by Gram's method and the pellicle with methylene blue. If necessary, make a second preparation from the broth, stained with carbol fuchsin for the identification of contaminating spore-bearing organisms. Discard any flasks found to be contaminated. Add 70 cc. of 5-per-cent phenol solution to each flask to give an approximately 0.5-per-cent solution. Mix thoroughly by rotating the flask, then allow the pellicle to settle. Pool the supernatant fluid from the flasks into sterile 8-liter bottles, pouring it through a large funnel. Transfer to each bottle the tag from one of the flasks. Filter, preferably the same day; if filtration is delayed until the following day, cover the bottles with a dark cloth and hold them in the draft-free room.

In handling toxin broth, observe, throughout, the usual precautions required with the material containing pathogenic microörganisms, since the preservative added is not sufficient to sterilize it immediately.

### *Filtration*

The toxin broth is passed through paper pulp in a Buchner funnel to remove the pellicle and then through a filter candle. For general directions, see "Filtration of Biologic Products," p. 511.

Filtration of toxins is carried on in a draft-free room with glass sides painted an ochre color to exclude those light rays which are said to be most active in hastening the deterioration of diphtheria toxin.

Supplies: Place orders for sterile 4- and 8-liter bottles several days in advance. On the day before filtration, sterilize in the autoclave a filter candle (2 by 10 in.) with connections. See "Final Filtration by Suction," p. 516. Keep on hand sterile 4-liter suction flasks made of Pyrex glass, and 20-cm. Buchner funnels fitted with rubber stoppers to fit the necks of the flasks.

**Filtration through Paper Pulp.**—Assemble all the required apparatus and supplies in the draft-free room. Set up a Buchner funnel in a suction flask and connect it with the negative pressure, using an empty bottle as a guard. Set the flask in a 13-liter pail for protection in case of breakage. Prepare the pulp filter as described in "Preliminary Filtration through Pulp," p. 515.

Insert a stopper with siphon attachment into one of the 8-liter bottles of pooled toxin, attach a screw pinchcock to the rubber tubing and set the bottle on the shelf above the funnel. Have the rubber connection long enough so that the glass tip extends about an inch below the top of the funnel. When not in use keep the tip in a test tube. Siphon

over enough toxin broth to a little more than cover the pulp. When the broth has passed through the pulp, disconnect the suction, and transfer the funnel to a clean 4-liter suction flask. Whenever a transfer is to be made, have an assistant hold the new flask close to the neck of the full one. After making the transfer and turning on the suction, fill the funnel with toxin broth; always replenish before the supply is exhausted. Take care that foam is not drawn into the side arm of the suction flask. Examine the filtered toxin frequently to make sure it has a clear, sparkling appearance.

Number the bottles of pooled toxin, and mark each suction flask with the number of the bottle from which it was filled, so that, later, an equal volume of toxin from each bottle can be poured into the glass container of the filter candle, thus insuring a uniform mixture in every bottle of filtered toxin. Should the lot of toxin to be filtered consist of two or more different batches, pass them through the same filters, unless other instructions are given.

**Filtration through Filter Candle.**—Follow the procedures given under "Final Filtration by Suction," p. 516. Fill the bottles as full as possible to avoid excess of air. State on the tag, the kind of toxin, lot and bottle numbers, and date (Diphtheria toxin 449A, 449B, etc., 12–1–25).

### *Sterility Tests*

Make routine sterility tests on each bottle of filtered material, following the directions given in the chapter "Sterility Tests of Biologic Products," p. 543. From one of the bottles, withdraw, besides the material necessary for sterility tests, 5 cc. for potency tests. After inoculating the tubes, place the remaining material in a sterile bottle labeled with the kind of toxin, the lot number, and the date.

### *Care of Materials and Apparatus*

Cover the stopper of each bottle with cotton dampened in 1-per-cent crude cresol, tie down securely, and cover with tinfoil and a paper cap. Store the toxin in the cold room apart from other supplies and protected from the light.

Clean the filter candle, following the directions given under the second method in the section "Filtration of Biologic Products," p. 521. Remove most of the moisture by connecting the filter candle with the vacuum intake and drawing air through it. Dismantle the filtering apparatus, replace the tag on the nipple, and dry the filter thoroughly before storing it.

Pool any discarded toxin and all contaminated liquid and sterilize

them in the autoclave, together with glassware and other apparatus which may have come in contact with the toxin. Remove supplies, etc. from the filtration room; clean it thoroughly, and wipe the shelves with 1-per-cent crude cresol.

Toxin for immunization of horses: Keep toxin for the immunization of horses in 4-liter bottles on the shelves reserved for toxin, from which it is removed as required by the antitoxin-production group. Since toxin over six months old is preferred, always maintain an ample supply.

Toxin for special purposes: Select toxin to be reserved for purposes requiring a stabilized toxin, as, for standardization tests, for Schick tests, and for toxin-antitoxin mixture, at times when toxin of high potency is being produced. Wrap the bottles in brown paper, mark each "Reserved," with the lot and bottle number, and place them on the shelves for reserved toxin. Enter "Reserved" on the record card.

Toxin for standard: When it is desirable to set aside a new supply of toxin for standardization tests, select from the reserve a bottle of toxin at least one year old and test it for the L+ dose. If this is satisfactory (L+ dose, 0.2 cc. or less), siphon a part of the toxin (avoiding light, exposure to air, and unnecessary shaking as much as possible) into sterile brown-glass bottles (500 cc.) with rubber stoppers. Cover the surface of the toxin in each bottle with about 2 mm. of albolene (C.P. previously sterilized in the autoclave), pipetting the oil carefully with aseptic precautions along the side of the bottle. Label each bottle "Standard Diphtheria Toxin," with the lot number, dates filtered and transferred to small bottles, and volume. Assign to the antitoxin-production group, and record the transfer on the record card.

Toxin for Schick tests: When tests of toxin in use for Schick tests give evidence of a change in the minimum fatal dose, make preliminary tests of each of two or three lots of toxin of high original potency to determine their approximate titers. Select the toxin approximating the required titer and test it further.

## STANDARDIZATION OF TOXIN

Diphtheria toxin is standardized by determining its minimum fatal dose as the measure of its potency or titer. The minimum fatal dose or M.F.D. is the least quantity of toxin which, when injected subcutaneously, will kill a guinea pig weighing 250 grams in ninety-six hours.

### PROCEDURE FOR DETERMINING THE M.F.D.

Standardization tests are made on each lot of toxin on the day the toxin is filtered. Three different doses are generally tested, one guinea pig being used for each. The doses are estimated from the titer of the preceding lots. At present, doses of 0.004, 0.003, and 0.002 cc. are given.[2] The dilutions are such that the doses are contained in not

[2] For a number of years the M.F.D. of most of the lots has been from 0.002 to 0.003 cc., the L+ dose, when tested, from 0.12 to 0.18 cc., occasionally 0.20 cc.

less than 1 cc., and not more than 2 cc. of the final dilution. For preparation of dilutions, see "General Instructions," p. 627. If toxin is refiltered for any cause, it is retested, two guinea pigs being used.

### *Special Glassware*

The burettes and pipettes used for measuring volumes must be accurately standardized[3] and all glassware must be perfectly clean.

Burette and siphon (for salt solution): Prepare and assemble the siphon and connections and sterilize them in the autoclave, following the directions given under "General Instructions," p. 622. Place the certified 50-cc. burette in strong cleaning solution; rinse in tap and in distilled water. Fill the burette with 80-per-cent alcohol, taking care that the alcohol penetrates between the stopcocks and sockets, and allow it to stand overnight. Then discharge the alcohol, smear the stopcocks with a sterile mixture of beeswax and vaseline, and connect the burette with the siphon, which has previously been placed in the bottle of salt solution. Whenever drops adhere to the burette, or the stopcocks stick, disconnect the apparatus and clean the burette as before.

Syringes: Use 3-cc. syringes (needle gauge 22, 1¼ in.), the plungers and needle connections of which have previously been tested to detect possible leakage.

Pipettes: Use standard "to contain" and certified graduated pipettes. After using, place them immediately in a cylinder containing tap water. At the close of the test, rinse them thoroughly in running water and place them in strong cleaning solution for at least one-half hour. Then remove the pipettes, rinse them very thoroughly in tap and in distilled water and draw up twice alcohol previously used for the final rinsing, and once 95-per-cent alcohol. Allow them to drain in a slanting position. If not used within a day, insert each "to contain" pipette into a sterile test tube with cotton at the bottom to protect the tip, and in the mouth of the tube. Store certified graduated pipettes with a sterile tube on each end. Make sure that the pipettes are absolutely dry before use.

### *Test Animals*

Use guinea pigs weighing between 230 and 280 grams. When the animals are received, weigh each and place an identification tag in one ear. Enter the date, source, ear-tag number, and weight of each animal,

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Retests of various lots stored at cold-room temperature for three or four years indicate only a very slight increase in the M.F.D., as a change from 0.002 to 0.003 cc. Retests of toxin exposed for varying intervals to higher temperatures (summer heat), also indicate marked stability. While the L+ dose of toxins prepared three or four years ago was not tested at that time, the present L+ dose of these toxins, ranging from 0.12 to 0.18 cc., suggests little deterioration.

[3] The graduated pipettes and burettes are sent to the Bureau of Standards in Washington for certification. "To contain" pipettes are standardized at the laboratory by determining the weight of water contained. For details, see Wheeler, M. W., Jour. Lab. & Clin. Med., 1919, 4, 498 (111).

and the name of the worker in charge of the group, on a manila tag. Place the animals in a cage and attach a "Normal animal" tag.

*The Test*

**Test Card.**—Before commencing the test, make out a "test card" (5 by 8 in.) shown in figure 32, giving the date, toxin number, dilutions, and doses to be tested. Have a second worker verify and initial each card before the dilutions are started. Enter on the test card also the numbers and weights and the source of the guinea pigs, and, on each animal tag, "Dip. toxin," and the number and test dose $\left(\frac{\text{No. 449}}{0.002}\right)$. Enter on the animal record card the lot number of the toxin, date and time of test; number, weight and source of each animal; and dose given

DIPHTHERIA TOXIN

Toxin No. 449 — 12-1-25, 4:00 p.m.

1 + 24 a (1st dilution)
1a + 19 b (2nd dilution)

| | | |
|---|---|---|
| 0.002–1.0 cc. | No. 5185 | 235 (grams) |
| 0.003–1.5 cc. | No. 5159 | 240 |
| 0.004–2.0 cc. | No. 5186 | 260 |

√J. E. D. (Checked by) — Laboratory stock — M. C. D. (Test made by)

FIG. 32. TEST CARD

each. Before starting the injections compare with the assistant the tags and cards. When the test is completed, copy the required data into the permanent-record book, and keep the cards a year for reference.

**Preparation of Dilutions.**—Select the required number of stoppered flasks and mark on each with a wax pencil the toxin number, volumes of toxin and of salt solution required, and a letter designating the position of the dilution in the series $\left(\frac{\text{No. 449}}{1+24\,a'}, \frac{\text{No. 449}}{1+19\,b'}, \text{etc.}\right)$. Fill the dilution flasks from the certified 50-cc. burette, following the directions given under "General Instructions," p. 624.

When all the flasks have been filled, adjust a 1-cc. "to contain" pipette in the special filling apparatus. (See "General Instructions," p. 622.) Draw up the toxin until the bottom of the meniscus just reaches the graduation. Wipe the tip of the pipette with filter paper, verify the reading, and deliver the contents into the first flask of dilut-

ing fluid.[1] After replacing the stopper, invert the flask two or three times. (This should be done gently to avoid air bubbles.) Remove the stopper and draw up exactly 1 cc. to rinse the pipette; discharge the contents again into the flask and mix as before. Rinse a second time in the same manner. (If two or more toxins are tested, make all first dilutions before making any of the second dilutions.) Taking a fresh pipette, draw up 1 cc. from the flask containing the first dilution and discharge into the flask prepared for the second; rinse twice as before. If more than two dilutions are to be made, continue in the same manner, using a fresh pipette for each dilution.

[1] Should the toxin go up in the pipette no more than 1 mm. above the required graduation, draw back the material by touching filter paper to the tip of the pipette. If, by accident, the toxin is drawn higher than 1 mm., use a fresh pipette.

When the dilutions are completed, transfer with a certified 2-cc. graduated pipette the required volumes, as indicated on the test card, to small amber bottles, each previously marked with the toxin number and the test dose $\left(\frac{\text{No. 449}}{0.002}\right)$. Before emptying the pipette, wipe the tip with filter paper and verify the reading; then touch the tip to the inside of the bottle to insure an even flow, and discharge. With an ordinary pipette add to each bottle enough salt solution to make a total volume of 3 cc. Rotate the bottle gently, taking care that no liquid touches the cork. Enter the time on the test card, and proceed at once to the injections.

**Injections.**—Before each injection have the assistant read aloud from the animal tag the toxin number and the dose; then read the entry on the bottle from which the dose is taken and the number of the guinea pig to be used, as given on the test card.

Draw the contents of the first bottle into a 3-cc. Luer syringe freshly sterilized by boiling; then, pipette into the emptied bottle 1 cc. of salt solution and rinse by rotating the bottle carefully. (Meanwhile the assistant picks out and stretches the guinea pig across his knee in the proper position for injection.)

Select as the site of injection an area slightly above and to the right of the umbilicus. Cleanse the area with cotton soaked in 0.5-per-cent crude cresol so that the hair is pushed back on all sides. Then, holding the skin at the site of injection between the thumb and forefinger, pierce it with a quick movement of the needle. Pointing the needle away from the head and obliquely toward the median line, insert it subcutaneously about 2 cm. or almost its entire length, drawing up the skin a little in advance. Keep the syringe close to the body. Avoid

undue stretching of the skin puncture, as it may later permit some of the injected material to escape.

Inject slowly, then withdraw the needle quickly, compressing the skin at the site of injection between the thumb and forefinger during withdrawal. (To prevent oozing, this pressure is continued by the assistant until the worker is ready to make the next injection.) Draw up from the bottle into the empty syringe the 1 cc. used for rinsing, then hold the syringe vertically with the needle upwards and draw down the plunger to beyond the 3-cc. graduation to rinse. Expel the air, taking care no drops of material are lost in doing so, and inject the rinsing fluid on the opposite side of the abdomen in the same manner as before. Always watch the sites of injection and record any oozing of the material that may occur. Place each guinea pig, after injection, in a second basket to avoid confusion. Use the same syringe for all doses. To rinse the needle and syringe between injections, plunge the needle into 5-per-cent phenol and draw up and discharge less than will fill it, then rinse both needle and syringe three times, twice with 3 cc. of salt solution and once with salt solution or with the water in which the syringe was boiled. When the injections are completed, return the animals to the cage, and attach the tags to the door. Initial the test card.

### *Observation of Animals*

Observe the animals for ten days, twice daily, at 9:00 a.m. and 5:00 p.m., or more often depending upon the severity of the symptoms. Enter on the animal record card (p. 451), the date, hour, and condition—slightly ill ("sl. ill"), ill, very ill, or dying; if the symptoms are very slight or doubtful, enter "v. sl. ill." Weigh the surviving animals at 9:00 a.m. on the first four days following injection, and record the weight on the record card. Place dead animals in the cold room; enter on the record card, the date and time found dead, and initial it. When animals are likely to die during the night, attach a green tag to the cage. (The cage will then be inspected by the night watchman.) Discharge animals still alive at the end of ten days. For further details, see "Care and Treatment of Animals—Small Animals," p. 574.

### *Autopsies*

Autopsy dead guinea pigs within twenty-four hours of death. (When a Sunday or a holiday intervenes, the interval may be extended to not more than forty-eight hours.) Autopsy those dying out of order, or sooner than expected, as soon as possible so that the results may be

controlled by satisfactory bacteriological tests. Record findings on the back of the animal record card.

Fasten the animal to a metal autopsy tray, ventral surface exposed, and wet the hair with 1-per-cent crude cresol. Make an incision along the median line and draw back the skin on each side. Record the extent and degree of induration at the site of inoculation as follows: "no indur.," "sl. indur.," "indur.," "mkd. indur." Open the peritoneal cavity with a longitudinal incision and examine especially the adrenals, recording the degree of congestion as "no C.," "sl. C.," "C.," "gr. C." Expose the thoracic cavity by cutting through the ribs on each side and along the upper margin of the diaphragm, and turning back the sternum. Record the degree of congestion of the lungs as for adrenals and note the presence of pleural fluid. Record also any unusual conditions. If infection is suspected, make a slide preparation and streak a blood-agar plate with the heart's blood, and with material from any other organ or area as indicated. (Special media may be substituted if advisable.) Should growth develop, make slide preparations and stain them by Gram's method. Record the cultural findings on the animal record card. Copy the data into the animal test book and keep the cards a year for reference. (A second person should verify the record.)

### *Estimation of the M.F.D.*

The least amount of toxin which killed a guinea pig in ninety-six hours is recorded as the minimum fatal dose or titer of the toxin. When death occurs a little before or after ninety-six hours, a − or + sign should precede the recorded dose. If the animal receiving the largest amount of toxin in the series survives, the test should be repeated with still larger amounts. It may also be necessary to repeat the tests if the original estimates of the titer prove to have been too low, or if the animals die out of order.

If all the animals of a series die within forty-eight hours, or live over five days, the toxin is always retested; otherwise, sufficiently close estimates can usually be made by taking into consideration the relative condition and the time of death of the guinea pigs receiving the different doses. Extreme temperature conditions and the physical state of the guinea pigs before injection may often affect the results, and should also be considered.

### *Permanent Records*

Standard strain: a. A card (5 by 8 in.) is kept for each subculture. On it are recorded any observations of special interest made in connection with the daily transfers or weekly tests. b. A special card (5 by 8 in.) is reserved for the results of the fermentation tests.

Standardization tests: The toxicity tests are recorded in a loose-leaf record book on sheets (8½ by 11 in.) with printed headings as follows: date, culture used, number of toxin, sources of guinea pigs, animal numbers, weight before injection, and weight and symptoms on the four days following if the animal is still alive, time of death (in hours if less than four days, in days and hours if over) and, under "Remarks," the condition after the fourth day, and the autopsy findings or date the guinea pig was discharged.

Toxin production: On a card (5 by 8 in. with printed headings) are recorded the following data in regard to each lot of toxin; lot number, strain, culture and medium used; hydrogen-ion concentrations (1) to which medium was adjusted and (2) after sterilization; total volume of broth inoculated; dates of inoculation, addition of preservative, and filtration; volume of filtered toxin, M.F.D. of toxin with time of death of guinea pigs on which estimate was based; results of sterility tests, and any observations of interest. On the reverse side of the card, are recorded lot and bottle number, M.F.D., and volume of each bottle of toxin.

The members of the antitoxin-production group consult these cards in selecting toxin to be used for immunizing horses, and record on them, at the time of shipment, date shipped, and numbers of the horses for which the toxin is to be used. When toxin is reserved for use in standardization tests, in toxin-antitoxin mixture or for other purposes, "Reserved" is entered in red on both sides of the card.

## CHAPTER 2

# PRODUCTION AND STANDARDIZATION OF TETANUS TOXIN

The bacillus of tetanus secretes a specific exotoxin which can be produced *in vitro*. The toxin is used in the immunization of horses for the production of tetanus antitoxic serum.[1] The procedures connected with the production of the toxin correspond closely to those described in the chapter "Production and Standardization of Diphtheria Toxin" to which the worker is referred for detailed instructions not given in the following text.

All procedures connected with the production of tetanus toxin, except the sterilization of contaminated material in the autoclave, are carried on in one room and the draft-free room connected with it. Since it is at present necessary to carry on the work in proximity to the general work of the laboratory, the head of the group must supervise the work with particular care, and assistants must be made to understand the reason for every precaution and the importance of observing each faithfully.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

### *Standard Strain*

The standard strain used in the production of toxin is:

*B. tetani:* "Pease Research." Collection No. 263A. Received from the New York City Research Laboratory, June, 1915. Isolated about 1904 at the state laboratory in Albany.

**Maintenance of Strain.**—Stock cultures of the strain are maintained in dextrose-free semisolid veal-infusion agar (150-by-15-mm. tubes, containing 15 cc. of medium). Transfers are made at least every three months by the worker in charge of the group, or by a specially designated assistant. Twelve or more tubes are inoculated each time. Two or more unopened cultures from each of the four previous transfers are kept in reserve. Stock cultures are labeled with the name, and number and date of transfer, and sealed with paraffin. The transfers are incubated and stored in a special basket covered with a dark cloth and distinctly marked "*B. tetani*, Pease Research Strain, Stock Cul-

[1] The tetanus toxin used in standardization of tetanus antitoxin is at present obtained as a dry powder from the U. S. Hygienic Laboratory in Washington.

tures for Toxin Production." Transfers are made by two workers. When making transfers, hold a piece of cotton dampened with 1-per-cent crude cresol under the tip of the pipette to catch any drops that may fall. When making slide preparations, place the slides in a Petri-plate cover before spreading the film, and fix the dried films with an inverted flame.

Place the tubes of medium to be inoculated, in a water-bath and boil them for fifteen minutes to drive off the absorbed oxygen. Then cool to about 50°C., taking care that the medium does not begin to solidify. Select one or two of the most recent semisolid cultures and heat them only enough to melt them, not over 70°C. Then pipette 1 cc. of melted culture into each of the fresh tubes of medium and rotate to mix the contents. Cool the tubes rapidly by placing them in cold water and label each. Incubate at 35°C. for seven days. Then remove the cultures to the draft-free room, and make two slide preparations from each. Stain one with steaming carbolfuchsin for five minutes, the other by Gram's method, and examine. Seal the tubes with paraffin and store the cultures in the cold room.

**Identification and Purity Tests.**—To protect against the presence of aerobic and anaerobic contaminating organisms in the stock cultures, twice a year (or more often if contamination is suspected), immediately after the routine transfers have been made, test the parent cultures for purity by inoculating three 0.2-per-cent dextrose-beef-infusion agar plates from each.

Two days before the tests, pour the plates and place them in the incubator. To inoculate, place a loopful of the culture upon the agar. With a sterile rod spread this thoroughly over the surface, and with the same rod streak two other plates in succession. Incubate the first plate under aerobic conditions for five days and examine it daily for evidence of growth. Incubate the last two in the hydrogen jar for anaerobic growth (See "Hydrogen-Jar Method," p. 6), for five days. Examine the plates both macroscopically and microscopically. Fish suspicious colonies and transfer to semisolid agar tubes and dextrose-agar plates for further study. If more evidence is required, prepare a tetanus-broth culture of the fishing, incubate five days, and inoculate guinea pigs intramuscularly as a test of toxin production.

Should contaminating organisms be found, discard the culture, and substitute one of the same series if pure. If necessary, go back to a preceding transfer. Resort to fishings only if the strain would otherwise be lost.

In addition to the characteristic forms, others of widely varying morphology are frequently met with in pure cultures of *B. tetani*.

## PRODUCTION OF TOXIN

For toxin production, the standard strain is passed through a preliminary series of daily transfers in tetanus-toxin broth (1-per-cent dextrose-veal-infusion broth in 175-by-22-mm. Pyrex tubes containing 30 cc.) and then grown for fourteen days in 2-liter Erlenmeyer flasks containing the same medium. The flasks are filled almost to the necks, but not so full that the plugs become moistened during sterilization. The toxin broth is passed through a preliminary as well as a final filter candle. All procedures are carried on in a draft-free room reserved for the purpose. Ten to twelve flasks are usually inoculated at one time.

Requisition for medium: Place orders for medium in flasks and tubes at least six days before the flasks are to be inoculated. Use the tubes of the preceding batch, if they are not more than six weeks old, for the preliminary cultures, and hold the fresh tubes for the succeeding lot. If the interval is more than six weeks, order a small lot of broth in tubes a week in advance of the main lot.

All apparatus and supplies which have come in contact with tetanus cultures must be marked "Tetanus" and placed in the autoclave under the supervision of a worker from the production group.

### *Preliminary Seed Cultures*

First day: Select two of the most recent semisolid stock cultures and melt them as previously described. Pipette from 5 to 8 cc. of the growth into each of two tubes containing broth freshly boiled for fifteen minutes to expel the air and cooled rapidly to about 50°C. Rotate to distribute the inoculum through the broth. Label the tubes with the name and date, protect them from the light, and incubate at 35°C. for twenty-four hours. Always return the parent cultures to the incubator.

Second day: Make microscopic preparations, using precautions previously described, stain with carbolfuchsin, and examine. If uncertain of purity, stain a second slide by Gram's method. Transfer 5 cc. of culture to each of three or four broth tubes, boiled and cooled as before. Incubate twenty-four hours.

Third day: After examining the cultures microscopically, transfer 5 cc. into each of as many tubes as are required (one or two more than the number of flasks to be inoculated). Incubate twenty-four hours.

Fourth day: Examine each culture microscopically before using the cultures to inoculate the flasks. Discard any doubtful cultures.

### *Inoculation and Incubation of Flasks*

After final sterilization the flasks are cooled rapidly to 50°C. in a sink, taken at once to the draft-free room, and inoculated. (See "Production and Standardization of Diphtheria Toxin," p. 310.) Do not add dextrose to the broth at this time, and rotate each flask after inoculating it.

Take care that the broth does not moisten the plugs. Always lift and carry the inoculated flasks with one hand underneath and the other steadying the neck. Incubate the flasks at 35°C. for fourteen days.

### *Addition of Preservative*

Remove the flasks to the draft-free room. After rotating each to bring the growth near the surface, make duplicate slide preparations from each flask, spreading not more than four films on each slide. Add 5 cc. cresol to each flask to give an approximately 0.25-per-cent solution. Add the preservative drop by drop with a rotary motion of the pipette to prevent the formation of large globules, and mix by rotating the flask. Stain one set of slides with carbolfuchsin and examine. If the purity of any flask is questioned, stain the duplicate slide with carbolfuchsin or by Gram's method as seems advisable. Discard any flask shown to be contaminated and pool the contents of the others in 8-liter bottles, placing each bottle in a 13-liter pail and pouring the toxin broth through a large funnel. Transfer a tag from a flask to each bottle. Place the empty flasks at once in the autoclave. Enter on the toxin card (p. 325) any observations of interest regarding growth, appearance of microörganisms, etc.

The toxin broth is usually held in the draft-free room until the following morning but may be filtered immediately.

### *Filtration*

Pass the toxin broth through paper pulp; then through a "preliminary" filter candle (coarse grained) before passing it through the final filter.

The broth appears somewhat cloudy after filtration through pulp (if very cloudy it is passed through a second time) but should be clear and sparkling after passing through the preliminary candle.

Prepare the preliminary candle in the same way as that used for final filtration. For procedure, see "Production and Standardization of Diphtheria Toxin," p. 311.

*Sterility Tests. Care of Materials and Apparatus*

For procedure, see corresponding headings under "Production and Standardization of Diphtheria Toxin," p. 312.

When all the toxin has been filtered through the preliminary filter, pass tap water through it immediately. Treat the second filter similarly. Then place both in the autoclave for sterilization, with the containers, mantles and connections, and the bottles containing washings. Always loosen the nuts on the filters and reattach the tags to the nipples before sterilization. After sterilization, clean the filter candles by the second method given in the chapter "Filtration of Biologic Products," p. 521.

## STANDARDIZATION OF TOXIN

Tetanus toxin is standardized by determining its minimum fatal dose as a measure of its potency or titer. The M.F.D. is the least quantity of toxin which, when injected subcutaneously, will kill a guinea pig weighing 350 grams within ninety-six hours. The procedures given in the chapter "Production and Standardization of Diphtheria Toxin," p. 313 are followed closely except as indicated in the following text.

At present doses of 0.0002, 0.0001, 0.00005 cc. are given.[2] (Dilutions 1 + 99 a, 1a + 99 b. The dose may be contained in less than 1 cc. of final dilution.) Guinea pigs weighing from 330 to 380 grams are used. Injections are made in the tetanus room.

After making the test pour the toxin dilutions and any water or salt solution used in rinsing the pipettes and syringes into a pail for sterilization. Fill the cylinder containing the pipettes, the empty dilution flasks, amber bottles, and other glassware with cleaning solution and allow to stand at least half an hour before handling them.

Do not weigh the guinea pigs after injection. Handle and disturb them no more than is necessary. Enter on the daily record card the symptoms as:

S—very slight: beginning contraction and rigidity of muscles of trunk felt on grasping it gently; slight curve of trunk to one side; stiffness of extremities.

SS—more definite: paralysis of extremities quite apparent; lateral deflexion of trunk and rigidity of muscles increased; slight intermittent tonic spasm on touching.

SSS—very marked: paralysis of legs extreme; head may be drawn back, but animal is still able to rise when placed on side; tonic spasm pronounced.

SSSS—whole body rigid: head thrown back; animal unable to rise to feet when placed on side; tonic spasms.

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[2] For a number of years the titer of the toxins prepared has been very uniform. The M.F.D. of most of the lots has ranged between slightly less than 0.00005 and 0.0001.

At autopsy make a rapid examination of the site of inoculation and of the abdominal and thoracic cavities (usually some congestion of the lungs is present). If the appearance is normal and there is no apparent reason for suspecting that death was due to infection or causes other than the toxin, enter "OK" on the card. If unusual conditions are found, record them.

### *Permanent Records*

Standard strains: A card (5 by 8 in.) is kept, on which are recorded date of transfer, and dates of parent cultures and semisolid medium used, number of tubes inoculated, results of microscopic examination and purity tests, and any further observations of interest.

Standardization tests and toxin production: The records are similar in form to those described in the chapter "Production and Standardization of Diphtheria Toxin," p. 319.

## CHAPTER 3

# PRODUCTION AND STANDARDIZATION OF STREPTOCOCCUS TOXIN (SCARLET FEVER)[1]

Streptococcus toxin, produced by cultivation of a single strain of hemolytic streptococcus isolated from scarlet fever, is used in the production of antistreptococcus serum in horses, in standardization tests of concentrated and unconcentrated sera, for the intracutaneous test for susceptibility, and for active immunization of persons against scarlet fever.[2] Although it is not possible by present methods to differentiate the hemolytic streptococci present in scarlet fever from those associated with other infections (113, 114), the practical value of the toxins and sera in the prevention and treatment of scarlet fever has apparently been demonstrated. The toxin is standardized by means of intracutaneous reactions induced in persons and in goats. The procedures connected with the production of the toxin correspond closely to those described in the chapter "Production and Standardization of Diphtheria Toxin," p. 308, to which the worker is referred for detailed instructions not given in the following text.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

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[1] The methods for the production and standardization of streptococcus toxin and antistreptococcus serum are necessarily tentative and subject to further change as additional data accumulate.

[2] Although the production of streptococcus toxin and antitoxin for the prevention and treatment of scarlet fever dates back many years to the early work of Moser, Gabritchewsky, etc., a renewed interest has developed as a result of the work of Dochez and the Dicks, and of the new methods of testing the action of toxins intracutaneously following the work of Schick with diphtheria toxin. Such methods have made it possible to secure a more convincing demonstration of the practical value of the use of these products. Streptococcus toxic filtrate quite similar to that now used in the active immunization against scarlet fever, has, however, previously been used in the immunization of the human subject as a vaccine treatment of streptococcus infection, and skin reactions following its use have been recorded. See Wadsworth, A. B., Jour. Amer. Med. Assoc., 1918, 71, 2052 (112).

*Standard Strain*

The standard strain used in the production of toxin is:

Hemolytic streptococcus: Dochez, N. Y. 5. Collection No. 14E. Received in May, 1924 from Doctor Dochez, Presbyterian Hospital, New York. This strain has been found to produce a highly potent toxin and to possess broad antigenic activity.

**Maintenance of Strain.**—The strain is maintained in "streptococcus-toxin broth" with passage through a mouse once a week.[3] At present the virulence is such that from 0.01 cc. to 0.001 cc. of a 16- to 18-hour broth culture injected intraperitoneally into a mouse weighing from sixteen to twenty-two grams kills within forty-eight hours.

For animal passage, seed from 0.1 to 0.2 cc. of the culture into a tube of broth. At the same time make a Gram-stained preparation of the stock culture, and streak a blood-agar plate. Incubate the seed culture for from sixteen to eighteen hours. In making the dilutions and injecting the mouse, use the same procedure as for the pneumococcus virulence control culture. (See "The Test" in "Production and Standardization of Antipneumococcus Serum," p. 422.) In general use two dilutions, injecting one mouse with each. Agitate the culture to insure a uniform suspension, before making the dilutions. At autopsy, after searing lightly the apex of the heart, snip off the tip and inoculate with one or two drops of the heart's blood broth tubes for stock cultures; at least two tubes from the mouse dying within forty-eight hours from the smallest dose which killed within that time, and one, for reserve, from the other mouse. Also streak a blood-agar plate with heart's blood from each mouse for control purposes. Incubate the cultures overnight and examine. If the plate and broth cultures are typical, store the broth cultures in the cold room. Should contaminating organisms be found on a plate, discard the corresponding broth cultures. Should cultures from both mice be contaminated, go back to the previous transfer for a subculture for animal passage. At least every three months, test the toxicity of the strain and the specificity of the toxin by toxin-neutralization tests with a known antitoxic serum.

[3] Until May, 1926, the strain was maintained in broth containing one or two drops of sterile, defibrinated, normal horse blood to 5 cc. of medium. If an interval between animal passages of more than a week should be substituted, the addition of blood might be advisable.

## PRODUCTION OF TOXIN[4]

For toxin production, the standard strain is grown for seven days in 700 cc. of streptococcus-toxin broth (0.02-per-cent dextrose-beef-infusion broth) in 2-liter Erlenmeyer flasks.[5] The preservative is then added and the toxin-broth culture passed through cotton and soft filter paper and then through a filter candle, to free it from organisms. For immunization of horses, about twenty liters of toxin are prepared at one time; for special purposes, from five to eight liters.

Requisition of media: Place orders for the streptococcus-toxin broth at least ten days before the flasks are to be inoculated. Order sufficient 20-per-cent dextrose solution to allow 0.7 cc. to each 700 cc. of broth to give approximately a 0.02-per-cent solution. For the seed culture, the medium for which should be from the same lot as that used in the flasks to be inoculated, order one or two 300-cc. Erlenmeyer flasks containing 50 cc. of medium to allow 2.0 cc. of culture for each large flask to be inoculated. Add 0.05 cc. of 20-per-cent dextrose to each 50 cc. of broth.

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[4] The present method was adopted following a study in this laboratory of toxin production in various media under different conditions. Kirkbride and Wheeler, Jour. Immunol., 1926, 11, 477 (113).

[5] When testing new strains for their toxin-producing power, prepare small amounts of toxin, as a rule from 15 to 150 cc. Transfer a loop of the culture to be tested (usually a blood-agar slant culture) to 15 cc. of streptococcus-toxin broth contained in a 50-cc. Erlenmeyer flask or, if more toxin is required, 50 cc. of broth in a 300-cc. flask or 150 cc. in a 500-cc. flask. Add the required volume of 6-per-cent dextrose solution to give a final 0.02-per-cent concentration (1 drop to 15 cc. of medium). Incubate the inoculated flasks for forty-eight hours at from 33 to 35°C. When the flask is removed from the incubator, make a slide preparation stained by Gram's method and streak a blood-agar plate. Add sufficient 5-per-cent phenol solution to give approximately a final 0.5-per-cent concentration (1.5 to 15 cc. of culture) and place the flask in the cold room until the following day when the plate is examined. Discard contaminated flasks. Pass the toxin-broth culture through a small filter candle (Berkefeld W.). Make sterility tests of the filtrate by inoculating two aerobic and two anaerobic tubes of sterility-test broth with five drops each and, if the material is to be tested on persons, inoculate in addition, one 40-cc. bottle of Hitchens' medium with 0.5 cc., or from 500 cc. or more of toxin one 160-cc. bottle, with 2 cc. The toxins are tested intracutaneously on goats, the general procedure being the same as for the standard scarlet-fever streptococcus toxin. Preliminary tests are usually made with heated toxin in a 1:200 dilution, with unheated toxin in a 1:200 and a 1:800 dilution and with the same dilutions of toxin mixed with the standard antistreptococcus goat serum, the amount of serum present in each mixture being sufficient to neutralize approximately 10 skin-test doses of standard scarlet-fever streptococcus toxin. Further tests with the toxins are later made in higher or lower dilutions as indicated. Filtrates are usually not tested in dilutions lower than 1:100, since experience has indicated that those which induce no reaction in this dilution seldom induce reactions in lower dilutions.

*Preliminary Seed Cultures*

Two days before the large flasks are to be inoculated, make a broth transfer from the latest culture from a mouse. The next day make a slide preparation stained by Gram's method and streak a blood-agar plate. Inoculate one or more small flasks of medium with from 0.5 to 1.0 cc. of culture.

*Inoculation and Incubation of Flasks*

On the following day, make slide preparations of the seed cultures, stain by Gram's method, and examine. Add 0.7 cc. of the 20-per-cent dextrose solution to each large flask and then 2.0 cc. of the seed culture previously agitated to insure a uniform suspension. Transfer the dextrose solution and culture to the flasks with separate 10-cc. graduated pipettes, using a fresh pipette for each 10 cc. Incubate the inoculated flasks for seven days at from 33 to 35°C.

*Addition of Preservative*

Examine each flask carefully as it is taken from the shelf of the incubator.

The supernatant broth is usually clear, the organisms having settled to form a heavy layer covering the bottom of the flask.

Make a slide preparation stained by Gram's method, and streak a blood-agar plate from each flask. Add 70 cc. of 5-per-cent phenol solution to each flask to give approximately a 0.5-per-cent solution. Mix thoroughly by rotating. Cover the flasks with a dark cloth and hold in the draft-free room until the plate cultures have been examined on the following day. Discard any flasks found to be contaminated.

*Filtration*

Pass the contents of each flask through cotton and filter paper, into 4-liter bottles, then through the final filter. For the preliminary filtration, follow the procedure for "Preliminary Filtration Through Soft Filter Paper and Cotton" as given in "Filtration of Biologic Products," p. 516. Filter into 4-liter or 2.5-liter bottles, depending on the amount prepared. Record on the tag the kind of toxin, lot and bottle number, and date, (Streptococcus Toxin (Scarlet Fever) 64A, 64B, etc., 12–9–25).

*Sterility Tests—Disposition of Toxin*

For toxin to be used in the immunization of horses, follow the procedure given in "Production and Standardization of Diphtheria Toxin," p. 313. (No data are as yet available as to the length of time toxin is satisfactory for use.) For toxin to be used for standard toxin, for the intracutaneous test, and for the active immunization of persons, test the sterility by inoculating from each bottle at the time of filtration or before preliminary human tests, three aerobic and three anaerobic tubes of sterility-test broth with a total of 2 cc. and two 160-cc. containers of Hitchens' medium with 3 cc. each. Siphon toxin which has been selected for standard into smaller bottles (200 to 500 cc.) as required. Make sterility tests on each bottle, inoculating one aerobic and one anaerobic broth tube. Label each bottle "Standard Strep. Toxin (Scarlet Fever)" with the lot number, dates filtered and transferred to small bottles, and the volume.

## STANDARDIZATION OF TOXIN

Streptococcus toxin is standardized in terms of its skin-test dose for persons. The skin-test dose is the least quantity of toxin, which, when injected intracutaneously into persons known to be susceptible to the toxin, will induce a reaction equal to that induced on the same persons at the same time by the injection of a skin-test dose of the standard toxin supplied by the U. S. Hygienic Laboratory in Washington.[6] The dose is contained in 0.1 cc.

Preliminary standardization tests of all toxins are made on goats. Toxins which are used only for the immunization of horses are not tested further. Toxins for use in the standardization of antistreptococcus sera, for the outfits for the intracutaneous test, and for active immunization are also standardized on human subjects.

### DETERMINATION OF THE SKIN-TEST DOSE—TEST ON GOATS[7]

When the goat is used as the test animal, the human skin-test dose is estimated from the reactions induced by the intracutaneous injection of 0.1 cc. of varying dilutions of the test toxin as compared with

[6] The standard test dose as at present used is based on that described by Dick, G. F., and Dick, G. H., Jour. Amer. Med. Assoc., 1925, 84, 1477 (115).

[7] This laboratory was the first to use the goat as the test animal for titration of streptococcus toxins and their antitoxins. The initial tests on goats were made in June, 1924. See Kirkbride, M. B., and Wheeler, M. W., Proc. Soc. Exper. Biol. and Med., 1924, 22, 86 (116); Jour. Immunol., 1926, 11, 477 (113).

the reactions induced by one-half, one, and two human skin-test doses of a standard toxin. A preliminary test of the test toxin is usually made with dilutions of 1:500, 1:1000, 1:2000, and 1:4000 and later, retests as indicated, are made with further dilutions at more frequent intervals, as 1:1500, 1:2000, 1:2500, etc. Control tests with heated toxin and with uninoculated toxin broth are made on each animal every time it is used.

### *Special Glassware*

The burette and pipettes used for measuring volumes must be accurately standardized[8] and all glassware must be perfectly clean.

### *Standard Toxin*

The toxin originally selected as a standard in this laboratory was prepared with the standard Dochez strain and the skin-test dose was carefully determined on a number of human subjects in comparison with the Hygienic Laboratory Dick toxin. New lots of standard toxin are standardized on persons against the laboratory standard toxin as well as against the federal standard toxin. Tests are made at frequent intervals to detect possible deterioration. When a slight decrease in potency is indicated, a larger skin-test dose is adopted. When marked deterioration occurs, a new standard toxin is substituted. The skin-test dose of the present standard toxin is 1/30,000 cc.

### *Test Animal*

White-skinned goats, preferably grade or pure-bred Saanen, from about one and one-half to four years old are used. The animals are numbered consecutively in the order of their receipt, a brass tag stamped with the number being attached to the leather collar. A preliminary test is made to determine the degree of susceptibility to the standard toxin and broth. Tests are made with one-half, one, two, and five, or

[8] Burettes from Arthur H. Thomas Company with factory certificate are used. One-cubic-centimeter graduated pipettes which flow fairly rapidly but which can be readily controlled are standardized in this laboratory by the method recommended by the Bureau of Standards (letter of July 12, 1926). Those found to be within the limits of volume error given in its circular, No. 9 (1916), p. 24 (117), are selected. Essentially, the method consists in weighing the water delivered by the pipettes at the intervals under test, observing the temperature, and applying the necessary corrections to obtain the true volumes at 20°C. The corrections are determined by reference to the tables in the Bureau of Standards Circular, No. 19, pp. 56–60 (118).

even ten skin-test doses of standard toxin, with heated toxin, and with uninoculated toxin broth. For general procedure see "The Test," p. 332. Animals which do not give a satisfactory reaction with two skin-test doses of toxin or which are definitely susceptible to heated toxin or broth should not be used. The same animal may be used repeatedly as long as the reactions induced by the standard toxin are definite and the controls are satisfactory. An interval of rest of at least two weeks should be allowed between tests.

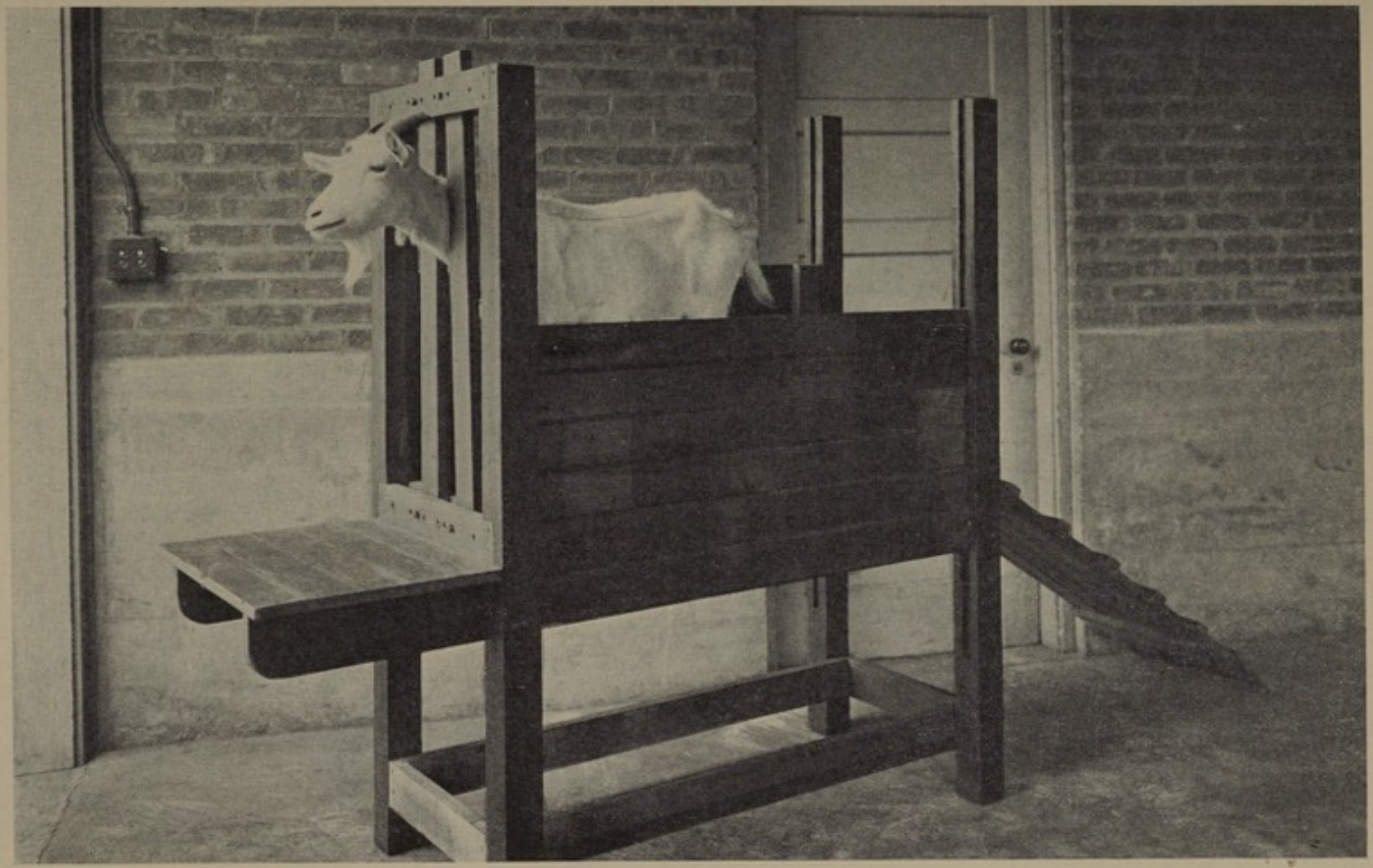
The hair on the sides of the goat is clipped short by means of an electric clipper, and then removed with a barium sulfide paste as described in the chapter "The Use of Experimental and Test Animals," p. 32. This is done several hours before the injections are made or preferably on the afternoon before, in order to avoid the possibility of irritation from the depilatory interfering with the interpretation of the reactions. After the hair has been removed the animal should be kept in warm quarters, especially during the period of the test when the temperature should be approximately from 68 to 70°F. The goat is placed in the special stocks for injecting.

Movable stocks for goats and calves: The stocks consist of an elevated platform and stancheon, dimensions over all, 67 by 24 inches, by 64 inches high. The platform, 50 by 20 inches, is set in 3-by-3-inch corner posts 28 in., from the floor, and extends a further 17 in. in front of the stancheon as a shelf. The stancheon, 36 in. high, is adjustable to give openings of 3 and 4 in. The sides of the stocks, sliding in grooves in the corner posts, are adjustable at heights of from 6 to 20 in. from the platform. A removable runway, 18 by 50 inches, may be placed at the rear of the platform, or when not in use, stored under it. See plate III.

### *The Test*

Before making the required dilutions prepare a dilution card giving the date, goat number, lot numbers of toxins, doses to be tested, and dilutions. (See: fig. 33.) Enter also the time when the material for the heated-toxin control is placed in the water-bath and the time of removal. (A second person should verify and initial the card before the dilutions are started.)

**Preparation of Dilutions.**—Use stoppered flasks for volumes over 10 cc., test tubes (150 by 15 mm.) for smaller volumes. After all the containers have been filled with the required volumes of salt solution, add with a 1.0-cc. graduated pipette the required amount of toxin to the first flask. Mix the contents thoroughly. With a fresh pipette transfer the required amounts to the next containers, usually tubes.



STOCKS FOR GOATS AND CALVES

With a fresh pipette mix thoroughly the contents of any tube containing material to be used for a further dilution by drawing up and discharging several times, then with the same pipette, transfer the required amount to the next container. When the dilutions are completed, transfer about 2 cc. of the required dilutions, as indicated on the dilution card, to sterile stoppered test tubes (100 by 15 mm.), each previously marked with the toxin number and dilution. With each toxin, start with the highest dilution, mix thoroughly with the pipette before transferring it as in preparing the dilutions. Then repeat the

STREPTOCOCCUS TOXIN (SCARLET FEVER)

12–15–25

Goat No. 36

Toxin No. 64

| (S.T. Dose) | (Dilution) | |
|---|---|---|
| | | 0.5 + 49.5 a |
| (1/5000 ) | (1:)500 | 1a + 4 b |
| (1/10,000) | (1:)1000 | 1a + 9 c |
| | 2000 | 0.5a + 9.5 d |
| | 4000 | 1c + 3 e |
| | 8000 | 1c + 7 f |

Standard Toxin No. 52

| | |
|---|---|
| | 0.5 + 49.5 a |
| 2000 | 0.5a + 9.5 b |
| 4000 | 1b + 1 c |
| 8000 | 1b + 3 d |

Broth (Uninoculated Toxin Broth)

| | |
|---|---|
| | 0.5 + 49.5 a |
| 500 | 1a + 4 b |

Heated Toxin Controls

No. 64—1:500
No. 52—1:2000
Time in 2:00 p.m.
Time out 3:30 p.m.

√P. P. M. (Checked by)

M. W. W. (Test made by)

FIG. 33. DILUTION CARD

procedure with each succeeding dilution. Use a fresh pipette for each product.

**Preparation of Heated-Toxin and Toxin-Broth Controls.**—For the heated test-toxin and standard-toxin controls transfer to stoppered test tubes (100 by 15 mm.) about 2 cc. of the lowest dilutions to be injected. Heat in a water-bath at 100°C. for one and one-half hours, immersing the tubes to at least two-thirds of their length. For the uninoculated toxin-broth control, prepare the dilutions as for the test and standard toxin. Use a dose equivalent to the largest amount of any toxin injected.

**Injections.**—Four or five rows of from five to eight injections, two or three inches apart, may be made on each side of the animal. The usual order is to make the first row of injections, starting at the shoulder and keeping about three or four inches below the median line of the back.

As each injection is made, enter the exact position and the material injected, on the record card. Use a card similar to that described under "Production and Standardization of Antistreptococcus Sera," p. 376. Indicate the side on which the injection is made by a letter, the row by its number, and the position of each injection in the row, starting from the front, by its number preceded by a dash (R1—1, R1—2, etc., L1—1, L1—2, etc.). Use 1-cc. tuberculin syringes (needle, 26 gauge, $\frac{3}{8}$ in.) and inject intracutaneously 0.1 cc. of the required dilution. Use separate syringes for the broth and heated controls and for the unheated toxin. Begin the injection of each product with the heated control, then the highest dilution of unheated toxin. Before injecting a dilution rinse the syringe twice with it. Between injections of different products change the needle and rinse the syringe three or four times with sterile salt solution or water, then twice with the first sample of the next product. In general, inject the standard toxin in the second or third row.

### *Readings and Estimation of Titer*

Readings are made in a bright light from eighteen to twenty hours after the injections. The time of reading, the degree of redness and swelling induced, and the size of the area in centimeters are noted on the record card. The reactions may fade rapidly after twenty hours.

Estimation of the skin-test dose (human) is based on a comparison of the reactions induced by the test toxin with that induced by the skin-test dose of the standard toxin. The broth and heated-toxin controls should induce no reaction. In case a slight reaction is noted with either or both, the fact should be recorded. The dose of test toxin which induces a reaction equal to that induced by the skin-test dose of standard toxin is recorded as the skin-test dose. If two dilutions induce reactions equal to that induced by the test dose of standard toxin, the estimated titer of the test toxin is based on the dilution containing the smaller amount of toxin. At least one dilution of the series should induce a reaction less than the standard toxin. The variations in susceptibility of different goats to the same test dose of the standard toxin must be taken into consideration. Thus, some animals may be highly susceptible to one-half a skin-test dose, and others only slightly susceptible to one or two skin-test doses.

## FINAL TESTS ON HUMAN SUBJECTS

When final tests of toxins are made on human subjects, at least two dilutions of test toxin, based on the results of the tests on goats, are used, and the reactions induced are compared with that induced by the test dose of the U. S. Hygienic Laboratory standard toxin injected at the same time. Heated controls of both test toxin and standard toxin are always used, but no broth control. Approximately ten persons previously found to be susceptible and ten previously found to be insusceptible should be tested. Persons giving pseudo reactions are not suitable.

### *Glassware*

The same special glassware is used as in the tests on goats. The pipettes are sterilized in the media department by hot air. The burette is specially sterilized and set up with a fresh supply of sterile 0.85-per-cent salt solution. For procedure see "Preparation of Outfits for the Schick Test," p. 489.

### *The Test*

Prepare the dilutions as in the test on goats, using special aseptic precautions. For injections use the syringes for the Schick test (MacGregor Instrument Co.), and follow the procedure given in the circular relating to the special outfit for the intracutaneous test. Inject the test toxin on the left arm, the standard on the right arm, placing the corresponding dilutions of test and standard toxins in approximately the same relative positions. Record the exact position and material injected, on the record card. For form of record, see "Production and Standardization of Antistreptococcus Serum (Scarlet Fever)," figure 40. Number the injections from the elbow down.

### *Readings and Estimation of Titer*

Readings are made in a bright light after from twenty to twenty-four hours, the degree of redness and of swelling induced and the size of the area in centimeters being entered on the record card. The dose of test toxin which induces a reaction equal to that induced by the test dose of the U. S. Hygienic Laboratory standard toxin is recorded as the skin-test dose. If all dilutions of the test toxin induce reactions equal to that induced by the standard toxin, the toxin is retested in higher

dilutions. The persons in whom the standard toxin induces no reaction should not react to the test toxin.[9]

*Permanent Records*

Standard strains: A card (4 by 6 in.) is kept, giving the following data in regard to the virulence tests; date of injection, source of the culture (heart broth and passage number), amount, time injected, date and time of death of mouse, date and result of autopsy, and passage number of new culture.

General record: The record is similar to that described in "Production and Standardization of Diphtheria Toxin," p. 319.

Standardization tests: The following cards are filed as permanent records:

Dilution card, by date.

Human record card, by date.

Goat record card, by consecutive animal numbers.

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[9] This does not apply to the exceptional persons (in our experience two out of seventy) who are highly susceptible to the laboratory standard toxin (Dochez), but insusceptible to the federal standard toxin (Dick). See Kirkbride, M. B. and Wheeler, M. W., Proc. Soc. Exp. Biol. and Med., 1924, 22, 85 (119); Jour. Immunol., 1927, 13, 19 (114).

# CHAPTER 4

# PRODUCTION AND STANDARDIZATION OF BOTULINUS TOXINS

Three specific types of toxins, produced by different strains of *B. botulinus*, have been differentiated by toxin-antitoxin neutralization tests. Toxins of types A and B, produced by strains associated with human botulism, are prepared in this laboratory for use in the immunization of horses for the production of monovalent antitoxic sera and in standardization tests of the sera. The type-C strains are apparently nonpathogenic for man. Small amounts of the toxins are prepared from time to time as required. The procedures for their production correspond closely to those given in the chapter "Production and Standardization of Tetanus Toxin," p. 320, which should be followed except as indicated in the following text.

All procedures connected with the production of botulinus toxin, except the sterilization of the contaminated material in the autoclave, are carried on in one room and the draft-free room connected with it. Since at present complete physical separation from the other work of the laboratory is not possible, the procedures must be closely supervised by a responsible worker.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

## *Standard Strains*

The standard strains used for toxin production are:

*B. botulinus*, type A: Strain A No. 16. Collection No. 175G. Received from the Harvard School of Public Health, July, 1920. Isolated in 1919 from olives responsible for an outbreak of botulism in Alliance, Ohio.

*B. botulinus*, type B: Nevin Strain. Collection No. 175B. Isolated in 1914 at the New York State Laboratory in Albany, from cottage cheese responsible for fatal cases of botulism.

Stock cultures are maintained in dextrose semisolid beef-infusion agar, pH 7.0 (150-by-15-mm. tubes containing 15 cc. of medium). Transfers are made at least every three months. The stock cultures of each type are kept in separate baskets or racks, distinctly marked, "*B. botulinus* Type A" and "*B. botulinus* Type B," and are transferred at different times. Slide preparations are stained by Gram's method.

Make identification and purity tests at least once a year, always before work with the strains is begun. Make anaerobic plate cultures on beef-extract blood agar to which 0.5 per cent dextrose has been added.

Make in addition tests to confirm the type of each strain. Inoculate a tube of van Ermengem broth. Incubate at from 33 to 35°C. for from four to five days. Add enough 5-per-cent phenol to make a final concentration of 0.5 per cent and filter about 20 cc. through a small filter candle, observing the usual precautions for handling such material. Inoculate subcutaneously three guinea pigs weighing from 250 to 300 grams; the first with about 0.05 cc. of toxin, the second with the same amount of toxin previously mixed with about 100 units of type-A antitoxin, and the third similarly with toxin and type-B antitoxin.

The guinea pig receiving the toxin and homologous-type antitoxin should survive, the others should die in from twenty-four to ninety-six hours with characteristic symptoms. (See "Standardization of Toxins".)

## PRODUCTION OF TOXINS

Type-A and type-B toxins are always prepared at different times. The cultures are passed through a series of three daily transfers in van Ermengem broth (180-by-25-mm. tubes containing 40 cc. of medium) and are grown for fourteen days in the same medium dispensed in 1-liter flasks in from 800- to 850-cc. volumes. (For preparation of van Ermengem broth see "Preparation of Media," p. 86.) All cultures are incubated at from 33 to 35°C.

In this laboratory, the broth is prepared without dextrose to avoid the increase in acidity which usually occurs during sterilization.

Just before inoculating the tubes for daily transfers and the flasks for toxin production, add sterile 20-per-cent dextrose solution to give a final 2-per-cent concentration; 4 cc. to each tube, from 80 to 85 cc. to each flask. Stain slide preparations by Gram's method only. Before filtration, pool the toxin broth and add enough 5-per-cent phenol to give a final 0.5-per-cent concentration (100 cc. preservative to 900 cc. toxin broth).

## STANDARDIZATION OF TOXINS

Botulinus toxin is standardized by determining its minimum fatal dose as a measure of its potency or titer. The M.F.D. is the least quantity of toxin which, when injected subcutaneously, will kill a guinea pig weighing 250 grams within ninety-six hours. The pro-

cedures given in the chapter "Production and Standardization of Diphtheria Toxin" (p. 313) are followed except as indicated in the following text.

The doses tested are at present 0.0003 cc., 0.0002 cc., and 0.0001 cc., for both types of toxin.

After making the test, pour the toxin dilutions and any water or salt solution used in rinsing the pipettes and syringes into a pail for sterilization. Fill the cylinder containing the pipettes, the empty dilution flasks, amber bottles, and other glassware with cleaning solution and allow to stand at least half an hour before handling them.

Enter the symptoms of the guinea pigs on the daily record card as follows:

Sl. ill: slight weakness, flabby abdominal muscles.
Ill: symptoms increasing to general weakness and prostration.
Very ill: increasing prostration, emaciation, paralysis of the throat muscles, exuding saliva.
Dying: extreme prostration. This condition may continue for some time before death.

At autopsy examine especially for the following lesions and record the findings.

Site of injection: usually no macroscopic changes.
Occasionally slight infiltration or oedema with or without hemorrhage.
Abdominal organs: general congestion with dark-colored liver and spleen (absent in acute intoxication).
Stomach: usually empty and distended.
Bladder: frequently distended.
Lungs: congested; may show large or small hemorrhages.

### *Permanent Records*

Records for type-A and type-B cultures and toxins are kept separate throughout, except that tests are entered in the test book consecutively without regard to type. The records are similar in form to those described in the chapter "Production and Standardization of Diphtheria Toxin," p. 319. In addition, the tests for confirmation of type are recorded in the "Standardization Tests" record book.

## SECTION II

## CHAPTER 1

# PRODUCTION AND STANDARDIZATION OF DIPHTHERIA ANTITOXIN

Diphtheria antitoxic serum for prophylactic and therapeutic use is produced by the immunization of horses or mules against the toxin of the diphtheria bacillus. The potency of the serum and of the purified concentrated product is determined by its neutralizing action when combined with diphtheria toxin.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

### PRODUCTION OF ANTITOXIN

#### *Diphtheria Toxin Used in Immunization*

Filtered toxin containing 0.5 per cent phenol and preferably over six months old, is used. The minimum fatal dose (M.F.D.)[1] of the toxin is determined at the time of preparation. (For procedure see "Production and Standardization of Diphtheria Toxin," p. 313.) The different lots are stored in the cold room in the order of their preparation. Toxin with an M.F.D. of from 0.003 to 0.004 cc. is generally used for immunization. Satisfactory results have been obtained, however, by using a weaker toxin (M.F.D. 0.005 cc.) during the early stages of immunization and later, during the period of production, substituting a stronger toxin. Toxin with an M.F.D. greater than 0.005 cc. is not used for immunizing purposes except in an emergency.

When toxin is required for immunization consult the toxin-production cards, which contain detailed information as to the supply available, and record on them the number and volume of the toxin selected, the numbers of the horses to be injected, and date of shipment to the farm. Enter on a duplicating order form the lot and bottle number of the toxin, its titer and volume, the date of shipment, and the numbers of the horses to be injected; and initial. Send the original with the toxin

[1] The adoption of the L+ dose in place of the M.F.D. as a basis for computing the antigenic value of diphtheria toxin used for the immunization of horses is under consideration.

to the farm. Enter the same data on the tag on the bottle. Pack the 4-liter green-glass bottles containing toxin in special wooden containers, or, if small volumes are to be injected, pipette or siphon the toxin into smaller bottles. Avoid any unnecessary exposure of toxin to light or heat. If not shipped immediately after packing, place temporarily in the cold room.

## IMMUNIZATION OF HORSES[2]

The method at present used[3] is briefly as follows: Horses whose serum has been tested for the presence of natural diphtheria antitoxic substance, are given a preliminary intravenous injection of diphtheria antitoxin followed by subcutaneous injections of toxin in gradually increasing doses at 3-day intervals. When whole bleedings are commenced the interval between injections is increased to four days. Two injections are usually given between bleedings, the first on the day following a bleeding. Bleedings are taken six days after the last injection of a series.

[2] The response of different horses to toxin varies greatly. For example, of two horses immunized at the same time with the same toxin, and injected and bled on practically the same schedule, horse 122 produced serum with a maximum titer of 2200 units per cubic centimeter; horse 120, serum with a maximum titer of only 600 units. The following data are taken from the protocol of horse 122: Gelding, twelve years old, weight about 900 pounds, previously used as a saddle horse by the State Department of Police; natural protection against diphtheria toxin equal to $\frac{1}{20}$ unit antitoxin per cubic centimeter. Injections of toxin commenced November 11, 1921. December 27, the titer of the serum was 230 units; January 30, 1922, 820 units; February 15 (at time of first bleeding), 1080 units. The dose of toxin given before this bleeding was 350 cc. of toxin with an M.F.D. of +0.004 cc. The titer of the serum on March 17 was 1450 units; April 21, 1720 units; May 5, 1880 units; June 23, the eleventh bleeding, 2220 units. Forty-seven bleedings were taken between February 15, 1922 and August 22, 1923. The titer during this period was at no time below 1080 units. Thirteen days before death from rupture of the liver, the titer was 1600 units.

[3] More rapid methods of immunization under study for two years have yielded very interesting results but none has as yet been adopted as a routine procedure. Certain horses, however, which it does not appear advisable to keep under treatment for the usual period, are given a series of very small initial doses of toxin without antitoxin, followed by a much more rapid increase in dosage. With this treatment the antitoxic content of the blood usually increases more rapidly, permitting earlier bleedings. But since the titer is seldom maintained over as long a period, the horse is usually bled out, after one or two whole bleedings at daily intervals, as soon as a satisfactory titer is reached; or the bleedings are continued for a short time with large doses of toxin between.

*Selection of Horses*

Since the immunity response to diphtheria toxin is relatively rapid, it is possible to use horses whose general condition or age make them undesirable for prolonged periods of immunization, such as are necessary in the production of tetanus antitoxin.

TABLE 26

*Schedule of first eighteen injections of toxin**

| INJECTION NUMBER | NUMBER OF M.F.D. | VOLUME OF TOXIN | REMARKS |
|---|---|---|---|
| | | cc. | |
| 1 | 250 | 1.0 | Preliminary injection of 3000 units of diphtheria antitoxin given intravenously on day preceding first injection of toxin |
| 2 | 350 | 1.4 | |
| 3 | 525 | 2.1 | |
| 4 | 750 | 3.0 | |
| 5 | 1,025 | 4.1 | |
| 6 | 1,350 | 5.4 | Injection of toxin every third day (seventy-two hours) subcutaneously |
| 7 | 1,750 | 7.0 | |
| 8 | 2,200 | 8.8 | |
| 9 | 2,750 | 11.0 | Doses of less than 20 cc. made up to that volume with 0.85-per-cent salt solution |
| 10 | 3,500 | 14.0 | |
| 11 | 4,500 | 18.0 | |
| 12 | 6,000 | 24.0 | M.F.D. of toxin, 0.004 cc. |
| 13 | 8,500 | 34.0 | |
| 14 | 12,000 | 48.0 | |
| 15 | 15,000 | 60.0 | |
| 16 | 19,500 | 78.0 | |
| 17 | 24,000 | 96.0 | |
| 18 | 30,000 | 120.0 | |

* This schedule has also given satisfactory results when a toxin with an M.F.D. of 0.005 cc. has been substituted and the dosage in cubic centimeters, as given, has been followed.

Horses which have been immunized with other bacterial products or cultures may be later assigned to toxins. Horses which have failed to respond satisfactorily to tetanus toxin, or whose serum has fallen below the required titer, may be transferred to diphtheria toxin, but the results are seldom as satisfactory as when fresh horses are used.

**Tests for Natural Antitoxic Substance.**—The serum of most horses contains minute amounts of an antitoxic substance. Before a horse is selected for immunization, a trial bleeding is always taken and the serum is tested by the intracutaneous method (for procedure see p. 353), for $\frac{1}{500}$, $\frac{1}{100}$, and $\frac{1}{20}$ unit per cubic centimeter. Horses whose serum

is found to contain $\frac{1}{20}$ unit or more per cubic centimeter are always selected, when available.[4]

*Injection and Dosage*

The schedule for the first eighteen injections of toxin is given in table 26. The general condition and the temperature curve of each horse is followed closely and the schedule varied when indicated by the reactions induced.

After a dose of 120 cc. has been reached, an increase of 25 cc. is usually made at each injection. Later, increases of from 30 to 50 cc. may be advisable. In no case should the increase be greater than 50 cc. Doses greater than 30,000 M.F.D. are recorded in cubic centimeters only.

Immunization of horses whose serum contains less than 100 units of antitoxin per cubic centimeter after eighteen doses, or in approximately eight weeks, is discontinued, as such animals are considered unsuitable for diphtheria-antitoxin production.

For reimmunization after a rest period, the initial dose is generally decreased, the extent depending on the length of the period. The doses are then increased rapidly, by 50 or 100 cc.

It is always desirable to give the least amount of toxin that will produce the best results. For many horses maximum single doses of 450 to 500 cc. of toxin with an M.F.D. of 0.003 or 0.004 cc. are ample. Increase in the volume injected may be avoided by the use of stronger toxin. Irrespective of the strength of the toxin, a dose of more than 800 cc. is never given.

Unnecessary changing of horses from one lot of toxin to another of the same potency is to be avoided. Unsatisfactory results may, however, indicate the desirability of substituting a new lot of the same or a higher titer.

At times, diphtheria horses which are later to be immunized for the production of tetanus antitoxin, have received the preliminary injections of tetanus antitoxin and tetanus toxin while still undergoing active immunization against diphtheria.

Injections at 3-day intervals are continued until whole bleedings are commenced, when a series of two (or occasionally three or four) injec-

[4] Of forty-seven horses whose sera were tested for natural antitoxic content, five with less than $\frac{1}{500}$ unit of natural antitoxin per cubic centimeter produced sera with an average maximum titer of 260 units; six with $\frac{1}{500}$ unit, an average maximum titer of 330 units; seven with $\frac{1}{100}$ unit, an average maximum titer of 453 units; and twenty-nine with $\frac{1}{20}$ unit or more, an average maximum titer of 658 units.

tions of toxin at 4-day intervals are given between bleedings. The first dose of a series is given on the day following a bleeding, which is taken six days after the last injection of the preceding series. During the 3-day schedule, when a trial bleeding is to be taken, the dose is delayed until the fourth day, and injected just after the bleeding. When an injection or a bleeding would fall on a Sunday or a holiday, it is postponed one, or even two days, and the schedule changed to conform with the new date.

*Temperature Reactions*

The daily morning temperatures of all horses undergoing immunization are taken. By following closely the general condition and febrile reactions, unfavorable results from injections, and early phases of suppurative or other processes may be noted. Frequently, variations from the regular schedule are shown to be advisable. Injections and bleedings are postponed if the temperature is above normal. If a whole bleeding is postponed for several days, it is often desirable to give one or more additional injections before bleeding.

*Trial and Whole Bleedings*

Trial bleedings are taken immediately before the fourteenth and again before every fifth injection or oftener, until whole bleedings are commenced. They are then taken at least once each month at the time of a whole bleeding; in some instances, when it is advisable to follow closely the rise or fall in unit content, at each whole bleeding. Trial bleedings are never taken sooner than the fourth day after an injection. For technic see "Care and Treatment of Animals—Large Animals," p. 594. The blood is sent to the laboratory where the serum is drawn off and the titer determined as soon as possible.

As far as possible the schedules of horses are so arranged that trial bleedings of different horses during the early stages of immunization are taken at about the same time so that they may be tested together; and monthy trial bleedings toward the end of the third week in order that the results may appear in the month's report.

Whole bleedings may be commenced when the titer of the serum reaches 250 units, unless, as frequently occurs, the tests of trial bleedings indicate that a further rapid rise in titer may be expected; in which case the whole bleeding is delayed, usually until the titer appears to have reached its upper limit. Bleedings are taken six days after toxin has been injected. While a horse is producing serum of a satisfactory

titer, bleedings are usually taken after every second injection, that is every eleventh day. They are generally continued until the antitoxic content of the serum approaches the minimum requirement (250 units), or until the condition of the horse makes further immunization inadvisable. Treatment is then discontinued after a series of two or three whole bleedings at 6-day intervals, or the horse is bled out, usually following a whole bleeding taken on the preceding day.

Occasionally the condition of a horse producing potent serum may make it advisable to omit injections and bleedings temporarily, the length of the rest period depending upon the recuperative power of the horse. On reimmunization the animal is likely to produce equally potent or usually only slightly less potent serum. Whether the immunization of a horse shall be discontinued temporarily or permanently, depends primarily on its condition and on the titer of the serum. The decision in the case of a horse producing serum of low potency must depend in part on the number and standing of the available horses, and the supply of antitoxin on hand. The supply should be enough to allow for an interval of at least twelve months between bleeding and distribution of the antitoxin.

Usually 9000 cc. of blood (from a horse weighing about 1200 pounds) are taken at each whole bleeding. When a horse is bled out, from 22 to 30 liters of blood are usually obtained. Nine parts of blood are bled into one part of 17-per-cent sodium-citrate solution ($(Na_3 C_6 H_5 O_7)_2 \cdot 11 H_2O$), giving a 1.7-per-cent citrated plasma. (See "Care and Treatment of Animals—Large Animals," p. 594.) The plasma is shipped to the concentration group. A member of this group checks the accompanying order form, places the material in the cold room and sends the form to the production group.

## TREATMENT OF PLASMA AND CONCENTRATED ANTITOXIN

### *Concentration of Antitoxin*

Antitoxic plasma is purified and concentrated by the concentration group as given in the chapter "Concentration of Antitoxin," p. 384. Instructions for pooling the plasma, based upon the relative standing of the horses as shown by tests of trial bleedings, are sent to the concentration group on an initialed duplicating order form. Samples of all lots of pooled diphtheria-antitoxic plasma are received from the concentration group. These, and samples of the concentrated antitoxin of the same lots taken at the time the protein content is adjusted, are tested by the intracutaneous method with the least possible delay, and the results reported to the concentration group in order that the "unit concentration" and the loss in total antitoxic content may be

controlled. Samples of tetanus-antitoxic plasma are received for similar determinations.

A duplicating order form giving the date and kind, number and volume of material, is received from the concentration group when plasma is taken from the cold room for concentration and when the concentrated product is returned.

**Dilution of Concentrated Antitoxin.**—Antitoxin is diluted (1) when the number of units required for a dose is contained in less than 2 cc., or (2) when the number of units per cubic centimeter is greater than 2500 in material intended for routine distribution. The antitoxin above 2500 is diluted to contain not more than 2300 units per cubic centimeter. When the intracutaneous test, before filtration, shows that an antitoxin requires dilution, the material is retested by the subcutaneous method, after which an order is sent to the concentration group, stating the kind, order number, and titer of the material to be diluted and the desired titer. Diluted antitoxin is filtered and tested in the usual way.

| Actual Quantities in Containers | | Labeled | |
|---|---|---|---|
| No. of cc. | 3.9 | Total Units | 6000 |
| Units per cc. | 1850 | Units per cc. | 1800 |
| Total Units | 7215 | Return | 8/18/27 |

Signed C.A.G.

FIG. 34. ENTRIES ON "SERUM AND ANTITOXIN RECORD" FORM

*Filtration of Antitoxin*

Antitoxin is filtered by the filtration group assisted by a member of the antitoxin-production group (see "Filtration of Biologic Products," p. 511). After filtration, samples for sterility and potency are taken, and the filtered antitoxin is returned to the cold room. (See "Sterility Tests of Biologic Products," p. 543.)

*Preparation for Distribution*

After the sterility tests have been satisfactorily completed and the titer has been determined, the antitoxin is ready to be bottled for distribution by the filling and boxing group. It is dispensed in 1000-, 3000-, and 6000-unit doses. In order to compensate for possible deterioration and for antitoxin remaining in the bottle or syringe at the time of injection, 20 per cent more antitoxin than the label indicates is always dispensed; thus, at the time of filling, a "6000-unit" bottle actually contains at least 7200 units. The number of units per cubic centimeter is given on the label in even hundreds, any number falling between two consecutive hundreds being recorded in terms of the lower (1220, 1280, etc. as "1200"). The actual number estimated from the test, how-

ever, is used in computing the volume required. Both the data on which the computations are based, and those for the labels, are entered on the duplicate "Serum and antitoxin record" form (See fig. 34) which is sent to the filling and boxing group. (See "Preparation of Biologic and Chemical Products for Distribution," p. 526.)

Diphtheria antitoxin is considered satisfactory for use, if kept under proper conditions, for eighteen months[5] from the date of the last satisfactory test. The return date is given on the bottle and the outside of the package.

Returned outdated antitoxin, if in satisfactory condition, is pooled and reconcentrated.

## STANDARDIZATION OF ANTITOXIN

Antitoxin is standardized by determining its neutralizing action when combined with diphtheria toxin previously standardized against the standard antitoxin supplied by the U. S. Hygienic Laboratory.[6] The antitoxic action is measured by the degree of reaction induced in guinea pigs by a mixture containing the antitoxin to be tested and the toxin, in comparison with that induced by a similar mixture containing standard antitoxin.[7] The antitoxic potency of the serum is expressed in units per cubic centimeter. Two methods are employed, the subcutaneous or official test in which the L+ dose of the toxin is used as the

[5] While it has been shown by repeated tests that antitoxin and other sera maintain their potency practically unimpaired for two or more years, it has been considered advisable to establish a conservative "return date" in order to guard against the use of material which may have become unsatisfactory due to clouding or to faulty storage conditions.

[6] For a detailed description of the methods and principles involved in the standardization of diphtheria antitoxin, see Hygienic Laboratory Bulletin, No. 21, 1905 (120). The antitoxic unit used in this country has the same value as that used throughout the world.

[7] Studies of the flocculation reaction for titration of toxins and antitoxins are in process. Wide variations have been found in the case of sera from certain horses, between the antitoxic titers as determined by the flocculation reaction and by the subcutaneous and intracutaneous tests upon animals. Thus, flocculation tests of trial bleedings from one horse, taken during the first four months of immunization, indicated unit values from 25 to 50 per cent less than by the subcutaneous and intracutaneous methods. During the next two months the antitoxic titer of the serum, as indicated by the flocculation test, became equal to or even slightly higher than was shown by animal tests. The flocculation test, when adequately controlled, however, has been found of considerable value, as when immediate data on the standing of a horse are desired. The method used for the test corresponds closely to that described by Bayne-Jones, S., Jour. Immunol., 1924, **9**, 481 (121), except that the test volumes of antitoxin or serum are increased progressively by 5 per cent.

measure, and the intracutaneous test based on the local reaction induced when diphtheria toxin is introduced into the skin of susceptible animals. Great accuracy is required in performing both tests.

The subcutaneous method is used for the final test on all lots of antitoxin distributed for prophylactic or therapeutic use, and as a check on the accuracy of the intracutaneous method. One guinea pig is required for each dose.

The intracutaneous method is used, ordinarily, for all tests other than those on finished antitoxin. Tests are made as a routine procedure on sera of normal horses, for content of natural antitoxic substance; on trial bleedings from horses undergoing active immunization; and on plasma before and during the process of concentration, that is, on every lot of pooled plasma before concentration and on all lots of material after concentration has been completed. These tests serve as a basis for computing unit concentration and loss, and aid in determining the disposition of the concentrated material, and in selecting the dilutions to be used in the subcutaneous test. Tests are also made from time to time on samples of filtrates and extracts taken during the process of concentration and on experimental material. The advantage of the intracutaneous method is that from ten to twelve, or even fourteen, doses may be injected on one guinea pig and when the test is completed the guinea pig may be used for complement. While the test has proved very satisfactory, the results should be controlled from time to time by comparison with the subcutaneous test. When the titer of the material is high, 2000 or more units per cubic centimeter, the readings are not so dependable.

### SUBCUTANEOUS TEST

Varying dilutions of the material to be tested are combined with the L+ dose of toxin, and injected subcutaneously into a series of guinea pigs weighing between 230 and 280 grams. At the same time, as a basis for comparison, two control guinea pigs are injected with a mixture containing one unit of standard antitoxin and the same L+ dose of toxin. The dilutions of the material to be tested are also made on the basis of one unit of antitoxin per cubic centimeter. That is, if an antitoxin is to be tested for titers of 1200, 1300, and 1400 units per cubic centimeter, dilutions of 1:1200, 1:1300, and 1:1400 are made. (The tables in the Hygienic Laboratory Bulletin No. 21, 1905, may be consulted in selecting the preliminary dilutions to be used to obtain the desired final dilution.)

The range to be covered by a test depends upon the material to be

tested and the data available from previous tests. For filtered antitoxin, the material usually tested by the subcutaneous method, it is based upon the results of the preliminary intracutaneous test before filtration; for serum, upon the standing of the horse when the preceding trial bleeding was taken; for pooled plasma, upon the titer of the trial bleeding taken from each horse nearest to the time of the whole bleeding; for concentrated antitoxin before filtration, upon the titers of the trial bleedings multiplied by the estimated unit concentration. It is usual to test three and occasionally four different dilutions of each sample of concentrated antitoxin. When sera are tested subcutaneously two dilutions 100 units apart are generally used. One guinea pig is generally used for each dilution, except that two guinea pigs are always used for the standard antitoxin control.

### *Standard Antitoxin*

Standard diphtheria antitoxin used in determining the L+ dose for standard toxin, and as a control in all tests on antitoxic material, is supplied in a glycerinated form by the U. S. Hygienic Laboratory in Washington, in approximately 5-cc. quantities. On each bottle are entered the expiration date and the dilution required to give 1 unit per cubic centimeter.

In all final tests of antitoxin before distribution, the Hygienic Laboratory antitoxin should be used as a control. In other tests, the state laboratory antitoxin, accurately standardized and reserved for the purpose, may be substituted if necessary. A fresh supply of antitoxin is forwarded by the Hygienic Laboratory from time to time. If the supply on hand becomes low, however, a request for a new lot should be placed at least one week before it will be required.

### *Standard Toxin*

Stabilized toxin to be used as standard toxin is supplied by the toxin production group, dispensed in 500-cc. bottles (see "Production and Standardization of Diphtheria Toxin," p. 313). The toxin, which is covered with a thin layer of albolene, is protected from light and disturbed as little as possible. Samples required for tests are withdrawn under aseptic precautions without removal of the bottle from the cold room.

**Determination of L+ Dose.**—The toxin is standardized by determining its L+ dose against the federal standard antitoxin. (Standard antitoxin which will soon become outdated should not be used.) The L+ dose is defined as the least quantity of toxin which, when added to one unit of antitoxin and injected subcutaneously, causes the death of

a guinea pig weighing 250 grams within ninety-six hours. The dose which kills in from seventy to eighty-five hours is selected, however, since in standardization tests all the control guinea pigs injected with one L+ dose of toxin and one unit of standard antitoxin should die within ninety-six hours, and allowance must be made for variations in susceptibility among the individual animals.

As a correctly standardized toxin is essential for the accurate standardization of antitoxin, it is generally necessary to use a large number of guinea pigs before the L+ dose of a new lot of toxin is definitely established. The L+ dose of the toxin is then determined for each new bottle of the same lot when first opened, and later if variations make it advisable. Control tests made with every routine standardization test of antitoxin serve as a further test of the potency of the toxin. When the control animals die somewhat too soon or too late in several consecutive tests, the L+ dose may usually be changed without re-standardization of the toxin.

The procedure is that used in testing antitoxins, except that varying doses of toxin are combined with one unit of standard antitoxin instead of varying doses of antitoxin with one L+ dose of toxin. For the preliminary test, the doses are selected at wide intervals on the basis of the M.F.D. Thus, a toxin having an M.F.D. of 0.002 cc. is usually tested for an L+ of 0.12, 0.16, and 0.20 cc. The final tests are made at intervals not greater than 0.01 cc. (0.12, 0.13, and 0.14 cc.; or 0.18, 0.19, and 0.20 cc.; etc.). The L+ dose is estimated in thousandths and occasionally a test may be made at intervals of 0.002 or 0.003 cc. (0.165, 0.168 and 0.170 cc.). While one guinea pig may be used for each dose in the preliminary test, in later tests, two, and in the final tests, three or four animals are used for each dose. The animals should weigh between 240 and 260 grams.

### *Test Animals*

Healthy guinea pigs weighing between 230 and 280 grams are used. Animals weighing not less than 250 grams and preferably not more than 260 grams are selected for the controls.

### *The Test*

Before commencing the day's tests, record on the test card (5 by 8 in.), shown in figure 35, the date, products, titers to be tested for, dilutions used, and the number, weight and source of each guinea pig. Enter the toxin number, its L+ dose, and the dilution required, at the bottom

of the card. Always have the card verified by a second person before making the dilutions. Later, add the time at which the control guinea pigs were injected, and the initials of the worker making the test. When the test is completed, copy the required data into the permanent record book, and have a second person verify the entries. Keep the cards a year for reference.

**Dilutions.**—The technic is that given under "Dilutions" in "Production and Standardization of Diphtheria Toxin," p. 315. The "to contain" pipettes must, however, be rinsed at least three times and the dilutions thoroughly mixed, since sera and antitoxins are more viscous than toxin.

DIPHTHERIA ANTITOXIN

12–4–25
4:30 p.m.

Anti. No. 480

1 + 49 a (1st dilution)
850 (units) 1a + 16 b No. 4247 – 242 (gm.)
950 1a + 18 c No. 4278 – 248
1050 1a + 20 d No. 4279 – 257

Standard control—Rec'd. 8–24–25

1 + 5
1. – No. 4277 – 250
2. – No. 4281 – 250

Anti. No. 481

1 + 99 a
1600 (units) 1a + 15 b No. 4294 – 240
1700 1a + 16 c No. 4295 – 262
1800 1a + 17 d No. 4253 – 268

Toxin No. 117 A-5 L+ dose 0.196 (3 + 12.3)
√E. G. B.
(Checked by)

R. F. C.
(Test made by)

Laboratory stock

FIG. 35. TEST CARD

Antitoxin: Place the dilution flasks in parallel rows with that for the first dilution of each sample in front. Arrange the rows from left to right in the order in which the tests are entered on the test card. Place in front of each row the bottle containing the material to be tested. Make the first dilution of all the samples, which should have reached room temperature, then go back and complete the dilutions of the first sample before going to the second. Continue with the other samples in order.

When all dilutions are completed, pipette 1 cc. of the final dilutions into small brown bottles previously marked with the product number

and number of units tested for. Use a 1- or 2-cc. certified graduated pipette for transferring all antitoxin dilutions. With dilutions of the same sample, transfer the highest dilution first, then wipe the tip of the pipette and draw up the next lower dilution and discard twice to rinse. Rinse between dilutions of different samples by drawing up and discarding salt solution twice and the dilution to be used twice. After all the dilutions have been pipetted, add 1 cc. salt solution to each bottle.

Use the same technic for diluting the standard antitoxin. Bring it to room temperature; open carefully and remove 1 cc. or 0.5 cc. in a "to contain" pipette. Add the antitoxin to the required volume of salt solution—at present 5 cc. for 1 cc. of antitoxin. Rinse the pipette at least three times on account of the glycerin present. Seal the rubber stopper of the antitoxin bottle with paraffin and return to the cold room without delay. Transfer 1 cc. of the dilution to a bottle as in the case of test antitoxins and add 1 cc. salt solution.

Toxin: Pipette from the stock bottle the necessary volume of standard toxin carefully into a sterile test tube. In pipetting, close the upper end of the pipette with the index finger and plunge the tip through the oil, draw up the toxin, withdraw the pipette, wipe the tip to remove oil adhering to the outside, and discharge. If any oil has been carried over, allow the tube to stand until the oil rises to the surface, then pipette the toxin carefully into another tube, taking care not to draw up any oil. Dilute the toxin so that 1 cc. of the dilution contains one L+ dose, using a 1-, 2-, 3-, or 4-cc. "to contain" pipette, depending on the amount of diluted toxin needed for the tests. Protect the toxin dilution from the light as far as possible and use soon after it has been made.

**Toxin-Antitoxin Mixture.**—Add to the antitoxin dilution in each of the bottles, 1 cc. of the toxin dilution, using a certified 1- or 2-cc. graduated pipette. Take care that the toxin pipette does not come in contact with the antitoxin, when touching the tip to the side of the container. Wipe the tip carefully after each drawing up and each emptying. Immediately after adding the toxin, shake gently and rotate the bottle to mix the contents thoroughly but avoid allowing any of the mixture to touch the cork. Place each bottle at once in a covered box, away from the light. Allow an interval of about one minute between the addition of toxin to each antitoxin dilution in order to approximate the interval between injections. Allow not less than thirty minutes, and not more than one hour, to elapse between combining the toxin and antitoxin and the injection.

**Injections.**—The method of injecting is the same as that given in the

chapter "Production and Standardization of Diphtheria Toxin," p. 316. The time when the control guinea pigs are injected is recorded on the card.

**Observation of Animals. Autopsies.**—The procedures are those given in "Production and Standardization of Diphtheria Toxin," p. 317.

### *Estimation of Unit Content*

Estimation of unit content should always be on the conservative side. To allow a safe margin, it is desirable that the control guinea pigs should die in from seventy to eighty-five hours, while the dilutions on which the titer of the material tested is based should protect for ninety-six hours.

In making the estimate, every factor must be considered, such as, the original weight and condition of the guinea pig, changes in weight from day to day, signs of illness, relative reactions of different animals of a series, autopsy findings, and particularly the condition and time of death of the two control guinea pigs. If the results of the test are indeterminate because the estimations on which the dilutions were based were too high or too low, or because the animals die irregularly, the test is repeated.

## INTRACUTANEOUS TEST

The intracutaneous test is based on the fact that mixtures of diphtheria toxin and antitoxin in which there is an excess of toxin, will produce, when injected into the skin of a guinea pig, a typical local reaction, the degree of which is, within certain limits, in direct proportion to the amount of uncombined toxin in the mixture. In testing antitoxins by this method, the reactions induced by mixtures of a test dose of standard diphtheria toxin and varying doses of antitoxin are compared with that induced by a mixture containing the same dose of toxin and a test dose of standard antitoxin injected at the same time on the same animal. The test dose of toxin used in this laboratory is the amount of toxin which combined in 0.1 cc. with $\frac{1}{1000}$ of a unit of standard antitoxin gives a mixture which injected intracutaneously into a guinea pig will induce an area of slight but definite redness beginning to fade after forty-eight hours. In practice, 1 cc. of serum dilution containing $\frac{1}{50}$ of a unit of antitoxin is mixed with 1 cc. of toxin dilution and 0.1 cc. of the mixture is injected. In testing material with very low antitoxic content, as serum containing less than one unit of antitoxin per cubic centimeter, the test dose of toxin is based on $\frac{1}{10,000}$ unit of antitoxin.

The worker should be thoroughly familiar with the procedure used

in the subcutaneous test before undertaking the intracutaneous test. The procedures should be carried out with the same attention to detail. The apparatus is in general the same, except that a burette which delivers more rapidly is used, and 1-cc. graduated pipettes standardized in this laboratory are used in place of the "to contain" pipettes.[8]

For routine determinations of natural antitoxic substances in normal sera, tests for $\frac{1}{500}$, $\frac{1}{100}$ and $\frac{1}{20}$ unit per cubic centimeter are made. (For the range for other material see directions under "Subcutaneous Test," p. 348.)

For routine determinations of material with a higher antitoxic content, three or four dilutions of each sample at 50- or 100-unit intervals are tested. For antitoxin having a titer of over 1500 units per cubic centimeter, it may be advisable to test at greater intervals.

### *Standard Antitoxin*

The standard antitoxin is the same as that used in the subcutaneous test. From the dilution containing one unit per cubic centimeter, the required dilutions ($\frac{1}{50}$ or $\frac{1}{500}$ unit per cubic centimeter) are made.

### *Standard Toxin*

The standard toxin is the same as that used in the subcutaneous test. The test dose is determined by preparing dilutions containing in the neighborhood of $\frac{1}{50}$ of an L+ dose per cubic centimeter, combining 1 cc. of each with an equal volume of a dilution of standard antitoxin containing $\frac{1}{50}$ unit per cubic centimeter, and injecting 0.1 cc. of the mixture intracutaneously into a guinea pig. The mixture which induces a slight but definite redness beginning to fade after forty-eight hours contains the test dose of toxin. At least three guinea pigs are used for the final test so that slight variations in the individual animals may be considered in making the final estimates. For retests, as when a fresh bottle of a given lot is tested, two guinea pigs are usually sufficient.

### *Test Animals*

White guinea pigs weighing between 350 and 450 grams are used. The hair is removed from the sides of the animal with barium-sulfide paste as described under "The Use of Experimental and Test Animals," p. 32, a narrow strip of hair over the spine and a wide strip along the median portion of the abdomen being left. The hair is removed on the

[8] See footnote 8, "Production and Standardization of Streptococcus Toxin (Scarlet Fever)," p. 331.

day before the test in order to avoid the possibility of irritation from the depilatory interfering with the interpretation of the reaction.

*The Test*

When the test dose is based on $\frac{1}{100}$ unit of antitoxin, first dilute each sample of material to be tested, 1:50 with salt solution; when on $\frac{1}{10,000}$, 1:500. From the initial dilution make dilutions such that the required amounts of the test antitoxin are contained in 1 cc. or less of one dilution. That is, if antitoxin is to be tested for 650, 700, and 750 units, make a 1:650 dilution of the initial 1:50 dilution. Doses of 1.0, 0.93, and 0.87 cc. respectively of the dilution will then be required.

DIPHTHERIA ANTITOXIN

12–18–25

Intracutaneous Test

| T. B. $\frac{128}{12/16}$ | Plasma Ord. 524 | Anti. Ord. 516 | Stand. control Rec'd. 8–24–25 |
|---|---|---|---|
| 1 + 49 a | 1 + 49 a | 1 + 49 a | 0.5 + 2.5 a |
| 1a + 49 b | 1a + 24 b | 1a + 49 b | 1a + 49 b |
| 1b + 12 c | 1b + 13 c | 1b + 27 c | |
| | | | Toxin No. 117A-5 |
| 650 = 1 cc. | 350 = 1 cc. | 1400 = 1 cc. | 1 + 9 a |
| 700 = 0.93 | 400 = 0.88 | 1500 = 0.93 | 1a + 38.9 b |
| 750 = 0.87 | 450 = 0.78 | 1600 = 0.88 | |

√E. G. B. (Checked by)

R. F. C. (Test made by)

FIG. 36. DILUTION CARD

Dilute the standard antitoxin so that 1 cc. contains $\frac{1}{50}$ or $\frac{1}{500}$ unit antitoxin. Prepare a dilution card (4 by 6 in.) and have it checked by a second person, before starting to make the dilutions. (See: fig. 36.)

In addition to the entries on the dilution card, enter on the record card (See: fig. 37), the guinea-pig number and weight; products, and titers to be tested for; and the test dose of toxin.

**Dilutions.**—In making the dilutions, follow the general procedure used in the subcutaneous test. Make all dilutions with 1-cc. graduated pipettes standardized in this laboratory. Use a clean pipette for each dilution. For the final measurements (fractions of a cubic centimeter), use federal certified 1-cc. graduated pipettes, selecting those which permit a more rapid delivery.

Antitoxin: When the dilutions of antitoxin are completed, pipette the

required volumes of the final dilution of each sample into brown-glass bottles previously marked with the product number, and number of units tested for. Use the same pipette for all samples, and between samples, rinse twice with salt solution and twice with the next sample. If less than 1 cc. of the dilution is used, add enough salt solution to make the volume 1 cc., using a fresh, certified graduated pipette.

Toxin: Dilute the standard toxin to contain twenty test doses in each cubic centimeter.

Toxin-antitoxin mixtures: Add to the antitoxin dilution in each of the bottles, 1 cc. of the standard-toxin dilution and mix. Allow not less

DIPHTHERIA ANTITOXIN—INTRACUTANEOUS TEST

Guinea Pig No. 4283 Date 12-18-25
Weight 432 Std. Toxin No. 117 A-5 Dil. 1/399 Time 4:00 p.m.

| SITE | PRODUCT NUMBER | DILUTION | 24 HOURS | 48 HOURS | 72 HOURS | REMARKS |
|---|---|---|---|---|---|---|
| | 12/16 | | | | | |
| F.R.D. | T. B. 128 | 650 | sl. rd. | v. sl. rd. | — | |
| C.R.D. | | 700 | sl. rd. | sl. rd. | v. sl. rd.√ | |
| H.R.D. | | 750 | mkd. rd. | rd. | rd. | |
| F.R.V. | Plas. 524 | 350 | v. sl. rd. | — | — | |
| C.R.V. | | 400 | sl. rd. | sl. rd. | v. sl. rd.√ | |
| H.R.V. | | 450 | rd. | rd. | sl. rd. | |
| F.L.D. | Ord. 516 | 1400 | v. sl. rd. | — | — | |
| C.L.D. | | 1500 | sl. rd. | v. sl. rd. | — | |
| H.L.D. | | 1600 | rd. | sl. rd. | v. sl. rd.√ | |
| C.L. | Standard Control | 1–50 | sl. rd. | sl. rd. | v. sl. rd. | |

R. F. C.
(Test made by)

FIG. 37. RECORD CARD

than thirty minutes and not more than one hour to elapse between combining the toxin and antitoxin and the injection.

**Injections.**—From five to six injections, at least one inch apart, may be made on each side of the guinea pig, depending on the size of the animal and the condition of the skin. Injections are usually made in the following order: front right dorsal, center right dorsal, hind right dorsal, front right ventral, etc. As each injection is made, the exact position is entered on the record card. The standard-antitoxin dilution is usually injected in the center of one side.

Use a 1-cc. tuberculin syringe with a $\frac{3}{8}$ inch, 26-gauge needle. Inject intracutaneously 0.1 cc. of the toxin-antitoxin mixture, beginning each

product with the mixture containing the highest dilution of serum. Before injecting a mixture rinse the syringe twice with it. Between injections of different products, change the needle and rinse the syringe twice with sterile water, then twice with the first mixture of the next product.

### *Readings and Estimation of Unit Content*

Make readings at the end of twenty-four, forty-eight, and seventy-two hours, noting the degree of redness, on the record card (fig. 37), as "v. sl. rd." (just perceptible); "sl. rd." (slight but definite redness); "rd." (redness more pronounced); "mkd. rd." (marked redness and induration, necrosis, scaling or pigmentation).

Estimation of unit content is based on the relative degree of the reactions induced at the 48-hour reading. The standard-antitoxin control used as the basis for comparison should show a slight but definite redness fading after forty-eight hours. Since slight variations in the reactions may occur in different guinea pigs, in interpreting the reactions on any one guinea pig, the control reaction on that animal should be used as the basis for comparison. Less reaction than the control, or no reaction, indicates an excess of antitoxin; more reaction, an excess of toxin. In the tests given, the titers would be estimated as: trial bleeding No. 128, 700 units; plasma No. 524, 400 units; anti. order No. 516, 1600 units.

### *Permanent Records*

Diphtheria toxin: The cards containing the data in regard to diphtheria toxin are described under "Production and Standardization of Diphtheria Toxin," p. 319.

Immunization of horses: A protocol is kept of each horse. For form and general data given see "General Instructions," p. 619. In the case of horses immunized for the production of diphtheria antitoxin, the following additional data are entered: dates and serial numbers of injections; number, titer, and amount of toxin injected; temperatures and any unusual conditions; and, in regard to each bleeding, date of bleeding, date tested, titer, bleeding number, volume, concentration order number of the lot in which the bleeding was included, and the serial number given the filtered antitoxin.

Plasma records: On a card are entered in regard to each bleeding; number, volume of plasma, date received at laboratory, date taken for concentration, order number in which pooled, date of final filtration, and lot number given filtered antitoxin.

Antitoxin record: On a second card are entered antitoxin lot number; volume; bleedings pooled and concentrated; date filtered and results of sterility tests on filtered material; units tested for, and titer; date filled, whether filled in therapeutic or prophylactic doses, volumes of doses, and results of sterility tests on

filled material; and date released. On the reverse side are entered lot number, number of bottles filled and size dose, date distribution starts, and date supply is exhausted.

Subcutaneous test: The following data are recorded in a loose-leaf record book (8½ by 11 in. sheets): date; concentration number, serial number of the filtered antitoxin; titers tested for; number and L+ dose of toxin; guinea-pig numbers; weights before injection, and weights and condition of the animals for four days following injections; time of death (in hours if less than four days, in days and hours if over); and, under "Remarks," condition after the fourth day, and autopsy findings or dates discharged; and initials of worker making the test.

Intracutaneous test: The dilution card (fig. 36) and the record card (fig. 37) are filed as permanent records.

## CHAPTER 2

# PRODUCTION AND STANDARDIZATION OF TETANUS ANTITOXIN

Tetanus antitoxic serum is produced for prophylactic and therapeutic purposes by the immunization of horses or mules against the toxin of *B. tetani*. The potency of the serum and of the purified concentrated product is determined by its neutralizing action when combined with tetanus toxin. The procedures connected with the preparation of the antitoxin correspond closely with those described in the chapter "Production and Standardization of Diphtheria Antitoxin," p. 340, to which the worker is referred for detailed instructions not given in the following text.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

## PRODUCTION OF ANTITOXIN

### *Tetanus Toxin Used in Immunization*

Fresh tetanus toxin containing 0.25 per cent cresol with a minimum fatal dose of 0.0002 cc., or a stronger toxin, is used for immunization. (For preparation of toxin see "Production and Standardization of Tetanus Toxin," p. 320.) Since freshly prepared toxin is required, a large supply cannot be maintained. The toxin-production group should, therefore, be kept informed as to the requirements. Toxin with an M.F.D. greater than 0.0002 cc. or more than three months old is used only in emergencies.

### IMMUNIZATION OF HORSES

### *Selection of Horses*

When available, relatively young horses of more than average weight, whose general condition appears to fit them for a long period[1] of active

[1] The first whole bleeding from horse 7 was taken in October, 1913, and the last in December, 1922. Three hundred and sixty-three injections of toxin were given and 161 whole bleedings taken. The maximum titer was over 700 units per cubic centimeter, the average, about 370 units. Horse 120 produced antitoxin of the required potency for nearly eight years. These horses were exceptional; more often the period is from two to four years.

immunization and production, are selected. No test is made for natural antitoxic substance.

### *Injection and Dosage*

The schedule of early injections and doses is given in table 27. Modifications are occasionally required. All injections are made subcutaneously.

During the past four years, satisfactory results have also been obtained by resting the horse for from four to six weeks after the tenth injection. When injections are resumed, 1 cc. is given as the first dose, but afterward the rate of increase is more rapid than that indicated in

TABLE 27

*Schedule of first sixteen injections of tetanus toxin*

| INJECTION NUMBER | M.F.D. | VOLUME | REMARKS |
|---|---|---|---|
| | | *cc.* | |
| 1 | 5,000 | 1 + tetanus antitoxin | Tetanus antitoxin is combined with the first two doses of toxin and allowed to stand one hour before injection. 5000 units are combined with the first dose, 1000 with the second |
| 2 | 10,000 | 2 + tetanus antitoxin | |
| 3 | 15,000 | 3 | |
| 4 | 25,000 | 5 | |
| 5 | 35,000 | 7 | |
| 6 | 55,000 | 11 | |
| 7 | 75,000 | 15 | |
| 8 | 105,000 | 21 | M.F.D. of toxin = 0.0002 cc. |
| 9 | 135,000 | 27 | Injections every third day (seventy-two hours) |
| 10 | 175,000 | 35 | |
| 11 | 215,000 | 43 | |
| 12 | 265,000 | 53 | |
| 13 | 320,000 | 64 | |
| 14 | 385,000 | 77 | |
| 15 | 450,000 | 90 | |
| 16 | 525,000 | 105 | |

the schedule. Doses larger than 525,000 M.F.D. are recorded in cubic centimeters only. If after from five to six months, a horse's serum contains less than 100 units of antitoxin per cubic centimeter, immunization is discontinued.

### *Trial and Whole Bleedings*

Whole bleedings may be commenced when the titer of the serum reaches 150 units. It is usual, however, to delay bleedings until after a titer of 300 units has been reached. Immunization is generally continued until the titer has dropped below 150 units. Two or three bleed-

ings of serum with a lower titer may be taken for the monthly prophylactic injections given to each horse not under active immunization against tetanus toxin. This material is filtered, tested for unit content, and filled in 2000-unit doses. It may be advisable to rest a good antitoxin producer for two or three months, at intervals of one or two years.

### TREATMENT OF PLASMA AND CONCENTRATED ANTITOXIN

Samples of the pooled plasma and of the antitoxin immediately after concentration are tested from time to time to determine unit concentration and loss. The finished product is dispensed in doses of 1500 units for prophylactic use, and 10,000 and 20,000 units for therapeutic use. Twenty per cent more antitoxin than the label indicates is added, as in the case of diphtheria antitoxin.

## STANDARDIZATION OF ANTITOXIN

Tetanus antitoxin is standardized against a standard tetanus toxin supplied by the U. S. Hygienic Laboratory. A standard antitoxin is also supplied for use in the control tests. The procedure followed is similar to that used in standardizing diphtheria antitoxin by the subcutaneous method (see "Production and Standardization of Diphtheria Antitoxin," p. 348).

### SUBCUTANEOUS TEST

#### *Standard Antitoxin*

Standard antitoxin is obtained as a glycerinated solution from the U. S. Hygienic Laboratory, upon request.[2] The dilution required to give 0.1 unit per cubic centimeter, is given on the label.

[2] For a statement of the methods and principles involved in the standardization of tetanus antitoxin, see Hygienic Laboratory Bulletin, No. 43, 1908 (122); for a further statement of the differences between the values of the American and European antitoxic units, the reports of the sub-committee on Antitetanic Serum presented at Paris, 1922; Reports of Serological Investigations, Health Organization, League of Nations, 1923 (123). This report gives the ratio of the values of these units as such that the volume of a given serum which contains one German unit contains from 60 to 66 American units, and in the neighborhood of 2500 French units. In October, 1926, the Permanent Standardization Commission established a new international unit. The relation of this unit to the American unit is two international units to one American unit. Report of Permanent Standards Commission, Health Organization, League of Nations, 1926, C. H. 517.

### *Standard Toxin*

Standard tetanus toxin is obtained from the U. S. Hygienic Laboratory upon request. It is sent in the form of a dry powder, in a sealed tube with a double arm. The identifying number and the amount required for the test dose are given on the label. The toxin is tested at small intervals (0.00002 cc.) on either side of the test dose given, two guinea pigs being injected with each dose; or, three or four control guinea pigs are injected in the first standardization test in which the toxin is used. Changes in the test dose are made if necessary. If kept under proper conditions, the toxin may be used for months, the extent of deterioration being determined by control tests against standard antitoxin and the test dose being changed as required.

**The L+ Dose.**—The L+ dose, which contains approximately 100 minimum fatal doses, is defined as the least amount of toxin which when mixed with one-tenth unit of antitoxin, will kill a 350-gram guinea pig within ninety-six hours. The test dose used, however, is that which kills in from sixty-five to eighty-five hours.

To preserve the toxin under favorable conditions, prepare a desiccator with fresh concentrated sulfuric acid, and vaseline the cover. Place the bottom of a Petri plate on the perforated plate. Record the date on the label of the new tube, open and place it at once on the Petri plate in the desiccator. Cover the latter and store in the cold room where it will not be disturbed. The slightest moisture must be guarded against; allow no cotton or other substance likely to retain moisture to remain in the desiccator. Replace the sulfuric acid after every fifth opening, or every three months. Record the date of replacing the acid and the dates the desiccator is opened, on a tag attached to it.

As tetanus toxin is one of the most powerful poisons known, caution should be exercised in filing and breaking off the end of the long arm of a new tube, and in handling the dry or diluted material. Typical tetanic symptoms from inhalation of the powder have been reported.

### *Test Animals*

Guinea pigs weighing between 330 and 380 grams are used. Animals weighing not less than 350 grams, and preferably not more than 360 grams, are selected for the controls.

### *The Test*

Consult the tables in the Hygienic Laboratory Bulletin No. 43, 1908, for the dilutions to be used, which are so made that varying volumes of the last dilution contain the amounts of antitoxin it is desired to test. As far as possible, select those dilutions which contain the desired doses in 2 cc. or less. For test card, see figure 38.

Weigh accurately a small amount (approximately that needed in the day's test) of the dry toxin and dissolve it in enough 0.85-per-cent salt solution to give one test dose per cubic centimeter. Record the weight and make the necessary calculations (later checked by a second worker) on the back of the test card.

Calculations are made as follows: assuming, (1) the weight of toxin to be 0.0182 gram and (2) the test dose to be 0.0008 gram, or expressed as a fraction reduced to its lowest terms, $\frac{1}{1250}$ gram.

Then: $\frac{0.0182}{0.0008}$, or more conveniently,

$0.0182 \times 1250 = 22.75$ cc. the volume of salt solution required.

---

TETANUS ANTITOXIN

12–30–25
3:30 p.m.

Anti. No. 164

1 + 99 a (1st dilution)
1a + 99 b (2nd dilution)

| | | |
|---|---|---|
| 833.3 (units) | 1.2 cc. | No. 4210 – 338 (grams) |
| 909.0 | 1.1 cc. | No. 4287 – 344 |
| 1000.0 | 1.0 cc. | No. 4208 – 366 |

Standard control—Rec'd 12–28–25
1 + 49

1 No. 4212 – 350
2 No. 4289 – 354

T. B. $\frac{12/14}{151}$

1 + 99 a
1a + 49

| | | |
|---|---|---|
| 333.3 (units) | 1.5 cc. | No. 4209 – 344 |
| 454.5 | 1.1 | No. 4206 – 364 |

√E. G. B.
(Card checked by)

R. F. C.
(Test made by)

Laboratory stock

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FIG. 38. TEST CARD

The procedure found most convenient has been: (1) removal of antitoxins and toxin from cold room, (2) preparation for antitoxin dilutions and making of first dilutions of each series, (3) weighing of toxin, (4) completion of antitoxin dilutions, (5) dilution of toxin (in short series this may be done immediately after the toxin has been weighed).

**Dilutions.**—Toxin: Remove the desiccator, carefully protected from light, to the balance room and allow it to come to room temperature.

Place a dilution flask inside the balance case and allow sufficient time for temperature and moisture equilibrium to be reached before beginning to weigh the toxin. When handling the flask, always use a piece of clean cheesecloth.

Make sure the analytical balance and the weights are in satisfactory condition. (It is essential that the worker making the test should be proficient in the use of the balance.) Weigh the flask, then add weights corresponding to the minimum amount of toxin required. (Not less than 0.01 gram of toxin should be weighed.) Remove the flask and place it on a piece of white paper.

Take the cover from the desiccator and remove the toxin tube, which should be handled with cheesecloth and held horizontally with the open arm upwards. Close the desiccator. Tap the tube gently until a little of the powder has shaken down toward the unopened arm. Remove the stopper from the weighing flask. Turn the tube so that the open arm is downward, and, holding the opening above the mouth of the flask, tap lightly on the tube until the estimated amount of toxin is transferred. Allow no toxin to fall on the outside of the flask. Do not touch flask or tube at any time with the fingers. Stopper the flask and return the tube immediately to the desiccator. Weigh the flask as quickly as possible to prevent absorption of moisture from the air. If insufficient toxin has been taken, reweigh the flask containing toxin, add more toxin, observing the same precautions as above, and weigh again. Add the weight of the added toxin to that first obtained. Record the date and return the desiccator at once to the cold room. Place the flask containing the toxin in the dark in the standardization room, until required.

When the antitoxin dilutions are completed, add the required volume of salt solution from a certified burette to the dry toxin. Invert the flask once or twice and place it in the dark until required. Before using, again invert the flask to insure a uniform dilution. Use the toxin dilution within one hour after it is made.

Antitoxin and toxin-antitoxin mixture: Prepare the antitoxin dilutions, pipette the required amounts into the brown bottles. If necessary, make the volume up to 2 cc. with salt solution. Then add 1 cc. of the standard toxin dilution.

**Injections. Observation of Animals. Autopsies.**—For procedures see "Production and Standardization of Tetanus Toxin," p. 324.

### *Estimation of Unit Content*

In computing the titer of tetanus antitoxin, it is advisable to leave an even wider margin for variations than with diphtheria antitoxin. The control guinea pigs should die in from sixty-five to eighty-five hours, while the animals on which the titer of the material tested is based should survive ninety-six hours or longer.

### *Permanent Records*

The records are similar in form to those of diphtheria antitoxin (see "Production and Standardization of Diphtheria Antitoxin," p. 357). In the records of the subcutaneous test the standard toxin is designated by the date received.

## CHAPTER 3

# PRODUCTION AND STANDARDIZATION OF ANTISTREPTOCOCCUS SERUM (SCARLET FEVER)

Antistreptococcus serum (scarlet fever) is produced in horses by subcutaneous injection into a mass of agar, of living hemolytic streptococci and by subcutaneous injection of the toxic filtrate of broth cultures of these organisms. The serum thus produced is distributed for therapeutic purposes. It may also, under special conditions, be used for passive immunization, and for the blanching test in doubtful cases of scarlet fever. The titer or potency of the serum is based on its neutralizing action when combined with "scarlet-fever" streptococcus toxin, as determined by intracutaneous tests on goats confirmed occasionally by tests on susceptible human subjects. For purposes of comparison and study, antistreptococcus serum is also produced in goats by subcutaneous injections of streptococcus toxin.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

### *Standard Strain*

At present one standard strain, Dochez N.Y.5, Collection No. 14E, is used for the immunization of horses and for the standardization of the sera.[1] For maintenance of strain, see chapter "Production and Standardization of Streptococcus Toxin (Scarlet Fever)," p. 327.

## PRODUCTION OF ANTISTREPTOCOCCUS SERUM

### IMMUNIZATION OF HORSES

Immunization of horses is commenced by the "agar-culture" method (Dochez) (124, 125). Living streptococci from an 18- to 20-hour

[1] Since a single strain producing a highly potent, stable toxin and possessing broad antigenic properties is obviously to be preferred, provided it is representative, the Dochez strain was selected as most nearly approaching such requirements. Serum produced with this strain has proved effective clinically. In order, however, to ascertain whether other strains of even broader antigenic activity may not be available, an immunological study of several carefully chosen strains is now in progress. Should no single strain, in the light of further experience, prove to be representative, it may be necessary to include other strains and their toxins in the immunization of horses.

broth culture are injected into a mass of agar previously injected in a fluid state under the skin of the horse and allowed to harden. Later, subcutaneous doses of toxin,[2] supplemented at intervals by injections of living organisms by the agar culture method, are given. The toxin and culture are obtained from the toxin-production group. Horses having discharging abscesses are isolated and special precautions taken.

*Preparation of Culture and Toxin*

**Culture.**—To prepare the culture used for immunization, select the latest heart-blood culture from a mouse, and transfer from 0.1 to 0.2 cc. to streptococcus-toxin broth, and incubate overnight. Streak a blood-agar plate and make a slide preparation stained by Gram's method and examine. Inoculate the required volume of broth with from 0.1 to 1.5 cc. of seed culture and incubate from eighteen to twenty hours. Make a slide preparation stained by Gram's method and examine.

If satisfactory, pipette the culture into sterile centrifuge tubes and centrifugalize for from twenty to thirty minutes, or until the supernatant broth is clear. Pour the supernatant broth into a jar containing 1-per-cent crude cresol. Suspend the sediment in from 2 to 3 cc. of broth and mix thoroughly. Transfer the suspension into a 5-cc. bottle. Rinse the centrifuge tubes with 1 or 2 cc. of broth and add the washings. Observe special precautions throughout to avoid contaminating the material. Streak a blood-agar plate with the suspension. (Should contaminating organisms develop on the plate, record the kind on the horse chart.) Close the bottle with a sterile stopper and cover with tinfoil, label with the horse and culture numbers, and send at once to the operating room with an initialed duplicate memorandum of shipment, giving the horse and injection numbers, culture number and amount of dose. Send with the culture suspension two bottles, each containing from 40 to 50 cc. of fluid one and one-half per cent agar previously melted by boiling and held, if necessary, in a 55°C. incubator. (The second bottle of agar is used as a temperature control.) In cold weather warm the copper container in which the agar is sent. When more than one horse is to be injected, dispense the suspension for each separately and send two bottles of agar for each horse to be injected.

[2] The method is similar to that used for the production of diphtheria and tetanus antitoxins. For its use in the production of streptococcus antitoxic serum, see Dick, G. F., and Dick, G. H., Jour. Amer. Med. Assoc., 1925, **84**, 803 (126).

**Toxin.**—The toxic filtrate from a 7-day broth culture is used. The toxin contains 0.5 per cent phenol. The toxin at present used has a titer of approximately 30,000 skin-test doses per cubic centimeter. No data are as yet available as to the length of time the toxin remains satisfactory, but potent toxins even up to nine months old have been used. For preparation of toxin, see "Production and Standardization of Streptococcus Toxin (Scarlet Fever)," p. 326; for directions for shipment see under "Diphtheria Toxin Used in Immunization," p. 340.

*Injection and Dosage*

Injections of living organisms into agar are made on the back of the horse near the saddle; those of toxin, subcutaneously by the usual method. For procedure see "Care and Treatment of Animals—Large Animals," pp. 589 and 590.

A series of two or three injections of living organisms is first given, the intervals between injections, usually two to three weeks, depending on the extent and severity of the abscess and the period during which it is actively discharging. An injection of culture may be given as soon as the abscess from the previous injection has ceased to discharge actively. The first injection of toxin is given from four to eight weeks after immunization is commenced. It may be given on the first evidence that the abscess from the last injection of culture is beginning to subside. Injections of toxin are then made at 3-day intervals, except when the date falls on a Sunday or a holiday, when the injection is postponed and the schedule changed to conform with the new date. The first injection is made on Wednesday so that the 3-day schedule may be maintained for six injections. A 4-day interval is substituted when the volume of toxin injected has reached 500 cc., usually in about six weeks. After treatment with toxin has been commenced, injections of living organisms are continued at 1- to 2- or even 3-month intervals, depending on the reactions induced and the potency of the serum. A series of two or three injections of culture at shorter intervals are at times advisable. Injections of culture and of toxin are usually given on the same day, but on different sides of the horse.

The initial dose is a broth suspension of living streptococci from 25 cc. of an 18- to 20-hour broth culture; the second, usually from 50 cc. For subsequent injections of culture the dose is increased, organisms from 50 to 100 cc. of culture being added each time; or, the same dose is repeated, depending upon the reactions induced. The maximum dose is organisms from 300 cc. of culture.

The dose for the first injection of toxin is 10 cc. (approximately

300,000 skin-test doses); for the second, 25 cc. For subsequent injections the dose is usually increased by 25 cc. or later even by 50 cc. until a total of 500 cc. is reached, after which no further increase is made.

*Temperature Reactions and General Condition*

The temperatures of horses undergoing immunization are taken each morning. (See "Care and Treatment of Animals—Large Animals," p. 583.) On the day following injection of live culture, a slight to moderate febrile reaction (101°C. to 103°C.) may be noted. This, however, is transient, usually subsiding within from twenty-four to forty-eight hours. Some oedema may occasionally develop following an injection, especially of toxin, but the general condition of the horse throughout immunization usually remains excellent.

*Trial and Whole Bleedings*

Trial bleedings are taken before immunization is commenced, just before the first injection of toxin, and then every month, or oftener when indicated. Trial bleedings are not taken sooner than the fourth day after an injection of toxin.

At the present time whole bleedings are taken for serum, and for plasma for concentration. Bleedings for plasma may be taken after the titer of the serum has reached 100 units per cubic centimeter; for unconcentrated serum, from 150 to 200 units, or preferably from 300 to 400 units or more.[3] The bleedings are taken on the sixth day after an injection of toxin, provided there is no actively discharging abscess. Injections are resumed on the following day. From one to three injections of toxin are given between whole bleedings, depending upon the titer of the serum and whether culture has been given. In one or two instances, two bleedings have been taken in succession, four or more days apart, with no evidence of appreciable drop in titer. For technic of bleeding, etc., see "Care and Treatment of Animals—Large Animals," p. 593.

IMMUNIZATION OF GOATS

Goats previously used for titration tests of standard streptococcus toxins and antistreptococcus sera are usually selected. Good-sized animals in satisfactory physical condition are chosen. Subcutaneous

[3] The highest estimated titer thus far obtained has been 600 units per cubic centimeter; that of the serum at present distributed is between 300 and 400 units.

injections of increasing doses of toxin are given at 3- to 4-day intervals. The initial dose is from 500 to 2000 skin-test doses. The doses are increased gradually at first, later by 5 and 10 cc. until 80 to 100 cc. is reached, after which the volume is not increased, though a more potent toxin may be substituted. Trial bleedings are taken just before immunization is commenced, at the end of two months, and then at least every month. Whole bleedings of from 300 to 700 cc. are usually taken when the titer of the serum has reached 10 units per cubic centimeter. Whole bleedings are taken six days after the last injection of toxin, and injections are resumed on the following day. After whole bleedings are commenced the interval between injections is increased to four days. Variations in the schedule may frequently be necessary. When the animal has apparently reached the maximum titer, it may be advisable to bleed it out after one or two whole bleedings have been taken. The procedures are in general the same as in the immunization of horses. The blood is, however, collected in cylinders (250 by 35 mm.) without weights. The serum is preserved with 0.3 per cent cresol.

### TREATMENT OF ANTISTREPTOCOCCUS SERUM[4]

Serum, or plasma, usually of approximately the same titer, from different bleedings from the same horse may be pooled, provided the bleedings were taken within a period of three months. Samples for potency tests are taken from each bleeding. Under certain conditions, the test of a single bleeding may be omitted, when there is definite evidence that the titer can be computed closely from that of the preceding and of the next bleedings and the material is to be pooled.

Serum is treated according to the procedure in the chapter "Production and Standardization of Antimeningococcus Serum," p. 433, except that samples for the final potency tests are not removed until after the material has been filtered.

Plasma, without preservative, is pooled by the concentration group on instructions from the production group. The method of concentration is similar to that used for diphtheria and tetanus antitoxins except that the salted plasma is heated to only 57°C. (See "Concentration of

[4] In view of its high antitoxic potency and possible antibacterial action, the unconcentrated serum has been preferred for general distribution, until further comparative data are available on the stability and therapeutic breadth of the purified and concentrated material. Some evidence has been obtained that the concentrated product may be less stable than the unconcentrated. Serum reactions from therapeutic injection of unconcentrated serum appear not to be excessive, rather the reverse.

Antitoxins," p. 384.) Samples of serum for potency tests are taken at the time of each bleeding. Samples are taken from the pooled plasma before concentration, and, if the concentrated material is to be held for some time before filtration, when the protein content is adjusted, in order that the "unit concentration" and total loss may be promptly determined. Final potency tests are always made after the material is filtered. For filtration, sterility tests, etc., see the chapter "Production and Standardization of Diphtheria Antitoxin," p. 346.

### *Preparation for Distribution*

Serum for therapeutic use is dispensed in approximately 3000-unit doses. In order to compensate for possible deterioration and for serum remaining in the bottle or syringe at the time of injection, a volume of serum containing 20 per cent more units than the label indicates is included in the dose dispensed. Thus a bottle labeled "3000 units" contains approximately 3600 units. The number of units, "3000," and the number of units per cubic centimeter are given on the label together with the product number and the return date. Until further data are available, serum, if kept under proper conditions, is considered satisfactory for use for one year from the date of the last test.

A limited supply of serum for prophylactic use, dispensed in doses of approximately 1500 units, is maintained for distribution on special request. A few bottles of serum in 1-cc. volumes for the blanching test in doubtful cases of scarlet fever are also maintained for special distribution.

## STANDARDIZATION OF ANTISTREPTOCOCCUS SERUM

The serum is standardized by determining its neutralizing action when combined with a standard scarlet-fever streptococcus toxin. This action is measured by the degree of reaction induced in goats by the intracutaneous injection of mixtures of the serum and the standard toxin, in comparison with that induced by similar mixtures containing a standard antistreptococcus serum (127). Tests of trial bleedings and preliminary tests of whole bleedings are usually made on one animal only. Final tests of sera to be distributed for therapeutic use are made on at least two animals and the results are controlled by tests on at least one susceptible human subject. The antitoxic potency of the serum is expressed in units per cubic centimeter. The protective value of the serum for mice, when tested against a culture of the standard strain, is also determined from time to time.

**Antitoxic Unit.**—One unit of antitoxin is the amount of antitoxin which neutralizes one hundred skin-test doses of toxin. This unit is that suggested at an informal conference in Washington in May, 1925.

### INTRACUTANEOUS TEST ON GOATS[5]

Varying doses of the test serum are combined in 0.1 cc. with a test dose of standard toxin and are injected intracutaneously into a goat. At the same time, as a basis for comparison, mixtures of the standard serum with the same test dose of toxin are injected. Control tests are also made with standard toxin heated and unheated, with toxin broth, and with each serum alone.

#### *Standard Serum*

The standard antistreptococcus serum used at present is a highly potent, unconcentrated serum prepared in this laboratory and carefully titrated both on human subjects and on goats against the U. S. Hygienic Laboratory standard toxin (Dick) and against a standard toxin prepared in this laboratory with the standard laboratory strain (Dochez). A portion of the serum has been preserved with an equal volume of glycerin and is tested at frequent intervals against the standard laboratory toxin to determine possible deterioration.[6] For purposes of control, tests are also made on the unpreserved serum. During a period of over ten months no appreciable deterioration has been noted in either. The titer of the glycerinated serum is 100 units per cubic centimeter (neutralizes 10,000 skin-test doses per cubic centimeter).

#### *Standard Toxin*

The standard toxin used for tests both on goats and on human subjects is a stable potent streptococcus toxin prepared in this laboratory with the standard strain (Dochez). The skin-test dose of this toxin has been accurately determined by comparative tests against the standard toxin (Dick) supplied by the U. S. Hygienic Laboratory. The standard toxin is selected carefully and tested at frequent intervals, since there is evidence that some toxins may deteriorate rapidly, or may induce reactions which reach their maximum and begin to fade before from twenty to twenty-four hours. Such toxins are unsatisfac-

[5] See footnote 7 under "Tests on Goats" in "Production and Standardization of Streptococcus Toxin (Scarlet Fever)," p. 330.

[6] Comparative titrations of the standard serum against the U. S. Hygienic Laboratory standard Dick toxin are also made at intervals.

tory for standardization tests. (For production and standardization see "Production and Standardization of Streptococcus Toxin (Scarlet Fever)," p. 326.)

**Determination of the Test Dose.**—The toxin is standardized by determining its test dose against the standard serum.[7] The test dose is the amount of toxin which combined in 0.1 cc. with a test dose of standard serum ($\frac{1}{100}$ of a unit) gives a mixture which injected intracutaneously into a goat will induce a very slight reaction in from eighteen to twenty hours. Equal quantities of varying dilutions of the standard toxin containing from one to four human skin-test doses of toxin in 0.05 cc. are mixed with an equal quantity of serum containing $\frac{1}{100}$ unit[8] of antitoxin in 0.05 cc., and 0.1 cc. of each mixture is injected intracutaneously into a goat. The mixture of toxin and serum which induces a very slight redness, about $\frac{1}{2}$ to 1 cm. in diameter, in from eighteen to twenty hours contains the test dose of toxin. The mixture containing the next higher dilution of toxin should induce no reaction. The procedure is the same as that used in testing the serum except that the dose of serum is constant.

### *Test Animals*

Goats previously found to be highly susceptible to the standard toxin and giving no or only very slight reactions to heated toxin, broth, and horse serum, are used. They are prepared as for the test for the standardization of toxin.

### *The Test*

Three or four dilutions of each serum are tested, the dilutions being made so that the desired dose based on $\frac{1}{100}$ unit will be contained in 0.05 cc. Thus, for a serum estimated as containing approximately 200 units of antitoxin per cubic centimeter, the dilutions prepared are 1:500, 1:1000 and 1:1500 so that 0.05 cc. contains respectively $\frac{1}{10,000}$ cc., $\frac{1}{20,000}$ cc. and $\frac{1}{30,000}$ cc. of serum. One cubic centimeter of each dilution of serum is then combined with 1 cc. of a dilution of standard

[7] It has been found more accurate to base the test dose of standard toxin on its combining power for a definite dose of standard serum, after it has been standardized as previously described, rather than to base it on a definite number of skin-test doses of toxin.

[8] When goats which are not highly susceptible to the toxin are used, a test dose of toxin based on $\frac{1}{10}$ of a unit or more of antitoxin may be advisable. The lower dilutions of serum may, however, frequently induce serum reactions in goats not sensitive to higher dilutions.

toxin containing one test dose in 0.05 cc., and 0.1 cc. of each mixture is injected intracutaneously into a goat. For purposes of comparison, 0.1 cc. of mixtures of standard serum containing $\frac{1}{50}$, $\frac{1}{100}$ and $\frac{1}{150}$ unit of antitoxin combined with one test dose of standard toxin are tested on the same animal at the same time. Control tests are also made

ANTISTREPTOCOCCUS SERUM (SCARLET FEVER)

| Serum No. 32 (Units per cc.) | Goat No. 34 (Final Dilution) | (Dilution before mixing) | 10-22-25 |
|---|---|---|---|
| | | | 0.5 + 49.5 a |
| (300) | (1:)3000 | (1:)1500 | 0.5a + 7 b |
| (400) | 4000 | 2000 | 0.5a + 9.5 c |
| | 5000 | 2500 | 0.5a + 12 d |
| | 6000 | 3000 | 1b + 1 e |
| Standard Serum No. 28 | | | |
| | | | 0.5 + 49.5 a |
| | 1000 | 500 | 1a + 4 b |
| | 2000 | 1000 | 1a + 9 c |
| | 3000 | 1500 | 0.5a + 7 d |
| Standard Toxin No. 52 | | | |
| | | | 1 + 9 a |
| | 2000 | — | 0.5a + 99.5 b |
| | *(4000 | 2000) | |
| | 4000 | — | 1b + 1 c |
| | 8000 | — | 1b + 3 d |
| Broth (Uninoculated Toxin Broth) | | | |
| | | | 0.1 + 19.9 a |
| | 2000 | — | 1a + 9 b |
| Heated Toxin Control Toxin No. 52 | 1:2000 Time in 2:00 p.m. Time out 3:30 p.m. | | |

√M. W. W. (Test checked by)

P. P. M. (Test made by)

* Test dose 1/40,000 cc.

FIG. 39. DILUTION CARD

with one-half, one, and two test doses of standard toxin, with a toxin control containing two test doses of toxin heated at 100°C. for one and one-half hours and with uninoculated toxin broth alone and each serum alone, the doses being equivalent to the largest amount of broth and of each serum respectively present in mixtures of toxin and serum.

Before starting the test, prepare a dilution card giving the date, product, titers to be tested for, dilutions of sera and toxins, and the number of the goat to be used. (See: fig. 39.) Always have the card verified by a second person, before starting to make the dilutions.

**Dilutions.**—The general procedure is that given under "Production and Standardization of Streptococcus Toxin (Scarlet Fever)," p. 332. The same accurately standardized burettes and pipettes are used.

Serum: Dilute the test sera and standard serum as required. When dilutions are completed, starting with the highest dilution of each product, pipette exactly 1 cc. of the required dilution into stoppered, sterile test tubes (100 by 15 mm.) previously marked with product number and dilution to be tested. Use the same pipette for all products. Between each dilution rinse the pipette with the next dilution—between each product rinse the pipette twice with salt solution and then twice with the highest dilution of the next serum.

Toxin: For the toxin-antitoxin mixtures, dilute the standard toxin to contain one test dose in 0.05 cc. or 20 test doses per cubic centimeter. For the control tests, prepare also dilutions containing one-half, one, and two test doses in 0.1 cc.; and for the heated-toxin control, heat a portion of the dilution containing the two test doses in a water-bath at 100°C. for one and one-half hours.

Broth control: Prepare a dilution of uninoculated toxin broth containing broth equivalent to two test doses of standard toxin in 0.1 cc.

Toxin-serum mixtures and controls: Add to the serum dilution in each tube 1 cc. of the standard toxin dilution and mix thoroughly. Pipette also the control dilutions of serum, broth and toxin into their designated tubes. Allow the mixtures of toxin and serum to stand for at least one-half hour before starting the injections.

**Injections.**—Four or five rows of from five to eight injections from two to three inches apart may be made on each side of the goat, the first row of injections being made about three to four inches below the median line of the back, starting at the shoulder. The control mixture of standard toxin and serum and the standard toxin alone are usually injected in the second or third row.

With a 1-cc. tuberculin syringe (needle 26 gauge $\frac{3}{8}$ in.) inject intracutaneously 0.1 cc. of each dilution. Use one syringe for the serum controls, a second for the mixtures of toxin and serum, and a third for the toxin-broth and toxin controls. With each serum to be tested inject first the serum control, then the toxin-serum mixtures beginning with the mixture containing the highest dilution of serum. Before in-

jecting each toxin-serum mixture, rinse the syringe twice with it. Between injections of different products, change the needle and rinse the syringe twice with sterile salt solution or water, then twice with the next serum control or toxin-serum mixture to be injected. Inject the controls of toxin broth and heated and unheated toxin in the order given. As the injections are made enter on the record card the material injected and the exact position. (See: fig. 40.)

SCARLET FEVER—STANDARDIZATION OF SERA

Goat No. 34 Intracutaneous Test Date 10–22–25

Dose 0.1 cc. Std. Toxin No. 52 Std. Serum No. 28 Time 3:30 p.m.

| SITE | SERUM | | TOXIN | | REACTION | REMARKS |
|---|---|---|---|---|---|---|
| | Number | Dilution | Number | Dilution | Time 9:00 a. m. 10–23 | |
| L 2–1 | 28 | 1000 | — | — | — | |
| 2–2 | " | 3000 | 52 | 4000 | sl. rd. v. sl. sw. 2 cm. | |
| 2–3 | " | 2000 | " | " | v. sl. rd. | |
| 2–4 | " | 1000 | " | " | — | |
| 2–5 | — | — | Broth | 2000 | — | |
| 2–6 | — | — | 52-Htd. | 2000 | — | |
| 3–1 | — | — | 52 | 8000 | sl. rd. sl. sw. 2 cm. | |
| 3–2 | — | — | " | 4000 | sl. rd. sl. sw. 3 cm. | |
| 3–3 | — | — | " | 2000 | sl. rd. sl. sw. 3½ cm. | |
| 3–4 | 32 | 3000 | — | — | — | |
| 3–5 | " | 6000 | 52 | 4000 | v. sl. rd. 2 cm. | |
| 3–6 | " | 5000 | " | " | v. sl. rd. 1 cm. | |
| 3–7 | " | 4000 | " | " | — | |
| 4–1 | " | 3000 | " | " | — | |

FIG. 40. RECORD CARD

*Readings and Estimation of Titer*

Readings are made in a bright light after from eighteen to twenty hours, the degree of redness and of swelling induced, and the size of the area in centimeters being noted on the record card. In estimating the titer of a serum, the reactions induced by mixtures containing the test serum are compared with the reactions induced by mixtures containing the standard serum and also with those induced by the toxin, serum and broth controls. The serum and broth controls are usually negative, but slight reactions may be noted especially with low dilutions of serum and these must be taken into account when interpreting the results. The potency of the test serum is based on the mixture of test serum and toxin which induces a reaction slightly less than, or equal to, that induced by the mixture containing $\frac{1}{100}$ unit of standard serum (the

test dose). At least one dilution of the test serum should completely neutralize the toxin. If two or more of the higher dilutions induce very slight reactions similar to that induced by the test dose of standard serum, the estimated titer is based on the mixture containing the greatest amount of serum. In titrating therapeutic sera, should the tests on the different goats show variations, further titrations are made on other goats. This is, however, seldom necessary. The titer is expressed as units per cubic centimeter or as the number of human skin-test doses neutralized by 1 cc. The titer of sera can usually be determined to within from 50 to 100 units with the present scale of dilutions. In the case, however, of sera with a titer considerably below 100 units the method has proved less satisfactory, due in part at least to serum reactions induced by the low dilutions of serum.

### CONFIRMATORY TESTS ON HUMAN SUBJECTS

At present confirmatory tests of each lot of serum for therapeutic use are made on at least one human subject previously tested and found to be susceptible to the toxin but not hypersensitive to heated toxin nor to the standard serum. In the preliminary test to determine susceptibility a test dose of standard toxin, the same dose of heated toxin, and at least $\frac{1}{100}$ unit of standard serum alone are injected. For the titration test of sera the test dose of standard toxin used is one human skin-test dose.[9] Two or three different doses of the test serum, based on the results of the tests on goats, and three doses of the standard serum containing respectively $\frac{1}{50}$, $\frac{1}{100}$ and $\frac{1}{150}$ unit of antitoxin are used. Tests are also made with a test dose of standard toxin, with the same dose of heated toxin and with the lowest dilution of the test serum and of the standard serum. The mixtures are prepared in the same way as for the tests on goats but with the same rigid aseptic precautions as used for preparing the toxins to be tested on persons (see "Production and Standardization of Streptococcus Toxin (Scarlet Fever)," p. 335). The method of injection is that used in the standardization tests of toxin. A separate syringe is used for each serum control (test and standard serum) for the toxin-serum mixtures of each serum and for the heated as well as for the unheated toxin controls. With each series of toxin-serum mixtures, the mixture containing the highest dilution of serum is injected first.

Readings are made after from twenty to twenty-four hours and

[9] Comparative titration tests with 5 skin-test doses of toxin indicate that the one skin-test dose is quite as, if not on the whole more, satisfactory.

again after from forty-four to forty-eight hours. The degree of redness and of swelling induced and the size of the area in centimeters are entered on the record card. The estimated titer of the test serum is based on a comparison of the reactions induced by mixtures containing the test serum with those induced by mixtures containing the standard serum. Mixtures with test and standard sera which give corresponding reactions are estimated as containing equivalent amounts of antitoxin. At least one mixture of the standard serum and toxin should induce no reaction and one mixture should induce at least a slight reaction. If all mixtures of a test serum and toxin induce reactions or all fail to induce any reaction, further tests must be made with other dilutions. If two of three mixtures of a test serum and toxin induce equal reactions, the titer is based on the mixture containing the larger amount of test serum. The titer of a serum is expressed as units per cubic centimeter.

### PROTECTION TESTS (MICE)

The protective value of the serum for mice is determined from time to time.[10] The serum is tested against a 16- to 18-hour broth culture of the standard strain. Protection tests of the serum of each horse are first made when the antitoxic content has reached approximately 100 units per cubic centimeter. The serum is then tested at least once in two months during the further course of active immunization, usually at the time of a whole bleeding.

The procedure followed is that given under "Production and Standardization of Antipneumococcus Sera," p. 420, with the following differences. The serum and culture before being combined are diluted with streptococcus toxin broth. Two mice are generally injected with each mixture. No standard serum is at present used. The virulence of the culture is determined by inoculating two or three mice with different dilutions of culture depending upon the extent of fluctuation in the lethal dose indicated by previous tests.

For the tests a first transplant from the latest stock broth culture is used. The dilutions of culture depend upon the virulence of the stand-

[10] Protection tests in mice have been suggested as an indication of the antibacterial activity of the serum. It is not, however, possible with present methods to differentiate antibacterial activity from antitoxic activity by a protection test, since antitoxic activity alone might conceivably suffice to protect the animal, or in the case of the animal's dying, the antitoxic activity might not be sufficient to save the animal. However, the test is generally, perhaps too generally, accepted as an indication of antibacterial activity.

ard strain and the titer of the serum from the previous bleeding tested. At present doses of from 0.1 cc. to 0.001 cc. of culture are tested against 0.2 cc. of serum.

At the time of autopsy of the virulence control mice, tubes containing streptococcus toxin broth instead of serum semisolid tubes are inoculated for stock cultures; at least two from the mouse dying within forty-eight hours from the smallest dose which killed within that time, one for reserve from each of the others.

### *Permanent Records*

Records for the "Immunization of horses," "Antistreptococcus sera," and the "Protection test" are similar in form to those described in "Production and Standardization of Antipneumococcus Sera," p. 427.

Standardization tests: A card (4 by 6 in.) is kept giving the product number, date tested, person or animal on whom tested, and titer. The following cards are also filed as permanent records.

Dilution card, by date.

Human record card, by date.

Goat record card, by consecutive animal numbers.

## CHAPTER 4

# PRODUCTION AND STANDARDIZATION OF BOTULINUS ANTITOXIC SERA

A small supply of unconcentrated, monovalent botulinus antitoxic sera, type A and type B, is maintained for therapeutic and diagnostic purposes. These sera are produced by the immunization of horses against the homologous toxins. The potency of the sera is determined by their neutralizing action when combined with their respective toxins.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

## PRODUCTION OF ANTITOXIC SERA

### *Botulinus Toxins Used in Immunization*

Satisfactory results have been obtained by using toxins of low titer (minimum fatal dose from 0.001 to 0.005 cc.) during the early stages of immunization and later, during the period of production, substituting a stronger toxin. No data are available as to the effect the age of a toxin has on its antigenic value. For directions for shipping toxin to the farm, see "Diphtheria Toxin Used in Immunization," p. 340. Type-A and type-B toxins are stored separately both at the laboratory and at the farm.

[1] Experience in this laboratory has been limited to five horses immunized by (1) frequent injections with rapid increase in dosage, (2) slow increase in dosage with intervals of rest, or (3) frequent injections with slow increase in dosage. The first method proved fatal to two out of three horses after the tenth and twelfth dose (100 to 140 M.F.D.). The third method, which is given in the schedule of doses, induced a more rapid rise in titer than the second without causing unduly severe reactions. A horse was immunized by this method with type-A toxin (M.F.D., 0.001 cc.). After three months, when the serum contained slightly less than one unit of antitoxin per cc., the doses of toxin were increased rapidly. The titer of the serum three months later was 1400 units per cubic centimeter. Toxin of higher titer (M.F.D., 0.0002 cc.) was substituted but no marked increase in potency of the serum resulted. The most potent type-B antitoxin which has yet been obtained contained 175 units per cubic centimeter. For further details, see Wheeler, M. W., Jour. Immunol., 1923, **8**, 501 (128).

## IMMUNIZATION OF HORSES

The method[1] corresponds to that given in the chapter "Production and Standardization of Diphtheria Antitoxin." No test, however, is made for natural protection against botulinus toxin. The initial

TABLE 28

*Schedule of injections of botulinus toxin up to 1000 M.F.D.*

| INJECTION NUMBER | NUMBER OF M.F.D. | VOLUME | REMARKS |
|---|---|---|---|
| | | *cc.* | |
| 1 | 1/20 | 1/20,000 | M.F.D. of toxin, 0.001 cc. |
| 2 | 1/10 | 1/10,000 | Injections subcutaneously every third or fourth day |
| 3 | 1/5 | 1/5,000 | |
| 4 | 1/2 | 1/2,000 | Doses less than 20 cc. are diluted to that volume with 0.85-per-cent salt solution |
| 5 | 1 | 1/1,000 | |
| 6 | 2 | 1/500 | |
| 7 | 4 | 1/250 | |
| 8 | 6 | 6/1,000 | |
| 9 | 8 | 1/125 | |
| 10 | 12 | 3/250 | |
| 11 | 16 | 16/1,000 | |
| 12 | 24 | 3/125 | |
| 13 | 32 | 4/125 | |
| 14 | 40 | 4/100 | |
| 15 | 50 | 5/100 | |
| 16 | 60 | 6/100 | |
| 17 | 80 | 8/100 | |
| 18 | 100 | 0.1 | |
| 19 | 130 | 0.13 | |
| 20 | 160 | 0.16 | |
| 21 | 190 | 0.19 | |
| 22 | 240 | 0.24 | |
| 23 | 290 | 0.29 | |
| 24 | 340 | 0.34 | |
| 25 | 390 | 0.39 | |
| 26 | 470 | 0.47 | |
| 27 | 550 | 0.55 | |
| 28 | 650 | 0.65 | |
| 29 | 1,000 | 1.00 | |

injection of toxin is very small ($\frac{1}{20}$ M.F.D.) and, until antitoxin has been demonstrated in the blood, increase in dosage is very gradual since the lethal dose for a horse approximates 100 M.F.D.

### *Injection and Dosage*

The schedule for injections which is given in table 28, has been tentatively adopted.[2] When antitoxin is demonstrated in the serum, the doses of toxin are increased from 40 to 50 per cent up to 100 cc., then increases of from 30 to 50 cc. are made, up to 500 cc., the maximum dose given. No definite requirements for potency have been established but immunization of horses producing less than 100 units per cubic centimeter after five months, should probably be discontinued.

When a more potent toxin is to be substituted for the one in use, the dose is reduced proportionately. When less than 1 cc. of toxin is to be given, a dilution containing one dose in each cubic centimeter is made at the laboratory on the day of injection. Three or four cubic centimeters of the diluted toxin are sent to the farm, where 1 cc. is further diluted to 20 cc. at the time of injection.

### *Trial and Whole Bleedings*

Trial bleedings are taken immediately before the fourteenth injection, then once each month or more often if advisable. When the titer of the serum is considered sufficiently high, whole bleedings are taken. The blood is not drawn into sodium-citrate solution.

## TREATMENT OF ANTITOXIC SERA

The procedure for the treatment of the sera is that given in the chapter "Production and Standardization of Antimeningococcus Sera," p. 433, with the following modifications: The label on each bottle for distribution gives, in addition to the lot number of the serum, the type, titer, and volume.

Botulinus antitoxic sera are distributed only on special order, for immediate use. When material is sent out, the return date, which is eighteen months from the date of the last satisfactory test,[3] is entered on the label and on the carton.

## STANDARDIZATION OF ANTITOXIC SERA

Botulinus antitoxic sera are standardized by determining their neutralizing action when combined with the homologous botulinus toxins

[2] Preliminary treatment with botulinus antitoxic serum may prove advisable, in which case larger doses of toxin could be given.

[3] Results of tests of both types of serum indicate but little deterioration during a period of from four to five years.

previously standardized against the federal standard sera supplied by the U. S. Hygienic Laboratory.[4] The procedure given in the chapter "Production and Standardization of Tetanus Antitoxin," p. 361, is followed closely, with the following differences.

Well-ripened botulinus toxins which have reached an equilibrium are used for the standard toxin. The test dose contains approximately one hundred times the M.F.D. or the amount of toxin which, when mixed with $\frac{1}{10}$ unit of the homologous standard antitoxin and injected subcutaneously, will kill a 250-gram guinea pig in about ninety-six hours. The test dose is determined by the procedure given for determining the L+ dose of diphtheria toxin (see "Production and Standardization of Diphtheria Antitoxin," p. 349), except that $\frac{1}{10}$ unit of antitoxin is used.

Guinea pigs weighing between 230 and 280 grams are used. The procedure of injections and the symptoms and lesions looked for in observing and autopsying the animals are the same as those described in the standardization of botulinus toxins (see "Production and Standardization of Botulinus Toxins," p. 338).

### *Permanent Records*

Separate records are kept for type-A and type-B material except that standardization tests are entered in the test book consecutively without regard to type.

Botulinus toxins: The cards containing data in regard to botulinus toxin are described under "Production and Standardization of Botulinus Toxins," p. 339.

Standardization tests: The record kept is that described under "Subcutaneous Test," "Production and Standardization of Diphtheria Antitoxin," p. 358.

Immunization of horses, and botulinus antitoxic sera: The records are similar in form to those given in the chapter "Production and Standardization of Antipneumococcus Sera," p. 427. The date tested and the titer of the antitoxic serum are entered on the serum record card.

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[4] The method is similar to that published from the U. S. Hygienic Laboratory, Bengtson, I. A., Amer. Jour. Pub. Health, 1921, 11, 352 (129).

## SECTION III

## CHAPTER 1

## CONCENTRATION OF ANTITOXINS

### DIPHTHERIA—TETANUS

Diphtheria and tetanus antitoxic plasmas are purified and concentrated[1] as a routine procedure. The method of concentrating is based on the fact that the antitoxin is associated with the globulins, chiefly the pseudoglobulin in the plasma. The removal of the antitoxin is accomplished by precipitating separately, first the euglobulin, and then the pseudoglobulin. The euglobulin is precipitated by adding ammonium sulfate $(NH_4)_2SO_4$ to the plasma to 30 per cent of saturation and heating. The precipitate is then removed by filtration and the pseudoglobulin and antitoxin are precipitated by increasing the content of $(NH_4)_2SO_4$ in the resulting filtrate to 48 per cent of saturation. Excess of moisture and some ammonium salt are removed from the precipitate by pressure, and the remaining ammonium salt is removed by dialysis. Preservative is added to the dialyzed material and its hydrogen-ion concentration is adjusted to that of the blood. The material is then stored at a low temperature for several months before filtration, to allow for the slow secondary precipitation which follows the addition of preservative.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

If desirable for experimental or other purposes, it is possible, by the fractional precipitation of pseudoglobulin to obtain the major part of the antitoxin in a more concentrated form. Experiments have shown that the fraction precipitated between 30 and 33 per cent of saturation with $(NH_4)_2SO_4$ constitutes only about 20 per cent of the total final product and yields antitoxin with a low titer. The second fraction, precipitated between 33 and 48 per cent of saturation, yields antitoxin with a higher titer than that obtained by the usual method. The increase in concentration has, however, not been considered sufficient to compensate, in routine production, for the greater total loss. Further studies relating to purification are in progress.

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[1] At present antistreptococcus plasma (scarlet fever) is being concentrated by the same method, except that the plasma after the first precipitation is heated to 57 instead of 60°C. Further studies are being made to determine the optimum precipitation limits and method of heating.

*Preliminary Treatment of Plasma*

Plasma when received from the farm is stored in the cold room until concentration is commenced, usually within a month. One lot of about sixty liters, representing about ten bleedings, is generally started each week. (Concentration of different kinds of antitoxic plasma is always commenced at different times.) The plasma is pooled in accordance with instructions received from the production group on a numbered order slip; the number being used to designate the lot until after filtration (Order No. 523). The hydrogen-ion concentration of the pooled material is adjusted immediately before the first precipitation to insure more rapid filtration. A sample of each lot of pooled diphtheria antitoxic plasma is taken for potency tests to determine the "unit concentration" and loss. These tests are made by the intracutaneous method. At least one sample of tetanus antitoxic plasma is taken each month.

For experimental and other purposes, bleedings may be concentrated separately. The procedure is the same as with pooled plasma.

**Receipt of Plasma.**—The citrated plasma (final concentration of citrate 1.7 per cent ($(Na_3C_6H_5O_7)_2 \cdot 11H_2O$) in 8-liter green-glass bottles, packed in special wooden boxes, is received by the concentration group. A tag on each bottle gives the kind of plasma, volume, horse and bleeding numbers, and date of bleeding. A duplicate memorandum of shipment giving horse and bleeding numbers, and volumes of the different bleedings, accompanies each shipment.

Upon receipt of plasma, verify and sign the memorandum of shipment and file with the production group. Store the plasma at once in the cold room, keeping each kind separate and apart from other material.

When plasma is taken from the cold room for concentration, and when the concentrated product is returned, an initialed duplicating order form, giving date, kind, number, and amount of material, is sent to the production group.

**Pooling of Plasma.**—Measure the volume of each bleeding in a graduated precipitating jar, and enter in the record book. Pool the bleedings in a 20-gallon crock, rinsing the bottles with a small volume of water, which is then added to the pooled plasma. Mix the plasma thoroughly by stirring. When the lot is to be tested for potency, remove 5 cc. before proceeding to the adjustment of the hydrogen-ion concentration.

**Adjustment of Hydrogen-Ion Concentration.**—The hydrogen-ion

concentration of the plasma is adjusted to pH 8 (Sörensen's notation) by the colorimetric method.

Special supplies and apparatus: Standard buffer solutions, pH values 7.2 to 7.4, phenol-red indicator, comparator block (for preparation of buffers and indicator and use of comparator, see "Determination of Hydrogen Ions," p. 404, roughly standardized solution of N/1 $NH_4OH$, and a N/10 solution prepared from it; 50-cc. burette; and test tubes (25 by 13 mm.).

Pipette 10 cc. of the pooled plasma into each of two test tubes and to one tube add 0.5 cc. phenol-red solution. Prepare two tubes con-

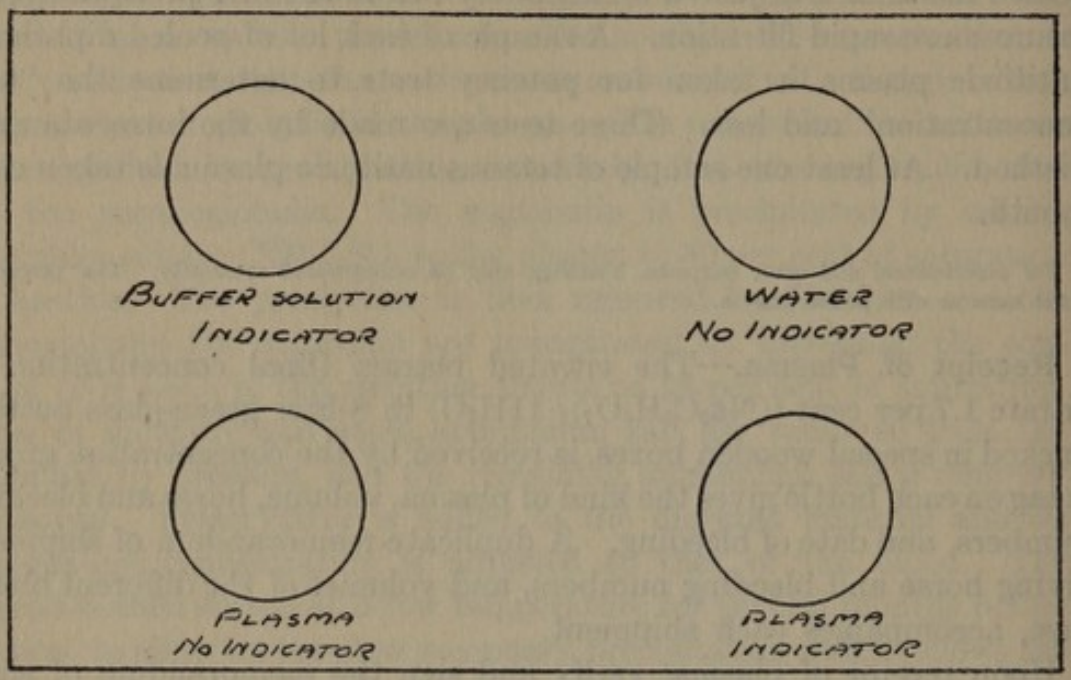


FIG. 41. POSITION OF TUBES IN COMPARATOR BLOCK

taining 10 cc. of buffer solutions, pH 7.2 and pH 7.4 respectively, and add 0.5 cc. phenol red to each. Place the 7.2-buffer tube, the plasma tubes, and a tube containing water in the comparator in the relative positions shown in figure 41.

From a burette add N/10 $NH_4OH$ drop by drop to the tube of plasma and indicator, shaking well after each addition and comparing the colors frequently. Continue until the color on the right side is slightly darker than that on the left, indicating that the pH of the plasma is greater than 7.2. Replace the 7.2-buffer tube with the 7.4, and again compare the colors. The right-hand color should now be lighter. If this is the case the pH of the plasma approximates 7.3 colorimetrically which is actually equivalent to the desired pH 8 because of the protein error of the indicator. From the volume of

N/10 $NH_4OH$ (usually from 0.4 to 0.8 cc.) used to adjust the plasma in the tube, calculate the volume of N/1 $NH_4OH$ necessary to change the whole volume of plasma as follows:

$$\text{Vol. of N/1 } NH_4OH = \frac{\text{Vol. of N/10 } NH_4OH}{10} \times \frac{\text{Vol. of plasma}}{10}$$

Add the calculated amount of N/1 $NH_4OH$ to the plasma slowly while stirring, and redetermine the pH, using the comparator. Should the adjusted plasma still be too acid, determine the additional volume of N/1 $NH_4OH$ required by titrating with N/10 $NH_4OH$ as above.

### *First Precipitation and Filtration*

The euglobulins and fibrinogen of the plasma are precipitated by adding $(NH_4)_2SO_4$ to 30 per cent of saturation and heating.[2] The heat also converts a small part of the pseudoglobulin into an insoluble state.

Test for optimum ammonium-sulfate concentration: While 30 per cent of saturation of ammonium sulfate is used as a routine procedure, it may occasionally be desirable to determine the optimum concentration for a given lot of plasma.

Pipette into each of three test tubes 4 cc. of plasma. Add 2 cc. of distilled water and enough saturated solution of $(NH_4)_2SO_4$ to make 28, 29, and 30 per cent of saturation (and additional percentages as desirable) determined by use of the following formula.

$$X = \frac{CV}{100 - C}$$

C = desired concentration
V = volume of diluted plasma
X = volume of saturated $(NH_4)_2SO_4$ solution

Shake the test tubes containing the precipitate, and filter the contents through soft filter paper. To the filtrate add two volumes of distilled water. Saturate with sodium chloride by adding an excess of salt and shaking well. Allow the salt to settle and note the appearance of the supernatant fluid.

The slight precipitate in suspension is euglobulin that has not been thrown down by the ammonium sulfate. A precipitate that permits the reading of ordinary type through the solution is regarded as satisfactory. It is generally considered better to allow some euglobulin to remain unprecipitated than to run the risk of precipitating pseudoglobulin.

Special supplies and apparatus. Bottles: Approximately sixty 8-liter flint-glass bottles; half of them graduated at 5500 cc.

Water-bath: An insulated copper water-bath, made from special design by Bramhall Deane Company, New York City. The inside dimensions are 2½ by

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[2] From October 23, 1918 to May 1, 1919, 0.35 per cent cresol was added to the plasma before precipitation. The correct amount of cresol was shaken with about 100 cc. of water until emulsified, and the emulsion then added slowly to the plasma with stirring (electric stirrer). This procedure improved the filtration but apparently increased the loss.

5 feet. The bottles rest on perforated metal trays raised 3 inches from the bottom, which permits the free circulation of water. The bath is filled and emptied by permanent water connections and is heated either by steam or gas. (See, plate IV.)

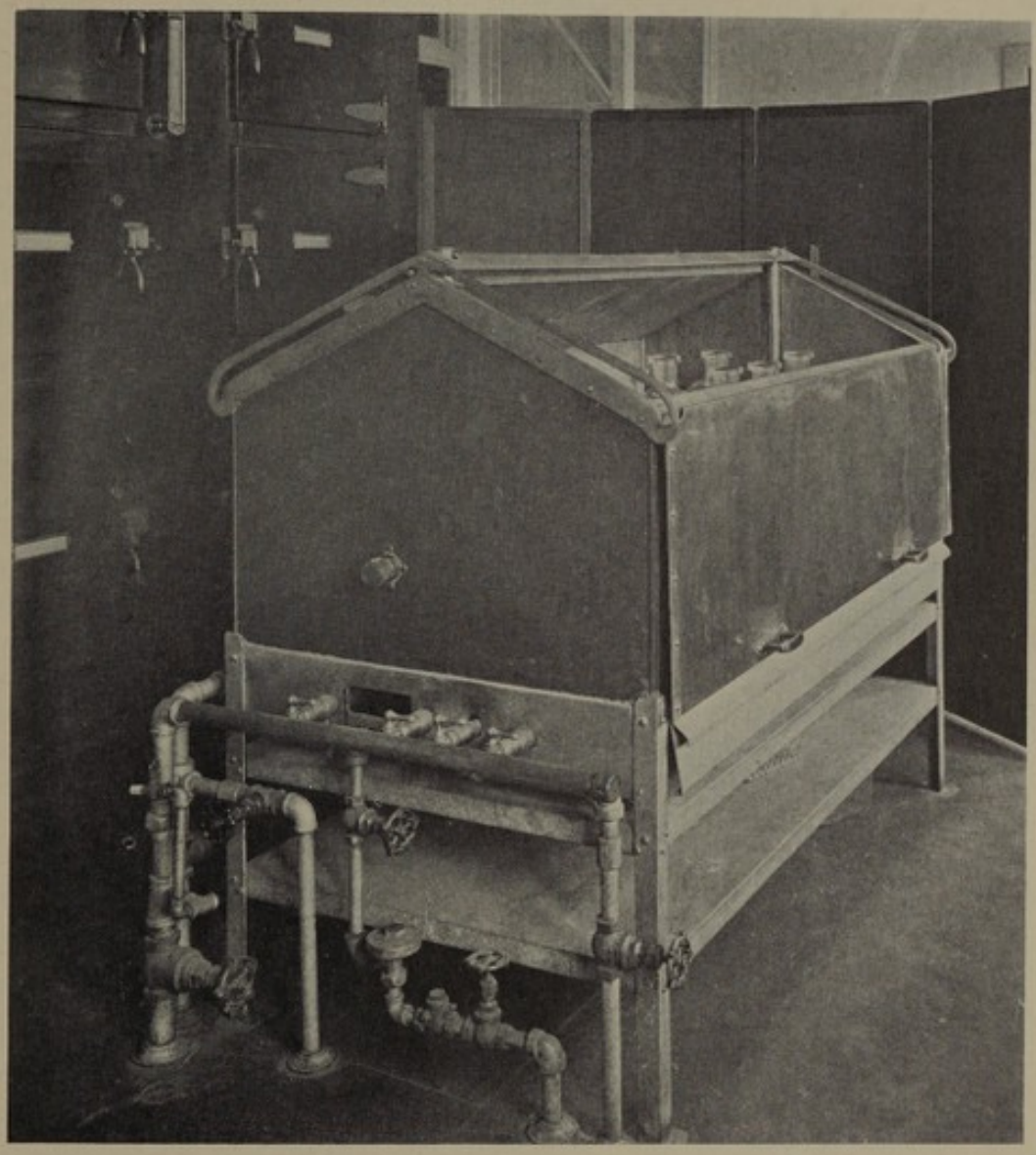
Saturated ammonium-sulfate solution: To prepare the solution place 30 kg. of powdered $(NH_4)_2SO_4$ (C.P.) in a 15-gallon crock. Add fifteen liters of tap water at from 70 to 80°C. Stir continuously for twenty minutes, using an electric stirrer with wooden paddle. Then add ten liters of water at about 80°C. and stir again for twenty minutes. Cool to room temperature. Determine the specific gravity with a hydrometer; if below 1.245, the saturation point of ammonium sulfate, raise the temperature by heating a part of the solution to boiling in an enamel pail and mixing with the remainder. Stir the residue from the bottom of the crock. Allow the solution to cool. Redetermine the specific gravity. After cooling, filter the saturated solution through soft filter paper into properly labeled bottles for future use. Cover the crock carefully to preserve the residue.

After several solutions have been made there will be considerable residue present, each liter of which may be considered as equivalent to a half kilogram of dry salt in the preparation of new solutions.

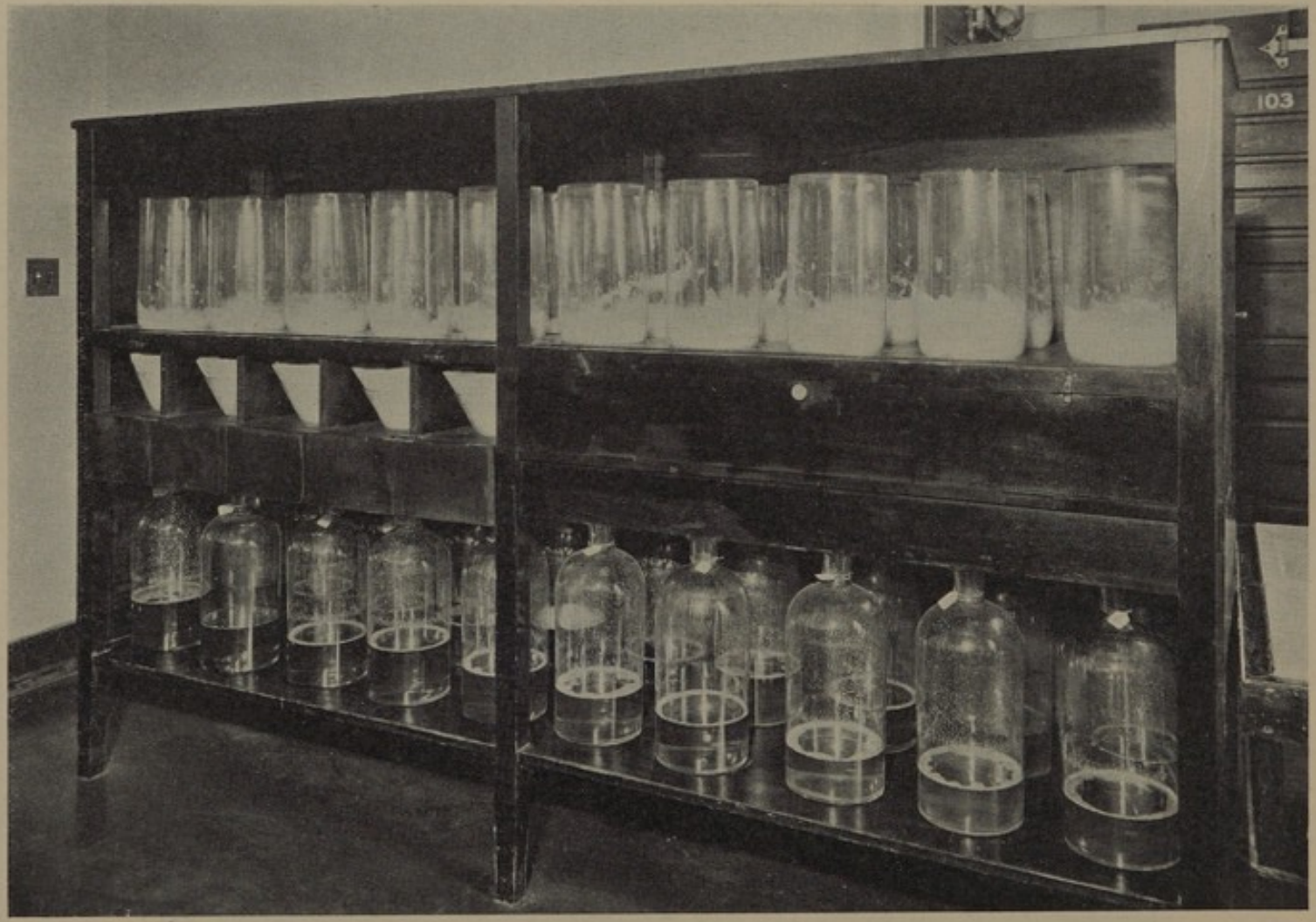
**Addition of Ammonium Sulfate.**—Distribute the adjusted plasma in 3-liter amounts in 8-liter bottles previously examined and found free from cracks. Tag each bottle, giving the kind of antitoxin and order number. Dilute the plasma with half its volume of tap water, add the required volume of saturated $(NH_4)_2SO_4$ solution (1928 cc. to 4500 cc. diluted plasma), and shake thoroughly.

**Heating of Salted Plasma.**—Place the bottles of plasma in the water-bath, and fill the bath with water to the level of the plasma in the bottles. Bring the temperature rapidly to 45°C. Then heat gradually by turning on the steam for a few minutes at a time so that the temperature of the plasma is raised to 50°C. in two hours, then from 50 to 59°C. in one and three-quarter hours, and finally from 59 to 60°C. in fifteen minutes. Hold at 60°C. for five minutes.[3] Keep the temperature of the water from 2 to 4°C. above that desired for the plasma until the latter reaches 57°C., then hold the water at 61.5°C. Never let the temperature of the water go above this point. Stir the plasma every fifteen minutes until the temperature reaches 57°C. and then every five minutes. Stir the water at the same intervals. Use for stirring heavy glass rods, each with a piece of soft rubber tubing on the end. After each stirring take the temperature and record on a tag

[3] In 1918, the following method of heating to 63°C. was tried: The temperature of the plasma was raised to 57°C. in about two hours, then to 59°C. in one hour, to 61°C. in thirty minutes, and to 63°C. in twenty minutes. In 1922, the same method, but with a final temperature of only 62°C., was used. These methods apparently increased the loss without improving the concentration.



WATER-BATH




FILTER RACKS

attached to the bath. When the heating is completed, remove the plasma, cool to room temperature, and filter.

If steam is not available gas may be used and the temperatures properly controlled by careful regulation of the supply.

**Filtration.**—By filtering through soft filter paper, the euglobulin, fibrinogen, and other proteins contained in the precipitates, are separated from the pseudoglobulin and albumin held in solution. Filtration is carried on in special filtering racks which permit it to proceed automatically. Different lots of plasma vary somewhat in the time required for filtration, but six liters should filter within eight hours. It is usually convenient to commence filtration in the late afternoon and to allow the filters to remain in place until the following morning. The filtrate should be clear and of a slightly greenish or brownish tint. If material is not clear, it should be refiltered through soft filter paper. Refiltration requires from three to four hours.

Special supplies and apparatus. Funnels: Heavy ribbed funnels, 21.5 cm. outside diameter.

Filter paper: Soft, round filter paper, 450 mm. in diameter. Eaton and Dykeman No. 615.

Filter racks: The filter racks are constructed on the principles of the bottled-water stand. They consist of a removable top shelf, a second shelf 6 inches lower, and a third 22 inches below the second. Circular openings in the top shelf, 6½ inches in diameter, are directly above openings in the second, 3½ inches in diameter. The funnels are protected in front by hinged doors. (See: plate V.)

Place the funnels containing fluted filter paper in the openings of the second shelf of the filter rack and adjust the top shelf. Under each funnel place a graduated 8-liter bottle and transfer the tags from the bottles containing material to be filtered to the empty bottles. Place the full bottle on top of the rack and grasp the upper part of it with the palms of both hands, the fingers pointing upward. Without changing the position of the hands, invert the bottle with a continuous and fairly rapid movement outward and over the funnel until the bottle rests on the rim of the upper opening. Filtration should now proceed automatically.

Should a paper become clogged and filtration stop, remove the bottle of filtered material from under the funnel and replace it by the bottle of unfiltered material. Make a hole in the filter with a glass rod, thus allowing the liquid contents to run into the bottle below. Remove the solid material, including the paper, to a beaker. Place new paper in the funnel and replace the bottles in their original positions, when filtration will proceed as at the beginning of the process. Keep the first filter paper and wash with the new paper and contents when filtration is completed.

**Washing of Precipitate.**—When filtration has ceased, wash the precipitate to remove any adhering pseudoglobulin. Remove a funnel from the rack and drop the filter paper and contents into a 3-liter precipitating jar. Add about one liter of $(NH_4)_2SO_4$ solution of a concentration 2 per cent higher than that used in the precipitation (32 per cent of saturation). Macerate the paper thoroughly with a rubber spatula and filter the material through a fresh filter paper into the original bottle of filtrate. Repeat the process for each funnel. Discard the precipitate when drained.

*Second Precipitation and Filtration*

The pseudoglobulin, containing antitoxin, is precipitated from the filtrate by 48 per cent of saturation[4] with $(NH_4)_2SO_4$, and separated by filtration from the albumin, which remains in solution.

To calculate the required volume of ammonium sulfate to give the desired saturation, the following formula is used:

$$X = \frac{V\,(C_2 - C_1)}{100 - C_2}$$

$X$ = volume of saturated $(NH_4)_2SO_4$ solution to be added to bring solution to required concentration
$V$ = volume of original solution
$C_1$ = initial concentration
$C_2$ = desired or final concentration

Hard filter paper, 500 mm. in diameter (Whatman No. 50) is used for filtration. This paper may be washed with water and used repeatedly.

Dispense the first filtrate in measured volumes, not exceeding 5500 cc. in 8-liter bottles, and transfer the tags. Add to each bottle, while stirring the filtrate, the required volumes of saturated $(NH_4)_2SO_4$ solution (1904 cc. to 5500 cc. of filtrate, if 30 per cent of saturation was used in the first precipitation), and shake thoroughly. Allow the mixture to stand at least two hours at room temperature. After again shaking at the end of this period, pour one-half of the contents of each bottle into a second bottle. Filter the mixture, as previously described, through fluted hard filter paper. (It is well to pour a little of the material into the funnel before inverting the bottle.) If the first filtrate that comes through is cloudy, return it to the original bottle and then invert the bottle. As the bottles empty, after about two to

[4] Prior to May 3, 1923, 50 per cent of saturation with ammonium sulfate was used for the second precipitation but experiments indicated that the antitoxic content of the fraction precipitated above 46 to 48 per cent was negligible.

three days, remove them and cover the funnels with a sheet of soft filter paper. Time may be saved by watching the filters and transferring material from a slow filter to one which has emptied more rapidly.

*Drying of Second Precipitate*

The excess of moisture in the precipitate containing pseudoglobulin and antitoxin, as well as some of the $(NH_4)_2SO_4$, is removed by means of filter paper and pressure. Usually from two to four hours are required for preparing the precipitate for pressing. To obtain the best results, pressing should not take over thirty hours. The work should be so planned that the material is ready for pressing by noon. It can then be pressed during the afternoon, left in the press overnight, and the pressing completed the next day.

Special apparatus and supplies. Absorbent papers: Soft filter paper 50 by 50 centimeters. With care the paper may be used repeatedly.

Muslin cloths: Heavy muslin cloths, 50 by 50 centimeters, with hemmed edges.

Boards for stacking: Hardwood boards, 18 by 18 inches by 1 inch thick.

Press: Cider press No. 4, manufactured by the Ames Plow Company, Boston, with the tub removed.

Drier for absorbent papers: At present a steam drier is used for this purpose.

**Preparation for Pressing.**—The object of this step is to work the precipitate into a consistency and shape suitable for pressing and at the same time to remove a part of the ammonium sulfate.

When the precipitates are drained, remove each filter paper with the material and lay it on a pad of about twenty-five sheets of absorbent paper. Remove the tags from the bottles to the top absorbent papers. Divide the precipitate of each third paper between the preceding two. Work the precipitates into flat disks, using a rubber spatula. Rework after about one hour and again if necessary. Take care to form perfectly flat disks of uniform diameter with sharp edges, otherwise difficulty will be experienced in pressing.

**Pressing.**—When sufficiently dry, the precipitates are placed in the press between layers of absorbent paper where most of the remaining moisture and some of the ammonium sulfate are removed.

When the material has dried to the consistency of vaseline, place the precipitates face to face in pairs. Place a board on the floor of the press and pile the precipitate on it between pads of about twenty-five sheets of absorbent papers. Use great care to keep the precipitates level, if necessary adding but five pairs at a time and applying moderate pressure after each addition. Lay a long strip of brown

paper, giving the kind of material and order number, beside each precipitate. Tighten the press from time to time during the first day's pressing, and leave the precipitates in the press overnight. On the following morning replace the hard filter papers with muslin cloths, to prevent breaking the papers as the pressure is increased. Work with one precipitate at a time. Place the papers with a precipitate between upon the table. Carefully peel off the top paper, removing with a rubber spatula any particles adhering to it. Place a single layer of muslin over the precipitate. Grasping the cloth and filter paper at their outer edges, invert the precipitate. Replace the second filter paper by a cloth in the same way. When all the hard filter papers have been replaced, return the precipitates, between fresh pads, to the press, adding five at a time and applying moderate pressure after each addition. Continue increasing the pressure about every hour until practically no more moisture is absorbed. The material is then ready for dialysis.

The degree of dilution during dialysis depends on the amount of ammonium sulfate left in the precipitate after pressing. If too little dilution occurs, as shown by a high protein content in the dialyzed material of successive lots, the absorbent papers may be changed less frequently. If, on the other hand, too great dilution occurs, it may be advisable to scrape away from time to time during drying, the crystallized salt formed around the edge of the precipitate.

### *Removal of Ammonium Sulfate by Dialysis*

The pseudoglobulin and antitoxin are freed from the ammonium salts by dialysis. Dialysis should be continued until not more than 0.2 per cent of salt remains, which usually requires nine days.

Special supplies and apparatus. Parchment for dialyzing bags: Extra heavy grade of parchment from Reeve Angel Company. A satisfactory grade is essential. Sheets 90 by 90 centimeters preferred. After use, the bags are scrubbed with a soft brush using Ivory soap, rinsed in water, and dried. With proper care, they may be used repeatedly.

Dialyzing bath: An alberene bath, 5 by 2 by 1 foot, with drain board, is built into the dialyzing room, which is kept at approximately 5°C. Tap water cooled to 5°C. enters near each end of the bath and escapes through an overflow. Crossbars of galvanized iron piping are placed parallel to the long sides of the bath.

**Preparation for Dialysis.**—Examine carefully each sheet of dry parchment for holes, by holding in front of a strong light. Soften the parchment by soaking in tap water, then lay it on a flat surface. Remove the upper muslin cloth from one of the precipitates. With a rubber spatula scrape away and discard all salt formed around the

edges. Loosen and scrape the precipitate on to the parchment paper. Gather the edges of the paper together to form a bag and tie with a strong cord. Attach a small tag stating the kind of antitoxin. order number, and the date dialysis is commenced. Repeat the procedure with each precipitate.

**Dialysis.**—Adjust the streams of water to give a slow but even flow. Tie the bags to the crossbars so that the bags are one-third submerged, and dialyze for nine days. If different lots are in the dialyzing bath at the same time, attach the bags of each lot to a separate crossbar. Clean the dialyzing bath once a week with Ivory soap.

Should the temperature of the bath ever rise to 15°C., dip the bags in a jar of 1.5-per-cent cresol each day, and add 2 cc. of chloroform to each bag on the third and the sixth day of dialysis.

While tests have shown that nine days are sufficient to remove practically all the ammonium sulfate, it is advisable occasionally to ascertain the efficiency of dialysis by determining the percentage of ammonium salt in the dialyzed material.

Transfer 1 cc. of the dialyzed material to a 100-cc. beaker and add 10 cc. of 2.5-per-cent trichloracetic acid to precipitate the protein. Filter the mixture through hard filter paper and wash with 10 cc. of 2.5-per-cent trichloracetic acid. Make up to about 200 cc. with ammonia-free water and add 10 cc. of a boiled solution of 10-per-cent NaOH. Distill for thirty minutes, collecting the distillate in 10 cc. of N/10 HCl. Titrate against N/10 alkali, using methyl orange as an indicator.

### *Neutralization of Antitoxin and Addition of Preservative*

The concentrated antitoxin is neutralized and adjusted to pH 7.4 by the addition of sodium carbonate ($Na_2CO_3$). Cresol is added as a preservative to give a final concentration of 0.3 per cent. In order to reduce the precipitation of protein by the cresol, the sodium-carbonate solution and cresol are mixed and added to the antitoxin together.

When concentrated material is being handled, the dialyzing bags, bottles, measuring flasks, and other containers are rinsed with distilled water and the rinsings added to a bottle labeled "Chemist's washings—diphtheria" (or tetanus), and stored in the cold room. The "washings" and residues from filtrations (see "Filtration of Biologic Products," p. 519), and the empty bottles (with tags) from which antitoxins have been dispensed, and the residues from fillings (see "Preparation of Biologic Products for Distribution," p. 534) are received from the filtration and filling groups. The bottles are rinsed and the rinsings pooled with the washings and residues. After sufficient material for a concentration (usually 40 to 60 liters) has accumulated, the production group is notified. When instructions are received, the washings and residues, together with returned outdated antitoxin, are reconcentrated by the procedure used for plasma, except that 33 per cent of saturation with ammonium sulfate is substituted in the first precipitation.

Special supplies and apparatus. Sodium carbonate: C.P. monohydrated salt is dissolved in sufficient water to form an 18.5-per-cent solution.

Container for pooling: Grey agate stock pot with two handles; 32-liter capacity.

Electric stirrer: This consists of a paddle driven by a 1/20 H.P. electric motor, at a speed of approximately 1200 R.P.M.

Pool the antitoxin from the dialyzing bags and measure. Add to the pooled antitoxin (usually about 10 to 12 liters) enough NaCl to give an 0.8-per-cent solution. To determine the volume of 18.5-per-cent solution of $Na_2CO_3$ required to adjust the reaction to 7.4, follow the directions given under "Adjustment of Hydrogen-Ion Concentration," p. 385, making the following changes:

1. Adjust the antitoxin to 7.3 colorimetrically, which is approximately equal to 7.4 electrometrically.
2. Use for a sample 2 cc. of antitoxin diluted with 8 cc. of an 0.8-per-cent salt solution. Dilute the buffer solution in a similar manner.
3. Use a solution obtained by diluting 5 cc. of 18.5-per-cent $Na_2CO_3$ to 100 cc. in place of the N/10 $NH_4OH$ solution.
4. Substitute 18.5-per-cent $Na_2CO_3$ for N/1 $NH_4OH$.
5. Use the formula:

$$\text{Vol. of 18.5-per-cent } Na_2CO_3 = \frac{\text{Vol. diluted } Na_2CO_3}{20} \times \frac{\text{Vol. antitoxin}}{2}$$

Calculate also the amount of cresol and emulsify it in the sodium-carbonate solution in a separatory funnel. Add the emulsion to the antitoxin in a small stream, stirring with an electric stirrer in such a way as to cause as little agitation as possible. Rinse the funnel with 5 cc. of distilled water and add while stirring. Determine the pH of the adjusted material. Pour the material into sterile, green-glass bottles (8 or 4 liter).

Close each bottle with a rubber stopper and attach a tag giving the kind of antitoxin, order and bottle number, date, volume of lot, and volume in bottle. Store in the cold room. Make out a duplicate memorandum slip for the production group giving the kind, order number, total volume, and date protein content will be adjusted.

### *Adjustment of Protein Content*

Determine the protein content of each lot of antitoxin and if above 19 per cent, adjust.

After the material has stood a month siphon off the supernatant fluid into the stock pot and place the residue which has settled to the bottom

of the bottle, with the "chemist's washings." Proceed to the determination of the protein content.

**Nephelometric Estimation of Protein in Antitoxin.**[5]—The nephelometric estimation of the protein is based on the comparison of the precipitate produced, by sulfo-salicylic acid, in a dilute solution of antitoxin with a similar precipitate formed in a standard solution of approximately the same protein content. A 1:2000-dilution of the antitoxin to be tested is made. Since the protein content of concentrated antitoxin is in the neighborhood of 20 per cent, this solution will contain approximately 0.01 per cent protein. The standard solution prepared from dry antitoxic plasma is made to contain exactly 0.01 per cent protein. Sulfo-salicylic acid is added to each and the resulting clouds compared in the nephelometer.

1. Preparation of standard solution: Pass some concentrated antitoxin to which no cresol or $Na_2CO_3$ has been added, through infusorial earth to remove shreds of filter paper or other foreign matter. Place the filtrate in a shallow dish in the vacuum oven and hold at 40°C. for two days. Grind in a mortar, then return to the vacuum oven. When the moisture content is below 1 per cent, place in a desiccator over $CaCl_2$. The powdered pseudoglobulin will keep indefinitely.

If a vacuum oven is not available, the antitoxin may be dried by pervaporation, that is, by placing the material in a parchment or collodion bag, which is then hung in a warm room and fanned with an electric fan.[6]

Weigh out 0.1 gram of the pseudoglobulin in a tared beaker. Add a few drops of water to make a paste, then 2 cc. of N/20 NaOH, and stir at intervals for about one hour. Transfer the material to a 100-cc. volumetric flask by rinsing the beaker several times with small quantities of distilled water. Add 5 cc. of a saturated solution of cresol and make up to the 100-cc. mark with distilled water.

This gives a 0.1-per-cent standard solution of protein which is reliable for three days. The solution is further diluted just before use.

2. Preparation of solution of antitoxin: With an accurate pipette, transfer 1 cc. of the antitoxin to be tested to a 100-cc. volumetric flask. Add two drops of a strong sodium-carbonate solution and make up to the 100-cc. mark with distilled water.

This solution is also further diluted just before use.

[5] The procedure is described by Kober, P. A., Jour. Biol. Chem., 1917, 29, 155 (130); Indust. & Engin. Chem., 1918, 10, 556 (131).

[6] For the method developed in this laboratory, see: Kober, P. A., Jour. Amer. Chem. Soc., 1917, 39, 944 (132).

3. Final dilution of solutions and addition of reagent: Dilute 10 cc. of the standard to 100 cc. in a volumetric flask. This gives a 0.01-per-cent solution. Dilute 5 cc. of the antitoxin solution to 100 cc. This gives an approximately 0.01-per-cent protein solution. Pipette 10 cc. of each solution into separate 250-cc. Erlenmeyer flasks and add 20 cc. of a 6-per-cent solution of sulfo-salicylic acid (C.P.) to each. Mix thoroughly by gentle rotation and allow to stand ten minutes.

4. Adjustment and use of the nephelometer: Place a cup on the stage and raise the stage until the bottom of the plunger just touches the bottom of the cup. Release the thumb screw on the front of the instrument and raise or lower the scale by means of the micrometer screw until the reading is zero. Repeat this on the other side of the instrument. Remove the cups, rinse each with a little of the standard solution, and then fill each about two-thirds full with this solution. Place the cups in the instrument, set the left side at 20 on the scale, and adjust the height of the right until the fields match. Take the reading. Remove the right-hand cup and rinse it with the unknown solution, then fill two-thirds full with this solution and replace in the instrument. Match the fields and take the reading.

When solutions are of nearly the same concentration the ratio between the strength of the unknown and that of the standard solution may be considered as inversely proportional to the nephelometric readings. If these readings differ by more than 15 per cent, the nephelometric formula (130) should be used. Since the solutions were so made that the standard is equivalent to 20 per cent protein in the undiluted antitoxin, this inverse ratio multiplied by 20 gives the percentage of protein in the antitoxin.

Example:

Average reading with standard = 19.8

Average reading with unknown = 21.6

$$20 \times \frac{19.8}{21.6} = 18.3 \text{ per cent protein.}$$

If the protein content is above 19 per cent, the antitoxin must be diluted with distilled water containing 0.8 per cent NaCl and 0.3 per cent cresol.

Formula:

$$\text{Final volume} = \frac{\text{per cent protein found} \times \text{first volume}}{19}$$

Final volume − first volume = volume of diluent required.

After dilution, redetermine the protein content with the nephelometer, using the same technic as in the first determination. Before

returning the antitoxin to bottles, remove about 3 cc. and send to the production group to be tested by the intracutaneous method. Enter on the tags the date, revised volume, and the protein content, and transfer them to the new bottles. Store the material in the cold room until required for filtration, which is carried on under the supervision of the production group.

*Dilution of Concentrated Antitoxin to a Desired Titer*

When it is necessary to dilute concentrated antitoxin (see "Production and Standardization of Diphtheria Antitoxin," p. 346), an order stating the kind, order number and titer of the material to be diluted,

| ORDER NUMBER | HORSE BLEEDING DATE | FIRST PRECIPITATION | | | | SECOND PRECIPITATION | | | DIALYSIS | | VOLUME AFTER DIALYSIS | TOTAL PROTEIN | FINAL VOLUME | NUMBER UNITS | | UNIT CONCENTRATION | PER CENT LOSS | PRODUCT NO. |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Date commenced | Volume plasma | Volume water | Volume $(NH_4)_2SO_4$ saturated | Total first filtrate | Volume $(NH_4)_2SO_4$ saturated | Date completed | Date commenced | Date completed | | | | Plasma | Antitoxin | | | |
| | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | | | | |

FIG. 42. CONCENTRATION OF ANTITOXIN RECORD (FRONT)

and the desired titer, is received from the production group. The material is diluted with cresolized salt solution (0.8 per cent salt and 0.3 per cent cresol) in accordance with the following formula:

$$\text{Final volume} = \frac{\text{first volume} \times \text{titer}}{\text{desired titer}}$$

Final volume − first volume = volume of diluent required.

Pool the concentrated antitoxin. Stir mechanically while adding the required volume of diluent. Enter "Diluted" and the final volume on the tags; the necessary calculations and data on the reverse side of the concentration record for the corresponding order number.

### *Determination of "Unit Concentration" and Loss in Total Number of Units*

The efficiency of the process of concentration is followed by frequent determinations of "unit concentration" (increase in potency due to concentration of the antitoxic plasma) and loss in total number of units. In any effort to improve the method both these factors must be taken into account.

Reports on the titer of selected lots before concentration and when concentration is completed, should be received from the production

| ADJUSTMENT OF pH VALUE OF PLASMA | | | | ADJUSTMENT OF pH VALUE OF ANTITOXIN | | | | | NEPHELOMETRIC ESTIMATION OF PROTEIN | | | | | REMARKS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Order number | Volume plasma | Volume N/1 $NH_4OH$ | Final pH | Volume antitoxin | Volume $Na_2CO_3$ | Volume cresol | Grams Na Cl | Final pH | Standard reading | Unknown reading | Per cent protein | Volume cresol solution | Final volume | |
| | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |

FIG. 43. CONCENTRATION OF ANTITOXIN RECORD (BACK)

group within two weeks of the time the protein is adjusted. To determine the "unit concentration" the units per cubic centimeter of the concentrated material are divided by the units per cubic centimeter of the plasma. To determine the loss in total units during the process of concentration, the total number of units in the concentrated material is subtracted from the total number in the plasma.

### *Permanent Records*

A complete record of each lot or batch of material is kept in a loose-leaf printed record book (8½ by 11 in. pages). The following entries are made on the front of the sheet (See: fig. 42): horse and bleeding numbers, order number, volume of plasma, volume of water added, volumes of saturated ammonium sulfate for first and second precipitations, date of completion of second precipitation, dates dialysis commenced and finished, volume after dialysis, final protein content, number of units in plasma (if determined) and in concentrated antitoxin, per

cent loss, and product number. On the back of the sheet are recorded data relating to the adjustment of the hydrogen-ion concentration of the plasma and of the final product, and nephelometric protein estimations. A blank space is reserved for all calculations relating to each lot. Each computation is dated and initialed by the person making it and checked and initialed by a second person. When a lot of antitoxin is diluted the antitoxin number, date, volume, calculations, final volume, and initials of the workers are entered on the reverse side of the concentration record of the corresponding order number. Data with special reference to research work may also be recorded on the back of the sheet, see figure 43.

## CHAPTER 2

## DETERMINATION OF HYDROGEN IONS

Two methods are used for determining hydrogen ions; the electrometric method for standardization and for special problems, and the colorimetric method for routine work. The electrometric determinations are at present carried on by workers in the concentration group, who also prepare the standard buffer solutions for use in colorimetric tests made by various other groups.

Hydrogen-ion determinations have become a part of the routine procedures of concentrating antitoxins and adjusting culture media. They are also used in any special investigations.

### THE ELECTROMETRIC METHOD

The electrometric method is based on the fact that when hydrogen ions come in contact with a platinum electrode coated with platinum black and saturated with hydrogen gas, an electromotive force is set up. This is measured by comparison with a calomel electrode of known value, the E.M.F. of the combination being determined by means of a potentiometer.

#### *The Electrometric Chain*

In this laboratory the following chain is used:

| Hg/HgCl·KCl (sat.) | / | KCl (sat.) | / | Solution under test | /$H_2$Pt |
|---|---|---|---|---|---|
| (Calomel electrode) | | (Connecting solution) | | (Hydrogen electrode) | |

The mercury is purified by treatment with mercurous nitrate and subsequent distillation in a Hulett still. The calomel is prepared by treating a solution of recrystallized mercurous nitrate with redistilled hydrochloric acid. The saturated potassium-chloride solution is prepared from C.P. material which has been recrystallized from ammonia-free water. To fill the calomel electrode vessel, add from a pipette 5 cc. of the purified mercury and then 5 cc. of a heavy suspension of calomel in saturated potassium chloride. Finally, fill the remaining portion, including the siphon, with saturated potassium chloride,

taking care that no air bubbles adhere to the wall at any place in the system.

The arrangement of the chain is shown in figure 44.

The hydrogen (over 99½ per cent pure) is supplied from a tank equipped with a pressure regulator. From the regulator it passes through copper tubing which extends to within one foot of the electrode. The copper tubing is connected to the electrode at "A" by a short piece of flexible rubber tubing.

1. Clark hydrogen-electrode vessel, consisting of a glass barrel 9 centimeters long by 1½ centimeters in diameter, having at either end a two-way stopcock (A and C) for the admission and expulsion of gas and liquids; 2, connecting vessel, containing saturated KCl and fitted with a one-way stopcock (E); 3, calomel-electrode vessel, containing mercury, calomel, and saturated KCl and connecting with 2 by means of a one-way stopcock (F).

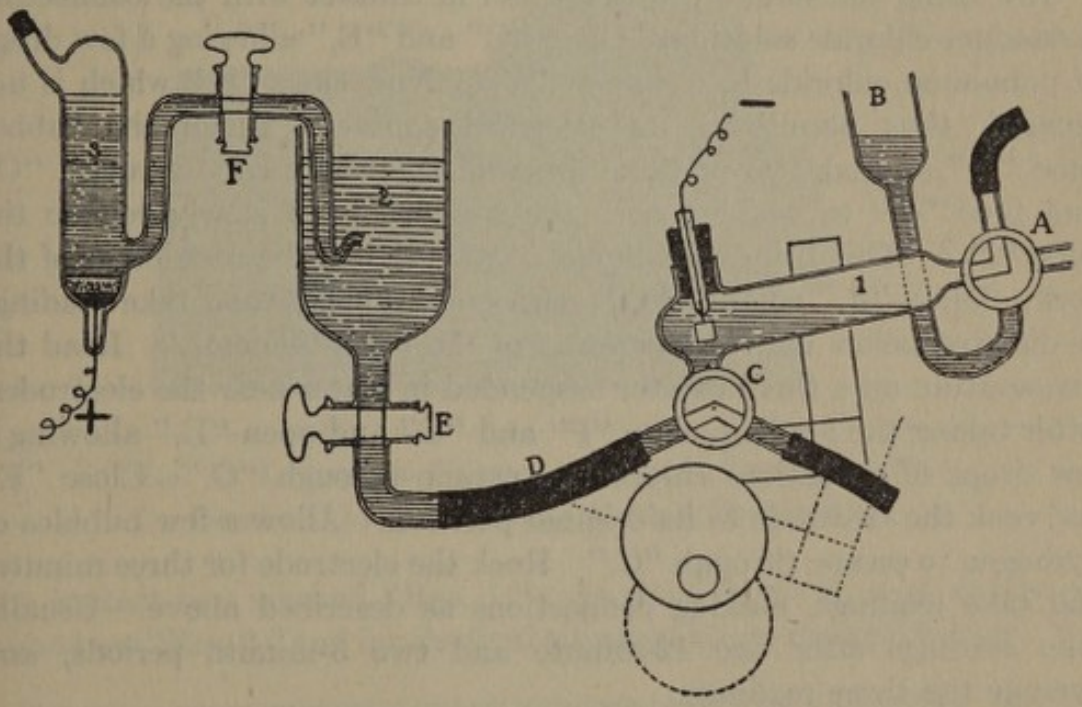



FIG. 44. ELECTROMETRIC CHAIN

*Manipulation of the System*

The system is manipulated as follows: Clean the platinum electrode by making it the cathode in a 5-per-cent solution of sulfuric acid for two minutes, using current from a 4-volt storage battery. Rinse thoroughly with distilled water and platinize by making it the cathode in a 2-per-cent solution of platinum chloride for two minutes. Rinse and repeat the sulfuric-acid treatment. Rinse thoroughly and place in the hydrogen-electrode vessel. Rinse the vessel with 5-cc. ammonia-free water (passing it in at "B" and out at "C"), then with 5

cc. of the solution to be tested. Flood the vessel with hydrogen, passing it in at "A" and out at "C."

Rock the vessel back until cock "A" is at its lowest point. Add a fresh supply (5 cc.) of the solution to be tested, through "B." Open "C" slightly and allow a few bubbles of hydrogen to pass through the solution. Now close "C," leaving "A" open so that the solution will be under a continuous slight pressure of hydrogen.

Rock the vessel for twelve minutes by means of a motor-driven rocker in such a manner that the platinum electrode is alternately immersed in the liquid and exposed to the hydrogen. Bring the vessel to rest in such a position that the platinum electrode is completely immersed.

Now bring the solution in the vessel in contact with the connecting potassium-chloride solution. Open "C" and "E," allowing a few drops of potassium chloride to escape at "C." Now close "E," which is ungreased, thus permitting an electrical contact. Pinch the rubber tube "D" so that two or three drops of solution escape through "C" and turn "C" to connect with the vessel. Then slowly release the tube "D." This brings the liquid contact below the restriction of the cock. Open "F," connect leads with potentiometer and take readings as directed below under "Operation of the Potentiometer." Read the temperature on a thermometer suspended in water near the electrodes. After taking the reading, close "F" and "C" and open "E," allowing a few drops of potassium chloride to escape through "C." Close "E" and rock the electrode to its original position. Allow a few bubbles of hydrogen to escape through "C." Rock the electrode for three minutes and take readings, making connections as described above. Usually take readings after one 12-minute and two 3-minute periods, and average the three readings.

### *The Potentiometer and Accessories*[1]

In the potentiometer method of measuring electromotive force, the unknown E.M.F. is measured by opposing it to a known E.M.F., usually a dry or storage cell, which is checked against a Weston standard cell immediately before each reading.

### *Operation of the Potentiometer*

The potentiometer is shown in figure 45. Connect galvanometer, standard cell, storage cell, and electrodes to indicated binding posts,

[1] For a discussion of the potentiometer see Clark. W. M., The Determination of Hydrogen Ions, 1923, Chapter 14 (133).

which are located at the rear of the potentiometer and are plainly marked. Set standard cell switch "SC" to correspond to the E.M.F. given on the certificate attached to the Weston standard cell. Place the plug located just at the right of the double-throw switch in the hole marked "1.0." Place the double-throw switch at point marked "Std. Cell." Adjust the regulating rheostat located on the right end of the instrument, as follows: Insert plug at $P_1$ when storage battery is fresh and at $P_2$ as it becomes weaker. Now turn the knurled head "R" for coarse adjustment and "R′" for fine adjustment, until the galvanometer shows no deflection. In making the first adjustment,

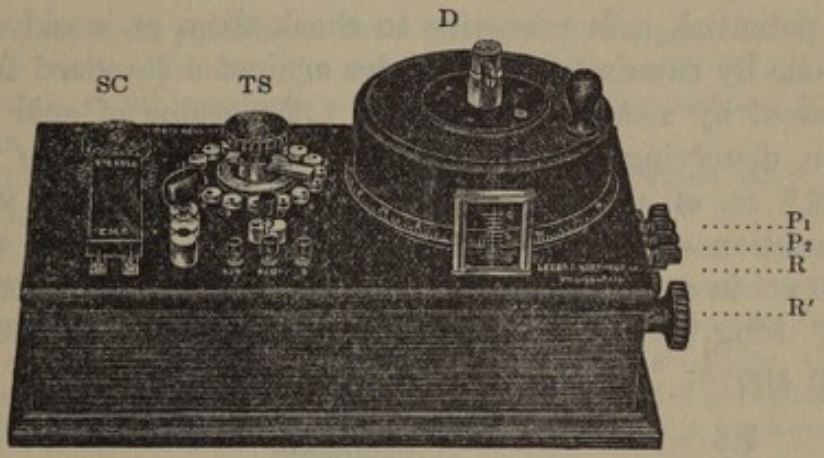



FIG. 45. LEEDS AND NORTHRUP TYPE K POTENTIOMETER
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use contact key marked "Res. 1." As the balance is more nearly obtained use "Res. 2," and for the final adjustment use the key marked "O."

These keys or buttons are located at the front of the instrument and are plainly marked. They make connection with the galvanometer. "Res. 1" and "Res. 2" are connected through resistance which tends to prevent excessive deflection of the galvanometer. "O" makes direct connection.

Next place the double-throw switch in the position marked E.M.F which makes connection with the chain. To obtain the balance for the chain, manipulate the switch "TS" located near the center of the top of the instrument for coarse adjustment, and rotate the contact drum "D" for the final adjustment. Make contact with the galvanometer with the keys "Res. 1," "Res. 2," and "O," as described above. Read the voltage of the chain from the position of the switch and the contact drum.

### *Calculation of pH*

In calculating the pH from the electromotive force, the following formula is used:

$$pH = \frac{E\ (observed) - E\ (calomel\ electrode)}{0.0001983T}$$

where E = electromotive force
T = absolute temperature

### *Checking Calomel Electrode*

As it is very difficult to produce calomel electrodes of absolutely constant potential, it is necessary to check them at weekly intervals. This is done by running the electrodes against a standard buffer solution prepared by accurately weighing 1.361 grams of acid potassium phosphate, dissolving it in about 100 cc. of ammonia-free water and adding 42.8 cc. of a very carefully prepared solution of N/5 NaOH (for preparation see colorimetric method). The volume is then made up to 200 cc. in a calibrated flask. The value of the electrode is obtained by using the following formula, which is a modification of the one given above:

$$7.6 = \frac{E\ (observed) - E\ (calomel\ electrode)}{0.0001983T}$$

The system is also frequently checked against M/20 potassium-acid-phthalate solution as recommended by Clark (134).

It should be noted that certain substances, such as arsenic, ammonia, hydrogen sulfide, and chloroform affect the hydrogen electrode, rendering results unreliable. In this laboratory it has been found that electrometric determinations of arsphenamine solutions can be accurately made if the platinum electrode is cleaned and replatinized after each determination. In determining the pH value of blood, special precautions to prevent loss of $CO_2$ must be taken (135).

## THE COLORIMETRIC METHOD

The colorimetric method is based upon the fact that stable buffer solutions can be prepared covering the whole range of pH values and that when the proper indicators are added to these solutions, a graduated series of colors is obtained. These serve as standards with which to compare unknown solutions, thus determining their pH values.

In this laboratory the following buffer mixtures, recommended by Clark, are used:

| *Buffer Mixture* | *pH Range* |
|---|---|
| Acid potassium phthalate + HCl | 2.2–3.8 |
| Acid potassium phthalate + NaOH | 4.0–5.6 |
| Acid potassium phosphate + NaOH | 5.8–8.0 |
| Boric acid and potassium chloride + NaOH | 8.0–10.0 |

### *Purification of Chemicals and Preparation of Stock Solutions*[2]

The following stock solutions are prepared from which the buffer mixtures are made. The water used in recrystallizing the salts and preparing the solutions is the ammonia-free water prepared in the laboratories for sanitary and analytical chemistry.

M/5 acid potassium phthalate: Use a high-grade salt, prepared especially for hydrogen-ion work. Confirm the purity by titration with M/5 NaOH, using phenolphthalein as an indicator. A fifth-molar solution contains 40.828 grams of the salt in one liter of solution.

M/5 acid potassium phosphate solution: Use a high-grade salt. Recrystallize once from ammonia-free water and dry at 110 to 115°C. for two days. A fifth-molar solution contains 27.232 grams of the salt in one liter of solution.

M/5 boric acid—M/5 potassium chloride: Use acid prepared especially for hydrogen-ion work, and high-grade potassium chloride. Recrystallize the KCl once from ammonia-free water and dry at 120°C. for two days. One liter of solution contains 12.405 grams of boric acid and 14.912 grams of potassium chloride.

M/5 sodium hydroxide solution: Dissolve 100 grams of a high-grade sample in 100 cc. ammonia-free water in a Pyrex flask. Stopper tightly with a rubber stopper and allow to stand for at least one month to permit the carbonate to settle out. Carefully pipette 25 cc. of the clear liquid and dilute to 250 cc. with ammonia-free water. This gives nearly a 2M solution. Withdraw 10 cc. with a pipette and standardize roughly. From this solution prepare two liters of a M/5 solution with as little exposure to the air as possible and place in a paraffined bottle to which a calibrated burette and soda-lime tubes have been attached. Standardize against benzoic acid supplied by the U. S. Bureau of Standards, following the directions accompanying the sample. Use phenolphthalein as an indicator.

M/5 hydrochloric acid solution: Dilute a high-grade hydrochloric acid to about 20 per cent and distill. Dilute to approximately M/5 and standardize against the M/5 NaOH solution.

### *Preparation of Buffer Mixtures*

In preparing the buffer mixtures, measure the salt solutions from calibrated pipettes, the acid and alkali from calibrated burettes. To 50 cc. of a M/5-solution of the buffer salt or salts add the amount of

[2] These directions are taken, with some modification, from Clark, W. M., The Determination of Hydrogen Ions, 1923, Chapter 6 (136).

acid or alkali indicated as follows and dilute to 200 cc. in a volumetric flask.

| *Phthalate-HCl Mixtures* | | *Phthalate-NaOH Mixtures* | |
|---|---|---|---|
| *pH* | *cc. M/5 HCl* | *pH* | *cc. M/5 NaOH* |
| 2.2 | 46.70 | 4.0 | 0.40 |
| 2.4 | 39.60 | 4.2 | 3.70 |
| 2.6 | 32.95 | 4.4 | 7.50 |
| 2.8 | 26.42 | 4.6 | 12.15 |
| 3.0 | 20.32 | 4.8 | 17.70 |
| 3.2 | 14.70 | 5.0 | 23.85 |
| 3.4 | 9.90 | 5.2 | 29.95 |
| 3.6 | 5.97 | 5.4 | 35.45 |
| 3.8 | 2.63 | 5.6 | 39.85 |
| | | 5.8 | 43.00 |
| | | 6.0 | 45.45 |
| | | 6.2 | 47.00 |

| *Phosphate-NaOH Mixtures* | | *Boric acid, KCl-NaOH Mixtures* | |
|---|---|---|---|
| *pH* | *cc. M/5 NaOH* | *pH* | *cc. M/5 NaOH* |
| 5.8 | 3.72 | 7.8 | 2.61 |
| 6.0 | 5.70 | 8.0 | 3.97 |
| 6.2 | 8.60 | 8.2 | 5.90 |
| 6.4 | 12.60 | 8.4 | 8.50 |
| 6.6 | 17.80 | 8.6 | 12.00 |
| 6.8 | 23.65 | 8.8 | 16.30 |
| 7.0 | 29.63 | 9.0 | 21.30 |
| 7.2 | 35.00 | 9.2 | 26.70 |
| 7.4 | 39.50 | 9.4 | 32.00 |
| 7.6 | 42.80 | 9.6 | 36.85 |
| 7.8 | 45.20 | 9.8 | 40.80 |
| 8.0 | 46.80 | 10.0 | 43.90 |

Transfer the solutions from volumetric flasks to 300-cc. Pyrex Erlenmeyer flasks fitted with 1-hole rubber stoppers bearing 10-cc. volumetric pipettes. When not in use, keep the flasks in a cold room at about 5°C.

Under these conditions the solutions will ordinarily keep for three months but they should be checked electrometrically at frequent intervals. Whenever the error in any solution reaches 0.05 pH, it is discarded and a new one prepared.

### *Preparation of Indicators*

The following indicators are used:

| *Name* | *Color Change* | *pH Range* |
|---|---|---|
| Thymol blue | Red-yellow | 1.2–2.8 |
| Brom phenol blue | Yellow-blue | 3.0–4.6 |
| Methyl red[3] | Red-yellow | 4.4–6.0 |
| Chlor. phenol red | Yellow-red | 5.0–6.6 |
| Brom cresol purple | Yellow-purple | 5.2–6.8 |
| Brom thymol blue | Yellow-blue | 6.0–7.6 |
| Phenol red | Yellow-red | 6.8–8.4 |
| Cresol red | Yellow-red | 7.2–8.8 |
| Thymol blue | Yellow-blue | 8.0–9.6 |

To prepare solutions, grind 0.1 gram of the dry powder in an agate mortar with the following quantities of N/20 NaOH:

| *Indicator* | *No. of cc. N/20 NaOH per Decigram* |
|---|---|
| Thymol blue | 4.3 |
| Brom phenol blue | 3.0 |
| Methyl red | 7.4 |
| Chlor. phenol red | 4.7 |
| Brom cresol purple | 3.7 |
| Brom thymol blue | 3.2 |
| Phenol red | 5.7 |
| Cresol red | 5.3 |

Dilute brom thymol blue, thymol blue, brom phenol blue, and brom cresol purple to 250 cc. to give a 0.04-per-cent solution. Dilute cresol red, phenol red, chlor. phenol red, and methyl red to 500 cc. to give a 0.02-per-cent solution. Keep these solutions in glass-stoppered bottles in the cold room. For routine use place small portions in 100-cc. Erlenmeyer flasks closed with rubber stoppers fitted with 1-cc. graduated pipettes.

### *Colorimetric Procedure*

The essential points of the colorimetric procedure are the addition of a suitable indicator to a series of tubes containing buffer solutions and to a similar tube containing the unknown. The pH value is then determined by matching the colors. In general, 0.4 cc. of indicator and 10 cc. of solution are used. These amounts may be varied to meet

[3] Brom cresol green suggested by Cohen as a substitute for methyl red has also been tried and found satisfactory.

various conditions. With colored or turbid solutions, such as medium or serum, a comparator is used. This is made from a block of wood. Six deep holes, large enough to hold 125-by-13-millimeter tubes are bored parallel to one another in pairs. Adjacent pairs are placed at intervals of one inch from each other. At right angles to these holes, and running through each pair, are bored smaller holes, through which the test tubes may be viewed. The central pair of tubes holds (1) the solution to be tested plus the indicator, and (2) a water blank. At either side are placed the standards colored with the indicator, each backed by a sample of the solution under test. The whole block, including the inside of the holes, is painted a dull black.

### *Errors of the Colorimetric Method*

Aside from color and turbidity, other difficulties of the colorimetric method are the so-called protein and salt errors. They occur in solutions rich in these constituents. They may sometimes be obviated by dilution, but in any event it is wise to determine the error by comparison of colorimetric and electrometric determinations. If necessary, proper allowance should be made for the error.

## SECTION IV

### CHAPTER 1

# PRODUCTION AND STANDARDIZATION OF ANTIPNEUMOCOCCUS SERA[1]

Antipneumococcus sera are produced by the active immunization of horses against representative strains of the three main types of the pneumococcus. Type-I serum is prepared for diagnostic and therapeutic purposes; types II and III for diagnostic use only, as sera of these types have not yet been produced of sufficient potency to be of therapeutic value. Type-I serum for therapeutic use is standardized by protection tests on mice. The potency or titer of diagnostic sera is determined by the agglutination reaction.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

### *Standard Strains*

At present the following standard strains are maintained.

Type I, "N" strain ("Neufeld"). Collection No. 5: Received June, 1914. This culture was originally secured by the Rockefeller Institute about 1910–11 from

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[1] The production of antipneumococcus serum is based fundamentally upon experimental studies in animals of the action of immune serum in pneumococcus infections published in 1912; Wadsworth, A. B., Trans. Assoc. Amer. Phys., 1912, 27, 72 (137); Jour. Exper. Med., 1912, 16, 78 (138). The recognition of type-I pneumococcus as distinguished from other types by Neufeld, and the studies of Cole and his collaborators, established the three principal types, and also the practical value of type-I serum in the treatment of type-I pneumonia. Since 1915, type-I antipneumococcus serum has been distributed for the treatment of type-I cases in New York State, and the local laboratories have very generally taken up the type diagnosis of pneumonia as a basis for serum treatment. Wadsworth, A. B., Amer. Jour. Hyg., 1924, 4, 119 (139).

The untreated serum is used. Although the methods of Huntoon, preparing antibody extract, and of Felton, precipitating globulin, offer opportunities of securing the active therapeutic substances free from other proteins, studies made in this laboratory have not indicated any greater specific therapeutic action with these products than with untreated antipneumococcus serum, and it has seemed unwise to change the established procedure until the advantage of doing so has been more clearly demonstrated.

Prof. Neufeld of Berlin. It has been used for years by this laboratory in experimental work, for immunization of horses, and in routine agglutination and protection tests.

"R. D." strain (Rockefeller D.). Collection No. 5B: Received October, 1916. This culture was isolated from a fatal case of lobar pneumonia by the Hospital of the Rockefeller Institute in 1916.

"H. L." strain (Hygienic Laboratory). Collection No. 5D: Received May, 1921. Standard type-I strain obtained from the U. S. Hygienic Laboratory.

Type II, "D" strain. Collection No. 5C: Received October, 1917. This culture was isolated by the Hospital of the Rockefeller Institute in 1916.

Type III. Collection No. 5A: Received August, 1915, from the Hospital of the Rockefeller Institute.

**Maintenance of Strains.**—Standard strains (when not in active use) are maintained in serum semisolid medium (125-by-13-mm. tubes). They are transferred to fresh medium once a month, and passed through mice every two months, or oftener if necessary to insure virulence. Unless otherwise specified the minimum virulence of all standard strains should be so maintained that 0.00,000,1 cc. of a 12- to 18-hour broth culture injected intraperitoneally into a mouse weighing from 16 to 22 grams invariably kills within forty-eight hours, preferably within thirty-six hours. The virulence of the type-I standard strain ("N" No. 5) used in potency tests on therapeutic serum should be such that 0.00,000,001 cc. kills under similar conditions.

For preparation of serum semisolid medium (140) or the special "pneumococcus broth" which is used wherever broth medium is mentioned in this chapter, see "Preparation of Media," pp. 87 and 110.

Serum semisolid cultures, from which cultures for distribution are prepared, are furnished regularly to the bacterial-collection group.

In transferring cultures, seed each tube of fresh medium with about 0.1 cc. of culture, and incubate from sixteen to twenty hours at 35 to 36°C. (If growth is scant, a transfer to broth between semisolid seedings will generally insure a richer growth.) Make a slide preparation stained by Gram's method, and streak a blood-agar plate, as tests of the purity of the new culture. Record on the label of each culture tube the strain number or name, pneumococcus type, source ("Ht" = heart, "SS" = semisolid culture), date inoculated, and initials of the worker. Store in the cold room with the cultures of the preceding six months. Keep all the cultures of each strain in a separate basket clearly labeled "Standard pneumococcus strain type I 'N' " (or type II or III). Whenever a culture has been opened, and is to be retained, record on the label the date of opening, and initials of the worker.

For animal passage, seed from 0.1 to 0.2 cc. of the semisolid culture

into a tube of broth, and incubate the tube for from sixteen to twenty hours. Inoculate a mouse intraperitoneally with the required dose of broth culture, following the procedure described under "Injections," p. 423. At autopsy, after searing lightly the apex of the heart, snip off the tip and with one or two drops of heart's blood, inoculate each of at least two serum semisolid tubes for stock. Also inoculate a broth tube, and streak a blood-agar plate, then make a slide preparation. For further procedure, see "Autopsies," p. 424.

**Identification and Purity Tests.**—In addition to the procedure already described, every four months, or oftener if necessary, test the broth culture made at autopsy for bile solubility, using 0.6 cc. of culture and 0.1 cc. of bile, and for agglutinative reactions with the three standard type sera. For technic, see "Agglutination Tests," p. 425. Also occasionally examine the cultures for capsules and test for fermentation of inulin. For procedure, see "Differentiation of Pneumococcus Types," p. 131. Always test thoroughly strains not in active use, before work with them is resumed.

Should contaminating organisms be found, discard the culture and go back to a previous semisolid transplant. Should the purity of type of any culture be questioned, fish at least five distinct colonies and test each against the three standard agglutinating sera. Resort to fishings for the stock culture only if the strain would otherwise be lost.

## PRODUCTION OF ANTIPNEUMOCOCCUS SERA

### IMMUNIZATION OF HORSES

Immunization is commenced with the injection of killed pneumococci in suspension, and continued with living organisms; either whole culture and blood (whole culture method[2]), or salt-solution suspension (vaccine method[2]). Twelve- to sixteen-hour broth cultures are used. All injections are given intravenously. The dosage depends largely upon the reactions induced. Usually from two to three months' immunization is required before the serum is of sufficient potency for whole bleedings to be taken. A rest period of from two to three months

[2] The whole culture method has been developed at this laboratory. For related experimental studies, see Wadsworth, A. B., Jour. Exper. Med., 1912, 16, 78 (138); Kirkbride, M. B., Jour. Exper. Med., 1915, 21, 605 (141); Wadsworth, A. B., and Kirkbride, M. B., Jour. Exper. Med., 1918, 28, 791 (142). The vaccine method is similar to that described by Cole, R., and Moore, H. F., Jour. Exper. Med., 1917, 26, 537 (143); and in Monograph No. 7, Rockefeller Institute, 1917 (144).

each year, or more frequently if indicated by the condition of the horse, is advisable.

*Selection of Horses*

Special care is taken in the selection of horses. Animals between seven and twelve years and weighing not less than eleven hundred pounds are preferred. They should be in good physical condition with no tendency toward enlarged or diseased joints. It is advisable to choose unused horses for production of type-I serum for therapeutic use; but, if necessary, horses previously immunized against diphtheria or tetanus toxin may be used for diagnostic sera.

*Preparation of Cultures*

Use for immunization the first or second broth transfer from a 12- to 24-hour broth culture inoculated directly from a mouse, or from a recent semisolid culture.[3] To prepare the culture make a slide preparation of the parent culture, stain by Gram's method, and examine. Transfer from 0.1 to 1.5 cc. of culture, depending on the volume of medium to be inoculated, and incubate for from twelve to sixteen hours. (The broth may be inoculated in the late afternoon and left at room temperature for the night watchman to place in the incubator. See "General Instructions," p. 620.)

Requisition of media: Place orders for broth at least a week before it is to be used. The broth is dispensed in 25, 50, 100, 150 cc. volumes in 100- and 200-cc. French square bottles, and 500 cc. in 1-liter Erlenmeyer flasks, plugged with cotton, and capped with tinfoil to prevent evaporation. Test a new lot of broth before using it for the cultures, to ascertain if it is favorable for growth.

The subsequent treatment of the culture depends on whether killed or living organisms are to be injected, and whether the whole-culture or "vaccine" method is to be used.

**Killed Culture for Preliminary Injection.**—A salt solution suspension of organisms, heated at from 52 to 54°C. for thirty minutes is used for the preliminary injections.[4] Enough heated suspension may be prepared at one time for a series of daily doses. For preparation of suspension, see "Live culture 'Vaccine' method," p. 414.

[3] Semisolid cultures made from broth transfers of 6- to 9-months-old stock cultures have been substituted when evidence of the increased virulence of the strain made such a procedure advisable.

[4] Culture filtrate has at times been injected with the later series of doses of killed organisms. Heated whole culture has occasionally been substituted for the heated suspension of organisms.

To heat, close with a rubber stopper the bottle containing the cotton-filtered suspension, cap with fishskin or rubber cloth held in place by elastic bands, and immerse it completely in a water-bath. Heat at from 52 to 54°C. for thirty minutes. If 20 cc. or more of suspension are heated, control the temperature by placing a thermometer in a similar bottle containing salt solution. Streak a blood-agar plate and inoculate one aerobic tube of "sterility-test" broth with the heated suspension. Dispense the suspension under aseptic conditions in single doses.

**Live Culture—Whole Culture and Blood Method.**—In the whole culture and blood method, defibrinated blood from one of the horses undergoing immunization whose condition is considered satisfactory, or from a normal horse, is added to the actively growing culture before the period of incubation is completed.

Four hours before the culture is to be taken to the operating room add quickly 1 or 2 per cent of its volume of defibrinated blood, and return the culture at once to the incubator. At the end of the incubation period make a slide preparation from the culture and streak a blood-agar plate. If any contaminating organisms are found on the film, discard the culture; if later any develop on the plate, record the kind of organism on the horse chart. Draw the culture through a cotton filter,[5] dispense the doses if more than one horse is to be injected, close the bottles with sterile rubber stoppers, label with the horse number and pneumococcus type, and send to the operating room for immediate injection, accompanied by an initialed duplicating order giving the kind and type of culture, horse and injection numbers, and amount of the dose. In cold weather, warm the copper container in which the cultures are sent to the operating room. In case the cultures are to be injected at the farm, pack the unfiltered cultures in a thermos jar or milk-sample container (see "General Instructions," p. 621), so that a temperature of approximately 36°C. is maintained, and have them filtered just before injection.

Defibrinated blood: Blood not more than four days old is used. It is collected under strictly aseptic precautions in a glass bottle containing glass beads, and defibrinated as described under "Care and Treatment of Animals—Large Animals," p. 595.

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[5] At times it is advisable, for intravenous injection, to reduce the number of organisms in the dose by passing part of the culture through a Buchner or other filter which will hold back a large part of the organisms; or most of the culture may be filtered through a filter candle and the filtrate and unfiltered portion combined. Subcutaneous injections of sterile filtrates have also been given.

On receipt at the laboratory, place the bottle immediately in the cold room. After adding the blood to a culture, streak a blood-agar plate with one or two drops from the pipette.

Suction filter: To prepare the cotton suction filter through which the culture is drawn into a second bottle, draw out a piece of 9-mm. glass tubing until the opening at one end is about 2 mm. in diameter. (The tube should then be from 8 to 10 cm. in length.) Place a loose wad of absorbent cotton in the small end, and insert this into a 12-cm. piece of thick-walled rubber tubing which is attached to a piece of bent glass tubing connecting the two bottles. Wrap sufficient cotton (secured by string) around the junction of the glass connecting tube to fit snugly into the neck of the bottle containing the culture to be filtered. Make the total length of the glass filter tube and the rubber tubing such that when the cotton plug is in place the lower end of the glass filter just touches the bottom of the bottle. Pass the other arm of the connecting tube through one hole of a two-hole rubber stopper of the proper size to fit a second bottle, into which the culture will be drawn after passing through the filter. Into the second hole of the stopper insert a bent piece of glass tubing plugged with cotton, to connect with the rubber tubing leading to the vacuum intake. Protect the filter and lower end of the rubber stopper with test tubes, wrap the whole apparatus in brown paper, and sterilize in the autoclave.

**Live Culture—"Vaccine" Method.**—In this method the broth cultures are centrifugalized and the sediment suspended in 0.85-per-cent salt solution.

After the culture has been incubated for from twelve to sixteen hours, make a slide preparation stained by Gram's method and examine. If satisfactory, pipette the culture into sterile centrifuge tubes and centrifugalize for from twenty to thirty minutes or until the supernatant broth is clear. Pour the supernatant fluid into a jar containing 1-per-cent crude cresol. If the sediment is for one horse, suspend it in 5 or 10 cc. of salt solution and mix thoroughly. Filter the suspension into a 20-cc. bottle through a sterile cotton filter (see "General Instructions," p. 624) previously moistened with from 1 to 2 cc. of salt solution. Rinse the centrifuge tubes and add the washings. When all the suspension has been filtered, pass 2 or 3 cc. more of salt solution through the filter. Streak a blood-agar plate with the suspension. (Should contaminating organisms later develop, record the kind of organism on the horse chart.) Close the bottle with a sterile stopper, cover with tinfoil, label with the horse number and pneumococcus type, and send to the operating room, accompanied by an initialed duplicating order form giving the kind and type of culture, horse and injection numbers, and amount of the dose. If the suspension is to be used for doses for two or more horses, filter the concentrated suspension through a cotton filter, and add sufficient salt solution to give a final

volume which can be divided readily into the required amounts, of which the largest is not more than 20 cc. For example, if three doses of suspension are to be given representing 100, 200, and 200 cc. of broth culture respectively, it is convenient to suspend the organisms from 500 cc. of culture in a final volume of 50 cc., in which case 10 cc. of suspension represents 100 cc. of broth culture, and 20 cc., 200 cc. Dispense the doses in 20-cc. bottles and treat as above.

### *Injections*

Injections are made intravenously.[6] (For technic, see "Care and Treatment of Animals—Large Animals," p. 590.) From three to five series of three daily injections of heated suspension are given, with a week's interval between each series. These are followed by injections of increasing amounts of whole blood culture, or unheated suspension, on two or three successive days, with a week's interval between. Two rather than three consecutive doses are preferable for most horses after the first two or three series of injections of living organisms.

### *Dosage*

The amount injected depends primarily on the temperature reactions and on the general condition of the horse. Some horses are much more susceptible than others. At present, by both the whole-culture and the vaccine method the doses of the first three series of killed organisms are the pneumococcus suspensions from 50, 100, and 100 cc.; from 100, 200, and 200 cc.; and from 200, 300, and 300 cc. of broth culture respectively. One or two additional series of killed organisms with further increase in dosage may be given. The first dose of live culture should not be over 25 cc. of whole culture, or sediment from more than 25 cc. of whole culture. If the temperature reactions are not excessive, the doses of a series may usually be increased from 15 to 25 cc. until a total of 150 or 200 cc. is reached, after which it is usually desirable to increase the doses more slowly or not at all. The dose given at the first injection in one series of live culture is usually the same as that given at the last injection in the preceding series. In the vaccine method the dose may frequently be increased more rapidly, but live organisms from more than 500 cc. of culture should not be given in one injection. The whole-culture method is more severe and the doses should, therefore, be smaller and very carefully gauged.

[6] No evidence has been obtained to show that the preliminary subcutaneous desensitizing dose which was formerly given when injections were resumed after a period of rest, was necessary. Filtered culture is sometimes given subcutaneously.

*Temperature Reactions*

The temperatures of all the horses undergoing active immunization are taken each morning. Temperatures are also taken from three to five hours after each injection; for the first one or two series of injections of live culture, each hour from the third until the maximum temperature has been reached and the decline has commenced. Reactions up to 106°F. and over may occur. A temperature over 105°F. is undesirable, however, and 103 to 104°F. is preferred. On the morning following an injection, the temperature should again be normal; if not, the day's injection is omitted. The general condition of the horse is followed carefully; the appearance of symptoms, such as loss of appetite and especially any evidence of stiffness in the joints or irregular heart action,[7] is reported at once to the production group, and injections are immediately reduced or omitted. The temperature following inoculation and the morning temperature are reported each morning so that the amount of the next dose may be gauged.

*Trial and Whole Bleedings*

Trial bleedings are taken before immunization, again immediately before the first inoculation with live culture, and then from time to time afterwards, preceding the first injection of a series—generally before every second series. When tests show the serum is of sufficient potency—usually in from eight to twelve weeks—whole bleedings of from seven to nine liters are taken seven days after the last inoculation. Injections are resumed five days after a whole bleeding; two series of injections are usually given between bleedings. (For technic of bleeding, etc., see "Care and Treatment of Animals—Large Animals," p. 593.)

Trial bleedings and samples from whole bleedings are kept in separate baskets according to type (pneumococcus I, II, or III). Trial bleedings are discarded

[7] Lesions of vegetative endocarditis have developed in a number of horses undergoing active immunization with living and highly virulent strains of pneumococci. From eight of the type-I horses showing such lesions, an atypical pneumococcus has been isolated from the blood stream and in some instances also from the synovial fluid from swollen hock joints and from the heart lesions at autopsy. All these organisms when first isolated gave nonspecific agglutination reactions in antipneumococcus sera of types I, II, or III, and, with the exception of two strains, failed to kill mice injected with 1 cc. of broth culture. The two strains which killed reacted specifically with type-I serum and killed in one millionth of a cubic centimeter after one or two animal passages. A detailed study of the lesions has been reported by Wadsworth, A. B., Jour. Med. Res., 1918–19, 39, 279 (145). Further studies of the organisms are to be published.

after six months; samples from whole bleedings, after the serum has been released for distribution. A bottle from each lot of serum distributed is held in reserve.

## TREATMENT OF SERUM

After the serum has been drawn off from the clot, samples for sterility and potency tests[8] are removed, and preservative is added. Later the material is filtered and bottled for distribution. Aseptic precautions are observed throughout the different procedures. Sera from different horses are usually kept separate.[9] Sera of each pneumococcus type are given separate series of consecutive numbers in order of their receipt. Type-I serum which falls below the requirement for therapeutic serum, or which has become outdated, or serum from a horse whose physical condition precludes its therapeutic use, may be used for diagnostic purposes if the agglutinative titer is adequate.

### *Preliminary Sterility Tests and Addition of Preservative*

On receipt of the serum, verify and initial the accompanying memorandum of shipment, and file; enter the serum number on the tag on the bottle, and record the required data on the permanent-record card. Inoculate the required media for sterility tests, following the instructions given in "Sterility Tests of Biologic Products," p. 543. From one of the bottles from each bleeding withdraw, besides the material required for sterility tests, from 5 to 10 cc. of serum for potency tests. After inoculating the tubes, place the remaining material in a sterile bottle labeled with the kind of serum, lot number, horse number, and date. Add about 10 cc. of chloroform (C.P.) to each four liters of serum (chloroform to saturation), and write "chloroform" on the tag.[10]

### *Filtration and Sterility Tests after Filtration*

Should the sterility tests show the serum to be contaminated, it is filtered at once. Otherwise it is held in the cold room for at least two weeks, preferably longer, to allow for precipitation before filtration.

[8] Our experience has been that, in general, filtration as carried out in this laboratory does not cause any appreciable deterioration in the potency of a serum as measured by protection or agglutination tests.

[9] For purposes of study, and because separate tests are made on serum from each bleeding, it has been customary to treat each as a separate lot. Sera from different horses or bleedings requiring refiltration after standing may be pooled and retested after filtration.

[10] The practice, followed during the last ten years, of adding chloroform has given satisfactory results. In the absence of more definite knowledge and despite certain opinions to the contrary, it has been deemed wise to continue its use.

The serum is filtered by the filtration group assisted by a member of the production group. (For technic see "Filtration of Biologic Products," p. 511.) Sterility tests are made upon the filtered product. Should contaminating organisms be detected, the serum is refiltered at once.

Batches of serum from different horses immunized against the same pneumococcus type, may be filtered in succession through the same filter, the serum having the highest titer being put through first.

*Preparation of Sera for Distribution*

As soon as the sterility tests are satisfactorily completed, the serum, if required, may be bottled for distribution by the filling and boxing group on written order from the production group. If not needed, it is held in bulk to allow for further precipitation. No serum should be bottled which has not first been passed upon by the head of the production group. For procedure of filling, see "Preparation of Biologic Products," p. 524.

Serum for therapeutic use is dispensed in 50-cc. volumes. On the label of each bottle are given the pneumococcus type, the serial number of the serum, the method of immunization, and the return date. The protective titer of the serum, and that of the standard control serum are also recorded. Serum, if kept under proper conditions, is considered satisfactory for use for eighteen months from the date of the last satisfactory test.[11] The return date is given on the outside of the package.

Since precipitation may continue to occur, the head of the production group should examine the bottled serum from time to time. Pooling and refiltration may be necessary.

Sera of the three main types for diagnostic use are dispensed in 10- and 20-cc. volumes. On the printed labels, which state the serum is "For diagnostic use only," are entered the pneumococcus type and the serum number. The serum dilutions to be used in routine tests for type differentiation are also entered in the following manner: "Use undiluted and diluted 1:10" (or 1:20, etc.). That dilution giving the most satisfactory agglutination and precipitation reactions is the one specified. Serum is distributed for diagnostic purposes as long as the agglutinative titer remains satisfactory. Samples from lots

[11] No definite period has been set after which serum should be withheld from distribution since, in practice, the supply of the various sera in storage has never been excessive, so that relatively fresh material has always been distributed, and sufficient data on which to base final judgment are not yet available.

in process of distribution are tested at least every two months, and the original dilution on the label changed if necessary. Workers in the diagnostic department are expected to report immediately to the production group any irregularities in the tests or deterioration in the titer of a serum.

## STANDARDIZATION OF ANTIPNEUMOCOCCUS SERA

The potency or titer of serum for therapeutic use (type I) is gauged by testing its protective value for white mice against a culture of the homologous standard type strain, as compared with the protection afforded by a standard control serum representing the minimum standard of potency.[12] The potency of the sera distributed for therapeutic use should exceed that of the minimum standard serum; under no circumstances is serum below the standard released for distribution. The development of protective power is followed during the earlier stages of immunization by tests on trial bleedings taken at frequent intervals.

Protection tests are made on trial bleedings from horses undergoing immunization for the production of therapeutic serum, just before the first inoculation with live culture, then before the first inoculation of each second series, and, as the serum approaches the required standard, before each series. Tests are made on each whole bleeding.

The protective titer and the agglutinative titer of a serum frequently do not parallel each other. A serum with high protective titer may have a lower agglutinative titer than one with a considerably lower therapeutic value, or the opposite may be true. The protection test is considered final for therapeutic sera, but the agglutination test is desirable as affording supplementary data. It is made on sera obtained before the first injection of live cultures and on every second or third serum from each horse which is tested for its protective value.

The potency or titer of sera for diagnostic use is determined by their agglutinative action upon the homologous standard strain used in immunization. The specificity of the reaction is controlled by simultaneous tests of the sera against the standard strains of the heterologous types. Recently isolated strains of the various types are included from time to time as a further control of the specificity and

[12] For further discussion regarding the problems of standardization, see Wadsworth, A. B., Kirkbride, M. B., and Gilbert, Ruth, Arch. Int. Med., 1919, 23, 269 (146); and also the reports of the sub-committee on antipneumococcus serum; Dr. Catoni, Dr. Griffith, Prof. Madsen, Dr. Wadsworth, Prof. Neufeld, Chairman; presented to the Second International Conference on the Standardization of Sera and Serological Tests, Paris, 1922; published in Reports of Serological Investigations; Health Organization; League of Nations, 1923 (123).

degree of agglutinative response. Serum selected for diagnostic use should give prompt and easily read agglutination with the homologous standard strain and recently isolated strains of the same type in from 1:5- to 1:20-serum dilution (1:10 to 1:40 final dilution) and no cross agglutination in low dilutions within two hours with heterologous strains. (In our experience cross agglutination practically never occurs.)

Agglutination tests are made on serum from trial bleedings taken from horses undergoing immunization for the production of diagnostic serum immediately before the first injection of live organisms, then before the first injection of each second series, or oftener if indicated. Tests are made on each whole bleeding. The protective antibody content of diagnostic sera is ascertained toward the end of the third month of immunization and from time to time afterwards.

Sera for diagnostic use are also tested for their precipitation reactions with the supernatant fluid from centrifugalized broth cultures of the homologous and heterologous types. The serum undiluted and in at least a 1:5-dilution should give immediate and definite specific reactions.

Since one or two lots of serum have been found to cloud temporarily when heated at 55°C. (Krumwiede's precipitation test), each serum is tested for fifteen minutes at that temperature before being released.

### PROTECTION TESTS[13]

Series of white mice weighing between 16 and 22 grams are inoculated intraperitoneally with mixtures containing a constant amount (0.2 cc.)[14] of the serum to be tested and varying amounts of standard culture. The serum and the culture before being combined are so diluted with "pneumococcus broth" that the required amount of each is contained

[13] The regulation in the Sanitary Code (Chapter IX, adopted 1917) relating to the potency of antipneumococcus and antimeningococcus sera is as follows: "No serum for the treatment of pneumonia or of meningitis shall be sold or offered for sale in the state of New York unless each package is accompanied by a label or circular on which is stated the potency of the serum as tested by the methods established by the rules and regulations of the state commissioner of health; and no such serum shall be sold or offered for sale the potency of which does not equal or exceed the minimum fixed in such rules or regulations. This regulation shall take effect February 15, 1918."

[14] Since 0.2 cc. of serum has been generally recognized in this country as the standard volume in protection tests, it has been adopted by the state laboratory. Comparative tests, however, have indicated that more satisfactory and uniform results can be obtained by the use of 0.1 cc. of serum. It may, therefore, at times, prove of value to run supplementary tests substituting 0.1 cc. for the standard 0.2 cc.

in 0.5 cc. Usually three or four mixtures containing different dilutions of culture are tested, three mice being injected with each. As a basis for comparison, corresponding tests are run at the same time on the minimum standard serum. The virulence of the culture is further determined by inoculating two mice with 0.00,000,001 cc. and 0.00,000,1 cc. respectively of culture without serum. The test should be completed within one hour from the time the culture is diluted.

It may occasionally be desirable, especially when new lots of broth are first used, to estimate the number of organisms in the culture.

Melt two tubes of 0.2-per-cent dextrose-beef-infusion agar and cool to 43°C.; enrich the medium with one or two drops of normal blood. Then inoculate the tubes with 0.5 cc. of a 1:500,000- and of a 1:5,000,000-dilution of culture respectively. Mix the contents thoroughly and pour into Petri plates. Count the number of colonies appearing at the end of twenty-four hours' incubation and calculate the number of organisms per cubic centimeter.

### *Culture*

For final tests on therapeutic sera, and preferably for all tests, the first transplant from a broth culture inoculated with heart's blood of a mouse dying within forty-eight hours from one millionth or less of culture, is used. The second broth transfer from a recent semisolid culture may be substituted for preliminary tests and trial bleedings. Twelve- to 16-hour growths are used throughout. Before the culture is used, a slide preparation stained by Gram's method is examined.

### *Standard Serum*

The standard control serum represents the minimum standard of potency for sera for therapeutic use. It is of such potency that 0.2 cc. protects for at least four days against at least 0.1 cc. of standard culture two-thirds or more of the mice inoculated with it. When comparative tests made with later sera previously tested with the standard, indicate that deterioration is becoming marked, a new standard is selected with a titer slightly higher than the old, so that the level of potency may be maintained. A serum not less than four months old is selected. Repeated tests are necessary in comparison with the standard and with other sera before final selection of a new standard is made.[15]

[15] The standard serum at present used is from a bleeding taken in November, 1918. It was adopted as the minimum standard of potency in July, 1919, after repeated comparative tests with sera prepared in this and other laboratories. No appreciable deterioration in the serum has been shown in the periodic comparative protection tests with more recent laboratory sera, and in tests with the U. S. Hygienic Laboratory standard control serum.

*Test Sera*

Sera from trial bleedings are tested as soon as possible after being taken, those from whole bleedings usually within one or two weeks from the date of bleeding. Sera which are held for six months or longer are retested before distribution.

*Test Animals*

Healthy white mice weighing between 16 and 22 grams are used.

*The Test*

Before starting the test enter directly upon the permanent-record card all available data, including serum and culture dilutions and mouse markings, for reference during the test. Since time is an important factor, assemble all the necessary supplies, mark the test tubes and small stoppered bottles to be used for dilutions, make the serum dilutions and pipette the broth for the culture dilutions, before starting to dilute the culture.

**Dilutions.**—1. Serum dilutions: Mark each bottle with its serum number (or for trial bleedings, the horse number) and the dose; $\left(\frac{\text{S } 140}{0.2}, \frac{\text{H } 101}{0.2}\right)$. Pipette 3 cc. of broth into each and then 2 cc. of the required serum.

2. Culture dilutions: Mark each bottle with the culture number and dose $\left(\frac{1\text{ N}}{0.3}, \frac{1\text{ N}}{0.2}, \text{ etc.}\right)$, and pipette the required amounts of culture and broth to be used into the bottles, adding the culture to the broth in each case.

| *Culture* | *Broth* | *Dose contained in 0.5 cc. of dilution* |
|---|---|---|
| 4 cc. | 1 cc. | 0.4 |
| 3 cc. | 2 cc. | 0.3 |
| 2 cc. | 3 cc. | 0.2 |
| 1 cc. | 4 cc. | 0.1 |

Should more than 5 cc. of any dilution be needed, increase the amounts of culture and broth proportionately. When doses below 0.1 cc. are required, as in testing early trial bleedings, use the intermediate dilutions made in preparing the virulence control-test dilutions. The second tube contains 0.01 in 0.5 cc., the third 0.001 cc., etc.

3. Culture dilutions for virulence control: Set up a rack with eight test tubes (150 by 19 mm.) and mark the dilution on each with a wax pencil. Pipette 2 cc. of broth into the first, 4.5 cc. into each of the

others. Add 0.5 cc. of culture to the first tube, mix thoroughly with a fresh pipette by drawing the suspension up and down a few times, then transfer 0.5 cc. into the next tube and repeat the step, transferring from each newly inoculated tube to the next tube. Use a fresh pipette for each dilution and mix each thoroughly as above before transferring it. (The sixth tube will contain 0.00,000,1 cc. and the eighth 0.00,000,001 cc. of culture in each 0.5 cc. of dilution.)

**Injections.**—Inoculate first the two virulence control mice and the series of mice receiving the standard serum and varying amounts of culture, then each series receiving a test serum and culture. In each series inoculate first the mouse receiving the highest dilution of culture. Make injections intraperitoneally. Immediately after injection, mark each mouse with a stain or picric acid, and record the markings as H (head); B (back); F. R. (right foreleg); H. R. (right hind leg); F. L. (left foreleg); etc. Identify the control mice by painting one or more rings around the tail.

Draw up in a 2-cc. syringe (needle 26 gauge, $\frac{1}{2}$ in. long) 0.5 cc. of culture dilution. (Always carefully revolve a culture or serum dilution just before drawing it up, to insure a uniform sample. Use only syringes which have been tested for accuracy.) Hold the mouse by the tail with the right hand, on a flat surface, and with the thumb and index finger of the left hand grasp quickly and firmly the skin at the back of the neck. Turn up the palm of the hand, and keeping the mouse stretched out, secure the tail and left hind leg against the palm with the second, third and fourth fingers, and let go with the right hand. Cleanse the site of inoculation with one or two drops of 80-per-cent alcohol from a dropper bottle or with a swab. Run the needle in subcutaneously from the right of the median line (parallel to the proximal end of the tibia) toward the center, then pierce the abdominal wall and inject. After inoculation, mark the mouse before letting go. Rinse the syringe with boiling water and allow it to cool before using again. (Three syringes are convenient.) Inoculate a mouse in a similar manner with the second culture dilution; then proceed to the injection of serum-culture mixtures. To inject the mixtures, draw into the syringe 0.5 cc. of culture dilution, then 0.5 cc. of serum dilution; mix carefully by tilting the syringe; allow an interval of from one to two minutes, and inject. Rinse the syringe between injections, and boil it before starting each new series. After injection, place each control mouse in a separate jar, and each series of mice in a separate box. Tag each box or jar giving the pneumococcus type, date of inoculation, and name of worker, and an identification number for the box.

**Observation of Mice.**—For the first four days inspect the mice twice during the day (usually at 9.00 a.m. and 5.00 p.m.) or more frequently if the mice are ill, and arrange for two inspections by the night watchman (at 11.00 p.m. and 5.00 a.m.). Then inspect only twice a day (at 9.00 a.m. and 5.00 p.m.) until the tenth day. Discharge surviving mice on the tenth day. When mice are found dead, wrap those from each box in paper, record on it the box number and the date and hour found dead, and place at once in the can for mice in the cold room.

**Autopsies.**—Autopsy the mice as soon as possible after death. Fasten the mouse, ventral surface exposed, to a wooden board by means of thumb tacks through the feet. Moisten the surface with 1-per-cent crude cresol. Make an incision along the median line and draw back the skin on each side. Open the peritoneal cavity with a longitudinal incision and make a rapid examination of the organs. Open the thoracic cavity by cutting through the ribs on either side, connecting the two cuts, and turning back the sternum. Raise the heart with hemostatic forceps. Sear the apex very lightly and cut off the tip. Streak a blood-agar plate with heart's blood and make a slide preparation. If later the plate culture shows no evidence of contamination, the direct film made at autopsy need not be stained. If the plate appears contaminated, examine it microscopically; also the direct film as a control if needed. Record the kind of contaminating organism and any additional data of interest on the permanent-record card. When autopsying virulence control mice, inoculate, before streaking the blood-agar plate, one or more serum semisolid tubes for stock from the mouse receiving the smaller dose, one for reserve from the other mouse, and a broth tube from each. If the plate culture is typical, and a slide preparation of the broth culture shows no contaminating organisms, store the semisolid cultures without opening, provided their appearance is satisfactory. Otherwise, streak a plate and make a slide preparation.

### *Estimation of Potency*

The protective titer of a serum is based upon the largest amount of culture against which 0.2 cc. of the serum protects for ninety-six hours or longer, at least two-thirds of the mice injected with it, provided that no deaths from pneumococcus infection occur among mice receiving the serum and smaller amounts of culture, and that the other conditions of the test are met. To determine satisfactorily the potency of the serum the following conditions are necessary. Control mice receiving culture only should die within forty-eight hours as the result of pneumococcus infection. The standard serum should protect for

ninety-six hours or longer, at least two-thirds of the mice receiving 0.1 cc. of culture. Death should occur, however, among mice receiving one or more of the larger amounts of culture and the standard serum. If these conditions are not met, the test on therapeutic serum for distribution is repeated. Tests on trial bleedings and preliminary tests on whole bleedings need not necessarily be repeated if a reasonably close estimation can be made.

If contaminating organisms are present in the culture from the heart's blood, the mouse is ignored in interpreting the test, unless the organisms, when grown on Andrade triple-sugar medium, suggest *B. coli*, in which case they are considered post-mortem invaders and, therefore, without significance.

### AGGLUTINATION TESTS

Three-tenths of a cubic centimeter of varying dilutions of serum are mixed with 0.3 cc. of uniform bacterial suspensions of organisms of the homologous and heterologous types. The serum, undiluted and in from two to five dilutions ranging from 1:5 to 1:80, is tested against the homologous-type culture; the serum, undiluted and in a 1:5- or 1:10-dilution only, against the heterologous-type cultures. A bile-solubility test is made as a check on the purity of the culture. The tubes containing the serum and the suspensions are incubated in a water-bath at 37°C. and examined at intervals for the presence or absence of clumping (agglutination).

#### *Procedure*

**Bacterial Suspensions.**—To prepare the suspensions, centrifugalize a 16- to 20-hour broth culture until clear. Pour off the supernatant fluid carefully. Add slowly 0.85-per-cent salt solution and mix until a uniform suspension is obtained. Standardize in a 150-by-19-millimeter test tube to the barium-sulfate suspension No. 4. (For preparation of the standard suspension, see "General Instructions," p. 625.)

**Serum Dilutions.**—Bring the serum to room temperature before preparing the dilutions. If precipitate is in suspension in the serum, centrifugalize it under sterile conditions. Use table 29 in making the dilutions.

Set up the required number of tubes (150 by 19 mm.) in a rack. Mark the first left-hand tube with the serum number and dilution and the remaining tubes with the dilution. Pipette 0.5 and 1 cc. of serum into the first and second tubes respectively. Add 0.85-per-cent salt solution, mix thoroughly, and transfer the required volume of the

1:10-dilution to each succeeding tube. Add the required volume of salt solution to each. Use separate pipettes for the undiluted serum, the salt solution, and the 1:10-dilution respectively.

**The Test.**—Set up a copper rack with the required number of sterilized, unplugged tubes (11 by 75 mm.). Record the serum number on the rack, the culture number on the first left-hand tube of each series, and the serum dilution on each tube. Use the front row of a rack for the agglutination test with the homologous-type strain and the back row for the precipitation test, and a second rack for the agglutination and precipitation tests with the heterologous-type strains. Pipette 0.3 cc. of serum dilution into each tube of a series, beginning with the highest serum dilution. (The same pipette may be used from higher to lower dilutions.) Now add 0.3 cc. of culture suspension to each tube. Make

TABLE 29

*Serum dilutions*

| VOLUME OF SERUM OR SERUM DILUTION | VOLUME OF SALT SOLUTION | FIRST DILUTION | FINAL DILUTION |
|---|---|---|---|
| *cc.* | *cc.* | | |
| 0.5 (undil.) | 2.0 | 1:5 | 1:10 |
| 1.0 (undil.) | 9.0 | 1:10 | 1:20 |
| 1.0 (1:10 dil.) | 1.0 | 1:20 | 1:40 |
| 1.0 (1:10 dil.) | 2.0 | 1:30 | 1:60 |
| 0.5 (1:10 dil.) | 1.5 | 1:40 | 1:80 |
| 0.5 (1:10 dil.) | 3.5 | 1:80 | 1:160 |
| 0.5 (1:10 dil.) | 4.5 | 1:100 | 1:200 |

a bile-solubility test on each suspension by adding 0.4 cc. of suspension to 0.1 cc. of sterile ox bile in a tube marked with the culture number. Before pipetting from a serum dilution or bacterial suspension, mix by drawing up into the pipette and discharging several times. When the suspension has been added to the serum or bile, shake the rack. Place all tubes in a water-bath at 37°C.

### *Reading and Recording Results*

Make readings against a dark background at the end of fifteen minutes, thirty minutes, and two hours. Record complete agglutination with clear, supernatant fluid and all large clumps, as 4+; clear, or nearly clear, supernatant fluid, with definite clumping, as 3+; supernatant fluid, not clear, but with definite clumping, as 2+; supernatant fluid, not clear, with very small clumps definitely visible to the unaided eye, as +; questionable reactions, as ±; and uniformly turbid suspension with no clumping, as —. Record a clear, bile solution as +; should the bile solution fail to clear, the entire test is considered

indeterminate. The agglutinative titer of a serum is the highest dilution in which distinct clumping ("+" reaction) is obtained within two hours. For dilutions to be recorded on bottles of diagnostic serum, see "Standardization of Antipneumococcus Serum," p. 418.

### PRECIPITATION TESTS

Prepare the serum dilutions and pipette them into 11-by-75-millimeter tubes as in the agglutination tests. Layer on to these slowly 0.3 cc. of the clear, supernatant broth obtained by centrifugalizing the 16- to 20-hour cultures of the three types used in the agglutination test, and look immediately for the formation of a ring. Shake the tubes and place them in a water-bath at 37°C. for two hours. Read, after fifteen and thirty minutes, and two hours.

### *Permanent Records*

Standard strains: A card (4 by 6 in.) is kept for each standard strain, giving the following data in regard to the virulence tests; date of injection, source of culture (Ht or SS); amount, time injected; date and time of death of mouse, date and result of autopsy.

Immunization of horses: A protocol is kept of each horse. For form and general data given, see "General Instructions," p. 619. In the case of horses immunized for the production of antipneumococcus serum, the following additional data are entered: the dates and serial numbers of the injections, kind and amount of material injected, the temperatures and any unusual condition, and, in regard to each bleeding or serum, the date of bleeding, date tested, titer, the volume of blood and serum, and the serial number given each.

Antipneumococcus sera: The following data in regard to the serum from each whole bleeding are recorded on a printed card (5 by 8 in.): the lot number of the serum, horse and bleeding numbers, date of bleeding, amounts of serum obtained at the first and second drawing off, and results of sterility tests on each; date filtered, volume recovered, and results of sterility tests on the filtered material; date of filling, number of bottles filled, number for distribution, and results of sterility tests on the filled products. Under remarks are entered the date of tests for potency, and any further observations of interest.

Protection tests: On cards (4 by 6 in.) are recorded the following data in regard to each test: date of test, horse and serum numbers and date of bleeding; source and age of culture; amounts of serum and culture used; mouse-identification records, time of inoculation, time of death or "lived," and autopsy findings; and initials of the worker. Tests of one date made with the same culture but with different sera may be entered on the same card. When more than one card is used, the virulence control tests are entered on each card. The cards are filed according to the pneumococcus type and date of test.

Agglutination tests: On a card (4 by 6 in.) are recorded the following data on each test: date, type, and number of serum; horse number and date of bleeding; culture; serum dilutions and culture suspensions; readings; and initials of the worker. The cards are filed according to the pneumococcus type and date of test.

Precipitation tests: The records for the precipitation test are similar in form to those for the agglutination test.

## CHAPTER 2

# PRODUCTION AND STANDARDIZATION OF ANTIMENINGOCOCCUS SERA[1]

Polyvalent antimeningococcus serum for therapeutic and diagnostic use is produced by immunization of horses against at least four standard strains representative of the main groups of the meningococcus. Monovalent sera for diagnostic purposes, and for use in controlling group specificity in routine standardization tests, or in connection with experimental studies, are produced by the immunization of rabbits against a single strain of the meningococcus. The titer or potency of a serum is determined by the agglutinative reaction.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

### *Standard Strains Used in Production of Serum*

At present the following four standard strains are used.[2]

"A" Group II. Collection No. 44A (original No. 46B). Received in 1916 from the Rockefeller Institute.

"B" Group III. Collection No. 44B (original No. 10B). Received in 1916 from the Rockefeller Institute.

"C" Group III. Collection No. 44C (original No. W30B). Received in 1918 from the U. S. Hygienic Laboratory, Washington.

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[1] For published studies made in this laboratory in connection with the production and standardization of antimeningococcus serum see: Wadsworth, A. B., Kirkbride, M. B., and Gilbert, Ruth, Arch. Int. Med., 1919, 23, 269 (146); Wadsworth, A. B., Gilbert, Ruth, Hutton, Alice, Jour. Exper. Med., 1921, 33, 99–105 (147); Wadsworth, A. B., Jour. Exper. Med., 1921, 33, 107 (148); Wadsworth, A. B., Kirkbride, M. B., Amer. Jour. Hyg., 1926, 6, 507 (149); Kirkbride, M. B., and Hutton, A. I., Jour. Immunol., 1926, 11, 393 (150).

See also reports of the subcommittee on antimeningococcus serum; Professor Dopter, Dr. Gordon, Professor Madsen, Professor Neufeld, Dr. Wadsworth, Chairman; presented to the Second International Conference on the Standardization of Sera and Serological Tests, Paris, 1922; published in Reports of Serological Investigations; League of Nations, Health Organization, 1923 (123).

[2] Groups I, II, and III correspond to the first three divisions of the Gordon classification and to the "Para," "Normal," and "Intermediate" or "Irregular Types" of the original Rockefeller Institute classification formerly used in this laboratory.

"D" Group I. Collection No. 44D (original No. W60B). Received in 1918 from the U. S. Hygienic Laboratory, Washington.

In addition a second group-I and a second group-II strain (Collection Nos. 44F and 44E, original Nos. 79B and 1B, received from the Rockefeller Institute about 1916) are sometimes used. Besides the standard cultures other strains of the same groups from various sources, and certain atypical strains, are maintained for use in special comparative tests of the agglutinative action of different sera. In order that recently isolated strains may be available, new cultures are added from time to time.

**Maintenance of Strains.**[3]—All strains in active use are maintained on dextrose-serum-agar slants (150-by-19-mm. tubes) and in ascitic semisolid medium (125-by-13-mm. tubes); all other strains in the semisolid medium only. Transfer the cultures on dextrose-serum agar every forty-eight hours, taking over a large amount of culture to insure growth. Transfer the cultures in semisolid medium every two months. To guard against loss, keep two complete sets of these cultures, each set in a different incubator. Arrange the dates for transferring the cultures so that one set is half as old as the other. To transfer, seed a large loopful of the semisolid culture to a dextrose-serum-agar slant (125-by-13-mm. tube). Incubate twenty-four hours. If the growth is abundant and apparently pure, transfer some of it to a fresh tube of semisolid medium. If the growth is scant, smear it with a loop over the surface of the agar and reincubate twenty-four hours before transferring. Stir the cultures of both sets once a week. Retain the parent cultures of each set until the next transfers are made. Maintain all cultures at a temperature of from 34 to 36°C. Resort to fishings only if the strain would otherwise be lost.

**Identification and Purity Tests.**—Dextrose-serum-agar cultures: Make slide preparations stained by Gram's method at least once each week, when a horse is undergoing immunization, preferably at the time the cultures for the first of the three consecutive injections are inoculated. Every six months, plate each strain on blood agar. Fish three colonies and test the agglutinative reaction of each fishing with the homologous and heterologous monovalent group sera. Test the cultures for fermentation, using maltose, dextrose, saccharose, and lactose. Inoculate the slant only of beef-infusion-agar slants containing Andrade indicator and the required sugar, and incubate for forty-eight hours. Make readings at the end of twenty-four and forty-eight hours, recording the results as ++ (strong acid); + (acid); ± (very slight

[3] The cultures are maintained by the group (bacterial-vaccine group) responsible for the standardization of the therapeutic polyvalent serum and for the production and standardization of monovalent diagnostic sera.

acid); and — (no reaction). Meningococcus cultures ferment maltose and dextrose and do not ferment saccharose and lactose. Plate and test similarly cultures not in active use before work with them is resumed.

The frequent agglutination tests which are made on trial bleedings and on therapeutic sera also act as a check on the group specificity of the standard strains.

Ascitic-semisolid-medium cultures: Confirm the purity of cultures maintained in ascitic-semisolid medium by making slide preparations stained by Gram's method from the growth remaining on each agar slant. If a contaminated culture is found, plate the parent culture on blood agar and fish colonies or make a transfer from the culture in the duplicate set. It is advisable to plate and test all stock strains once a year.

## PRODUCTION OF ANTIMENINGOCOCCUS SERUM

### IMMUNIZATION OF HORSES[4]

The four standard meningococcus strains, consisting of one group-I, one group-II, and two group-III cultures, are always used in the immunization of horses. In addition a second group-I and a second group-II strain are usually included. The cultures, grown on dextrose-serum-agar slants, are suspended in salt solution, pooled, and injected intravenously. Series of three daily injections are given with a 7-day interval between series. Live cultures are used and the doses are increased gradually. Whole bleedings are commenced after three months if the serum has reached the required potency. They are taken six

[4] From July, 1916, when immunization of horses against the meningococcus was commenced, to May, 1918, the treatment was based on the method published from the Rockefeller Institute (Amoss, H. L., and Wollstein, M., Jour. Exp. Med., 1916, 23, 403 (151)). Each horse during the course of immunization received a large series of cultures (usually between fifty and sixty strains) representing the so-called regular, para, and irregular or intermediate groups. Strains were added from time to time, especially if the serum failed to cause their agglutination in high dilutions. The method at present in use was developed by this laboratory in an effort to simplify the procedure, while at the same time retaining or even increasing the potency of the serum as shown by the agglutinative titer. In May, 1918, experimental immunization of two horses was commenced. Results showed that the agglutinative titer was much higher than that of serum produced by the original method, not only against the homologous cultures, but also against the heterologous strains; moreover, the time required for immunization was shortened. This method was adopted as the routine procedure in June, 1919.

days after the last injection of the previous series. A rest period of two to three months each year, or more frequently if indicated by the condition of the horse, is advisable.

### *Selection of Horses*

Horses meeting the usual physical requirements are selected. The general condition of the horse usually remains good during treatment if the dosage is carefully gauged. If unused horses are not available, those previously immunized against diphtheria and tetanus toxin may be used. Antimeningococcus serum of high agglutinative titer has been obtained from such animals.

### *Preparation of Cultures*

On the afternoon preceding the inoculation, make transplants from 24- or 48-hour cultures of the required strains to dextrose-serum-agar slants (150-by-19-mm. tubes). Plan the work so that the horse will receive cultures between seventeen and nineteen hours old. On the following morning, when notified that the worker in charge of injections is nearly ready to inject, examine the cultures and if the growth is abundant and there is no evidence of contamination, prepare the suspensions. Place the tubes in a rack and add, with a 10-cc. pipette, 3 cc. of salt solution to each culture. Loosen the growth carefully with a wire loop and stir until a uniform suspension is obtained, taking care not to break the agar. Pass the suspensions through a sterile cotton filter (for preparation see "General Instructions," p. 624), previously moistened with a volume of salt solution equal to 1.5 cc. for each culture. Then pass through, 1.5 cc. of salt solution for each culture to wash the filter. Pipette the dose prescribed for each horse into a separate sterile bottle and add enough salt solution to bring the volume to 20 cc. When the dose of suspension is over 20 cc., do not dilute further. Send the suspension at once to the operating room accompanied by an initialed duplicating order form giving the horse and injection numbers, kind of culture, and amount of the dose.

The suspensions are injected as soon as prepared. If severe reactions are induced by an injection the method followed until 1925 may be used for subsequent injections, namely, that of giving the required dose in two injections an hour apart, in which case the cultures must be divided and the suspension for the second injection prepared separately immediately before the injection. It may occasionally be necessary to inject the cultures at the farm, in which case they are sent in a special container (see "General Instructions," p. 621), and the suspensions are made by the worker in charge of injections.

### *Injections*

Injections are made into the jugular vein. (For technic see "Care and Treatment of Animals—Large Animals," p. 590.) They are given on three successive days with a 7-day interval between each series. To avoid unduly severe reactions the dose for a given day may be divided and injected in two portions, one hour apart.

### *Dosage*

No definite schedule can be followed, since the reaction induced in different horses varies widely. Each dose is based largely on the temperature reaction after the previous injection and on the general condition of the horse. Immunization is begun with very small doses of the living cultures (not more than 0.2 cc. of the pooled suspension on the first day). The doses may be increased during the early series by from 0.2 to 0.5 cc. In later series the increase is greater, the rate depending on the virulence of the cultures and the reactions induced in individual animals. Maximum increases have varied from 3 to 10 cc. Usually the first dose of a new series is the same as the last dose of the preceding series. On resuming injections after each of the first two or three whole bleedings, one-half the previous dose is given; later in the course of immunization, if the condition of the horse permits, three-quarters of or even the entire preceding dose may be injected. Care is taken not to increase the dose so rapidly that the general condition of the horse is markedly impaired.

### *Temperature Reactions*

Temperatures are taken every morning and also from three to five hours after each injection. For the first two or three series, temperatures are taken from the third hour until the highest point has been reached and the decline has commenced. The aim should be to give a dose that will produce a reaction of from 104 to 105°F. in from four to five hours after inoculation, with a return to normal by the following morning. The morning temperature and the temperature after inoculation are reported each morning in order that the next dose may be gauged. If the temperature remains high (over 101°F.), the treatment is postponed until it returns to normal and the same, or a smaller dose, is given at the next inoculation.

### *Trial and Whole Bleedings*

Trial bleedings are taken immediately before the first and the fourth series of inoculations, then before every second series, and when tests

indicate the serum is approaching the required potency, before every series. Whole bleedings are commenced after three months' immunization, or as soon afterwards as the titer is satisfactory. While serum may reach a titer equal to or above the minimum standard after a few series of injections, it has not been considered advisable to distribute serum which has been obtained from a horse under immunization less than three months. Whole bleedings (seven to nine liters) are taken on the sixth day after the last inoculation. Inoculations are resumed on the fifth day after bleeding. Two or three series of inoculations, depending on the titer of the serum, are given between each whole bleeding. For technic of bleeding, see "Care and Treatment of Animals—Large Animals," p. 593.

### TREATMENT OF POLYVALENT ANTIMENINGOCOCCUS SERUM

The procedures are those given under "Treatment of Antipneumococcus Sera," p. 417, with the following differences.[5]

No sample for potency tests is withdrawn before the preservative is added. Three cubic centimeters of cresol per liter (0.3 per cent) are added as a preservative. The cresol is added in the form of an emulsion as follows: Transfer the serum to a graduated precipitating jar, previously filled with 1-per-cent crude cresol for several hours and then rinsed with sterile distilled water just before use. Emulsify the required volume of cresol in a volume of distilled water equal to 2 per cent of the total quantity of serum. Shake the mixture thoroughly in a separatory funnel and add it at once, but very gradually, to the serum, stirring constantly by means of an electric stirrer. Pour the cresolized material into fresh sterile bottles. Withdraw from one bottle 5 cc. for potency tests and store the material in the cold room until filtered. Since precipitation may occur even after several months, sterile serum is usually not filtered until shortly before bottling, otherwise a second filtration is often necessary.

Serum for therapeutic use is dispensed in 20-cc. volumes. A record of potency (fig. 46) is enclosed in each package. The form for this is filled out and sent to the filling and boxing group. The serial number of the serum and return date are entered on the labels. The serum is considered satisfactory for therapeutic use for eighteen months from the date of the last satisfactory test if kept under proper conditions.

[5] Sera from different bleedings from the same horse or from two or three horses are now frequently pooled on the basis of their agglutinative titers, provided the bleedings were taken within a period of three months.

Serum that has been stored for some time should be carefully examined before being sent out. While some precipitation is unavoidable, serum which shows a considerable amount on being shaken should be refiltered before distribution. Potency tests need not be repeated, unless the previous tests showed the titer to be near the minimum standard.

Serum for diagnostic use is dispensed in 5-, 10-, and 15-cc. volumes. Outdated therapeutic serum, or occasionally, serum, the titer of which is below the standard for therapeutic purposes, is usually available for diagnostic use. The outdated serum is pooled, refiltered, tested, and dispensed. Each bottle is labeled "For diagnostic use" and with the kind and lot number of the serum.

ANTIMENINGOCOCCUS SERUM NO. 106

Potency as determined by the agglutination titer
Tested 6-23-24

| STANDARD STRAIN | MINIMUM STANDARD | STANDARD SERUM NUMBER 58 | TEST SERUM NUMBER 106 |
|---|---|---|---|
| A | 5000 | 12000 | 12000 |
| B | 2000 | 3000 | 5000 |
| C | 3000 | 3000 | 4000 |
| D | 1500 | 2000 | 4000 |

*Note:* The standard serum approximates the minimum titer required in New York State.

FIG. 46. RECORD OF POTENCY

## PRODUCTION OF MONOVALENT RABBIT SERA

Standard monovalent sera, groups I, II, and III, are produced for use in routine diagnostic and standardization tests. A small supply of monovalent sera produced against atypical strains (at least three or four, including a strain classified by Gordon as type IV) is maintained. Sera against additional strains are produced as required.

**Immunization of Rabbits.**—Use normal rabbits weighing preferably from 1800 to 2200 grams. Before commencing immunization, take about 5 cc. of blood from the ear vein, and hold for comparison if required. Follow the schedule of doses[6] and bleedings given below, making all injections intravenously and using live culture throughout.

1st and 3rd days: $\frac{1}{50}$ of a slant
5th and 12th days: $\frac{1}{25}$ of a slant

[6] With certain strains of meningococcus it may be necessary to vary the dosage.

14th day: $\frac{1}{15}$ of a slant
16th day: $\frac{1}{10}$ of a slant
21st day: Take a trial bleeding
22nd day: Take a whole bleeding if titer of trial bleeding is sufficiently high.

To prepare the cultures, inoculate a dextrose-serum-agar slant (150 by 19 mm.) with the strain selected for immunization and incubate from sixteen to twenty-four hours. Make a slide preparation, stained by Gram's method, and examine. If satisfactory, inoculate two dextrose-serum-agar slants and use the remainder of the growth for agglutination tests. After from sixteen to eighteen hours' incubation make a slide preparation, stained by Gram's method, from one transplant. Add 4 cc. of salt solution (0.85 per cent) to the culture and loosen with a loop. Filter 2 cc. of suspension through a cotton filter previously moistened with 1 cc. of salt solution. Wash the filter with 2 cc. of salt solution. Dilute the filtered suspension (corresponding to the entire slant in 10 cc.) so that the required dose is contained in 1 cc. (for $\frac{1}{10}$ of a slant 1 + 4; $\frac{1}{25}$, 2 + 3; $\frac{1}{15}$, 2 + 1). Inject 1 cc. of the dilution. Sixteen to eighteen hours before the next dose is to be prepared, inoculate two dextrose-serum-agar slants from the second culture, one for the rabbit dose and one as a seed for further transplants.

Bleed out the rabbit as described under "Use of Experimental and Test Animals," p. 40. Allow the clot to form, and if necessary, rim with a sterile pipette, and place in the cold room overnight.

**Treatment of Serum.**—When the serum has separated from the clot, usually on the following day, draw off the serum into 50-cc. centrifuge tubes and if necessary centrifugalize to throw down the cells. For further treatment see "Production and Standardization of Immune Sera," p. 498.

**Standardization of Serum.**—The procedures followed are those given under "Standardization of Polyvalent Serum," p. 436. The serum is generally tested in dilutions from 1:200 to 1:2400 at intervals of about 200. The four standard strains are always used in these tests. In specifying the titer of a serum, allowance is made for differences in technic and for possible deterioration.

### *Permanent Records*

Immunization of rabbits: A card (4 by 6 in.) is kept for each animal, giving rabbit number and weight, group and number of the culture used, date of each inoculation, and dose, and date of each bleeding.

Standardization tests: The records are similar to those for standardization

tests of polyvalent serum (p. 440), except that no standard serum is used, and the test on each serum is put on a separate card which is filed with the immunization card.

## STANDARDIZATION OF POLYVALENT SERUM

Polyvalent antimeningococcus serum is standardized by determining its agglutinative action against the four homologous standard group strains in comparison with the agglutinative activity of a standard control serum. The titer of the serum distributed for therapeutic use should exceed that of the standard control serum; under no conditions is serum distributed which fails to agglutinate the standard cultures A, B, C, and D in dilutions of 1:5000, 1:2000, 1:3000, and 1:1500 respectively (the minimum requirement).[7]

While the agglutinative reaction is inadequate as a measure of the therapeutic strength, it suggests the degree of polyvalency, and sera of high agglutinative titer have proved effective as therapeutic agents. The reaction has, therefore, been adopted by this laboratory as the standard of potency in the absence of a more satisfactory index. The bacteriotropic action of sera from different horses is tested occasionally for purposes of study and comparison, but the results obtained with this test in this and other laboratories are not considered sufficiently uniform to warrant its inclusion as a routine test. There is no satisfactory protection test and the practical value of the complement-fixation test has not been demonstrated.

### AGGLUTINATION TESTS

Varying dilutions of the sera to be tested and of the standard control serum are combined with equal volumes (0.3 cc. each) of uniform suspensions of the strains used in immunization. The reactions are controlled by combining the suspensions with monovalent group sera, normal horse serum, and salt solution. The tubes containing the mixtures are incubated at 55°C. for twenty-four hours at the end of which time readings are made. The agglutinative range of the sera is further controlled by occasional tests using meningococcus strains other than those used for immunization, particularly atypical strains and strains recently isolated.

[7] The standard of potency required for therapeutic serum was established after repeated comparative tests of different sera. Following the precedent established with the unofficial standard guiding practices in the standardization of the polyvalent 60-strain sera, the first or original standard, established in 1918, conservatively designated as a minimum of potency, agglutinative action in a 1:800-dilution against the four standard cultures when tested and compared with the standard serum. In October, 1922, a new and more potent control serum was selected and the present minimum standard of potency, adjusted for each standard culture, was adopted.

Four or five sera, including the standard, can conveniently be tested on one day. Usually nine dilutions of a serum are tested, the same dilutions being used for all cultures. Sera for therapeutic use are frequently tested in dilutions of from 1:200 to 1:12,000 or higher (as 1:200, 1:500, 1:1000, 1:1500, 1:2000, 1:4000, 1:6000, 1:8000, 1:10,000, and 1:12,000). The dilutions used for the normal horse serum control are 1:200 and 1:500; for monovalent rabbit control sera, such dilutions (generally 1:200 and 1:800 or 1:1000) as will give characteristic reactions with their homologous standard strains, and will indicate any cross agglutination.

*Standard Serum*

For the standard control, a serum not less than four months old, which has been shown by repeated tests to have a titer closely approximating (but never below) the minimum requirements is chosen. Since the decrease in agglutinative titer is usually very gradual, the serum will probably remain satisfactory for several years.[8] When comparative tests with later sera, previously tested with the standard, indicate that deterioration is becoming marked, a new standard is chosen.

*Test Sera*

Sera from trial bleedings are tested as soon as possible after being taken. A preliminary test is made of a sample from each whole bleeding taken after the preservative is added. The final standardization test of a serum is made on the filtered material shortly before it is bottled for distribution. If the serum is distributed within three months of a bleeding, the test on the filtered material may be omitted.

*Procedure*

**Bacterial Suspensions.**—Use 16- to 18-hour cultures grown on dextrose-serum-agar slants. If the growth is good and there is no evidence of contamination, add 2 cc. of salt solution to each culture and loosen the growth with a loop. Then transfer the suspension into previously sterilized, unplugged tubes (150 by 19 mm.) and proceed to dilute each suspension so that all are of a uniform turbidity as shown by comparison with barium-sulfate standard No. 3 (corresponding approximately to a 2000-million meningococcus suspension). For preparation of standards see "General Instructions," p. 625. Always shake the standard suspension well just before using, as barium-sulfate settles

[8] The standard serum now in use is from a bleeding taken in May, 1920. It was adopted as the standard in October, 1922, and is still satisfactory.

rapidly. Add salt solution to the concentrated bacterial suspension and mix thoroughly by drawing it up and down in a pipette. Repeat the procedure until the bacterial suspension corresponds to the standard.

TABLE 30

*Serum dilutions*

| VOLUME OF SERUM OR SERUM DILUTION | VOLUME OF SALT SOLUTION | FIRST DILUTION | FINAL DILUTION |
|---|---|---|---|
| *cc.* | *cc.* | | |
| 0.5 (undil.) | 4.5 | 1:10 | |
| 1.0 (1:10 dil.) | 4.0 | 1:50 | 1:100 |
| 1.0 (1:10 dil.) | 9.0 | 1:100 | 1:200 |
| 0.1 (undil.) | 9.9 | | |
| 2.0 (1:100 dil.) | 1.0 | 1:150 | 1:300 |
| 1.0 (1:100 dil.) | 1.0 | 1:200 | 1:400 |
| 1.0 (1:100 dil.) | 1.5 | 1:250 | 1:500 |
| 1.0 (1:100 dil.) | 2.0 | 1:300 | 1:600 |
| 1.0 (1:100 dil.) | 2.5 | 1:350 | 1:700 |
| 1.0 (1:100 dil.) | 3.0 | 1:400 | 1:800 |
| 1.0 (1:100 dil.) | 3.5 | 1:450 | 1:900 |
| 1.0 (1:100 dil.) | 4.0 | 1:500 | 1:1,000 |
| 1.0 (1:100 dil.) | 4.5 | 1:550 | 1:1,100 |
| 1.0 (1:100 dil.) | 5.0 | 1:600 | 1:1,200 |
| 1.0 (1:100 dil.) | 5.5 | 1:650 | 1:1,300 |
| 1.0 (1:100 dil.) | 6.0 | 1:700 | 1:1,400 |
| 1.0 (1:100 dil.) | 6.5 | 1:750 | 1:1,500 |
| 1.0 (1:100 dil.) | 7.0 | 1:800 | 1:1,600 |
| 1.0 (1:100 dil.) | 7.5 | 1:850 | 1:1,700 |
| 1.0 (1:100 dil.) | 8.0 | 1:900 | 1:1,800 |
| 1.0 (1:100 dil.) | 8.5 | 1:950 | 1:1,900 |
| 1.0 (1:100 dil.) | 9.0 | 1:1,000 | 1:2,000 |
| 0.5 (1:100 dil.) | 7.0 | 1:1,500 | 1:3,000 |
| 0.5 (1:100 dil.) | 9.5 | 1:2,000 | 1:4,000 |
| 0.2 (1:100 dil.) | 4.8 | 1:2,500 | 1:5,000 |
| 0.2 (1:100 dil.) | 5.8 | 1:3,000 | 1:6,000 |
| 0.2 (1:100 dil.) | 6.8 | 1:3,500 | 1:7,000 |
| 0.2 (1:100 dil.) | 7.8 | 1:4,000 | 1:8,000 |
| 0.2 (1:100 dil.) | 8.8 | 1:4,500 | 1:9,000 |
| 0.2 (1:100 dil.) | 9.8 | 1:5,000 | 1:10,000 |
| 0.2 (1:100 dil.) | 10.8 | 1:5,500 | 1:11,000 |
| 0.2 (1:100 dil.) | 11.8 | 1:6,000 | 1:12,000 |

**Serum Dilutions.**—If precipitate is in suspension in the serum, centrifugalize the serum. Make the dilutions required according to table 30.

Set up the necessary number of tubes (150 by 19 mm.) in racks. Record the serum number or horse number and date of bleeding, and

serum dilution on the first tube of each series; the serum dilution on the other tubes. With a 1-cc. pipette, put the serum (0.5 cc. for the first dilution of horse serum, 0.1 cc. for rabbit serum) in the first tube and discard the pipette. Add the required volume of 0.85-per-cent salt solution. With a fresh pipette, mix the dilution thoroughly by drawing up and discharging several times, then transfer the required volumes for the rest of the dilutions. Finally add the required volume of salt solution to the tubes.

**The Test.**—Use unplugged tubes (11 by 75 mm.) free from cloudiness or scratches, previously sterilized in covered cans. Set up the tubes in copper racks. Arrange the racks so that all the tubes to contain a given culture are in a row from left to right; those to contain a given serum dilution in a row from front to back. Begin at the extreme left with salt solution, followed by dilutions of normal horse serum, monovalent rabbit sera, standard control serum, and finally the dilutions of the sera to be tested, starting each series with the lowest dilution at the left. Mark the limits of each serum by red lines across the tops of the racks and write the serum number on each rack between the lines. Write the culture number at the extreme left of each row. Mark the ends of the racks that come together, with identifying numbers.

Beginning at the extreme right with the highest dilution of the last test serum, mix thoroughly each serum dilution in turn and add 0.3 cc. to each tube in a row from front to back. (The same pipette may be used from higher to lower dilutions of the same serum.) In each tube of the extreme left-hand row, put 0.3 cc. of salt solution. Add 0.3 cc. of one of the bacterial suspensions to each tube in the front row from left to right. Repeat with the second suspension in the second row and continue with the other suspensions. After flaming the mouths of the tubes carefully, shake the racks well, and place them in the incubator for twenty-one hours at 55°C. Then shake the racks again, and return them to the incubator for three hours before making readings. (To prevent evaporation a pan of water is always kept in the incubator.)

### *Reading and Recording Results*

Always read the reactions by electric light against a dark background to insure uniformity. Lift one or two tubes at a time from the rack, and holding them at an angle, examine before and after shaking. Record complete agglutination with clear supernatant fluid and all large clumps, as 4+; clear, or nearly clear, supernatant fluid with definite clumping, as 3+; supernatant fluid not clear, but definite clumping, as 2+; supernatant fluid not clear with very small clumps

definitely visible to the unaided eye, as +; questionable reactions as ±; and uniformly turbid suspension with no clumping, as −. Enter the readings as made directly on the permanent-record cards. (It is convenient to have a second person make the entries.)

The titer of the serum is the maximum dilution in which clumping definitely visible to the unaided eye when read by electric light against a dark background ("+" reaction), occurs. If there is any uncertainty regarding the test, it should be repeated promptly; when two or more tests have been made on one serum it may be advisable to take the average of the titers obtained as the potency of the serum. The return date is then based on the first of the tests. Should irregularities in the reactions of a culture with the monovalent sera or salt solution occur, the test with that culture is considered indeterminate.

### *Permanent Records*

Standard strains: A separate card (4 by 6 in.) is kept for each culture on which are recorded the group, number, and history of the strain, as well as any observations of general interest. The results of the agglutination tests made on cultures recovered from colonies are recorded on a card (4 by 6 in.); those of the fermentation tests made on the same cultures, on a second card. These cards are filed together according to dates.

Immunization of horses; serum records: These records correspond to those described under "Permanent Records" in "Production and Standardization of Antipneumococcus Sera," p. 427.

Standardization tests of polyvalent sera: On cards (4 by 6 in.) are recorded in regard to each test: kind of product, dates tested and read, numbers of the control sera, horse and serum numbers of the test sera and whether filtered or unfiltered (in the case of trial bleedings "T.B." and the date of bleeding are substituted for the serum number), serum dilutions, and culture numbers. The salt solution and serum dilutions are arranged from left to right across the top of the cards, and the culture numbers in a column at the left of each card, in the order used in setting up the tubes. The readings are recorded directly on the cards and each card is initialed by the workers preparing and reading the test.

Immunization of rabbits; standardization tests of monovalent sera: See "Permanent Records," p. 435.

## CHAPTER 3

# PRODUCTION AND STANDARDIZATION OF ANTI-DYSENTERY SERA

Polyvalent antidysentery serum for therapeutic and diagnostic use is produced by the immunization of horses against strains selected as representative of the principal groups of the dysentery bacillus. Monovalent sera for diagnostic use and for controlling the type specificity of cultures in routine standardization tests are obtained by the immunization of rabbits and goats against a single strain. The potency of a serum is gauged by its agglutinative action and also, in the case of therapeutic serum, by its neutralizing properties for the toxins of organisms belonging to the Shiga group. The neutralizing action is measured by protection tests in animals.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

### *Standard Strains*

At present the following standard strains are used in the production of sera.

Shiga group: "Shiga F." Collection No. 114F. Received in 1915 from the New York City Research Laboratory.

"Pasteur Shiga." Collection No. 114V/1. Received in 1923 from the Pasteur Institute, Paris.

Mannite-fermenting group: "Flexner D." Collection No. 114D. Received in 1915 from the New York City Research Laboratory.

"Mt. Desert E." Collection No. 114E. Received in 1915 from the New York City Research Laboratory.

In addition to the standard cultures a number of different stock strains obtained from various sources are maintained for use when required. New cultures are added occasionally so that recently isolated strains may always be available.

**Maintenance of Strains.**[1]—All strains are maintained on beef-infusion agar by monthly transfers.

Incubate the fresh transfers at from 35 to 37°C. for from eighteen

[1] The standard strains are maintained by the group (bacterial-vaccine group) responsible for the standardization of the therapeutic polyvalent serum by the agglutination method, and for the production and standardization of monovalent diagnostic sera.

to twenty-four hours. Make slide preparations stained by Gram's method and examine, then store the cultures in the cold room. Always retain the cultures from the preceding month and resort to fishings only if the strain would otherwise be lost.

**Identification and Purity Tests.**—Every six months plate the standard cultures and other cultures in active use on Endo's medium; fish two colonies from each to triple-sugar Andrade slants. Select at least one fishing, make a slide preparation, and stain by Gram's method. Then inoculate tubes containing dextrose, mannite, and maltose, serum water for fermentation tests, a broth tube for a motility test, and a beef-infusion-agar slant for agglutination tests. Read the fermentation tests after twenty-four and forty-eight hours' incubation (see "Production and Standardization of Diphtheria Toxin," p. 309). Shiga strains ferment dextrose only; Mt. Desert, dextrose and mannite; and Flexner, dextrose, mannite, and maltose. Make hanging-drop preparations for the motility test as described in "General Bacteriological Technic," (p. 11). Use monovalent Flexner, Mt. Desert, and Shiga F rabbit sera for the agglutination tests. At least once each year plate and test the special stock strains, always before work with them is resumed.

As a partial control of the identity of cultures in active use, make frequent transfers to triple-sugar Andrade medium. Agglutination tests of trial bleedings and therapeutic sera also act as controls of the type specificity of the strains.

## IMMUNIZATION OF HORSES

The two standard Shiga strains and the standard Flexner and standard Mt. Desert strains are always used in the immunization of horses. More strains may be added if the potency and polyvalency of the serum is found to be inadequate.[2] The bacterial suspensions of the Shiga strains may be supplemented by toxic filtrates, prepared from broth cultures, injected subcutaneously if the antitoxic properties are deficient. The cultures, grown on beef-infusion-agar slants, are suspended in salt solution and the suspensions pooled and injected intravenously. Series of three daily injections are given with an interval of seven days between series. For the first three series, killed organisms are used. The doses

[2] Until June, 1923, fourteen representative strains of the Shiga and mannite-fermenting groups were used in the production of polyvalent antidysentery serum. In the light of results obtained by a radical reduction of the number of strains used in the production of antimeningococcus serum, the number of dysentery strains was reduced to four. Equally potent sera have been produced by the latter method.

are increased very gradually. Whole bleedings may be commenced after ten weeks if the agglutinative titer is equal to the control serum. (See also footnote 3, p. 444.)

### *Preparation of Cultures*

On the day before the first injection of a series, make slide preparations from the parent cultures (which should not be more than one week old), stain by Gram's method, and examine. If satisfactory, inoculate beef-infusion-agar slants (150-by-16-mm. tubes). On the following morning prepare the doses for the horses as described in the chapter "Production and Standardization of Antimeningococcus Sera," p. 431, except that 2 cc. of salt solution is added to each slant instead of 3 cc., and the filter is moistened with a volume of salt solution equal to 1 cc. for each culture and is washed with the same volume.

**Heated Suspension for Preliminary Injections.**—Heat a suspension of the organisms prepared as above, in a water-bath at 60°C. for thirty minutes (see "Production of Antipneumococcus Sera," p. 413). Streak a blood-agar plate and inoculate one aerobic tube of sterility-test broth with the heated suspension. Dispense the suspension in single doses as required, under aseptic precautions.

When heated cultures are given, enough vaccine for the three doses of a series may be prepared on the day preceding the first inoculation.

### *Injection and Dosage*

Injections are made intravenously. Series of three daily injections are given with an interval of seven days between series. For the first series, a heated suspension of the standard Flexner culture is used. The dose on the first day is 1 cc. On the two following days it may be increased slightly, depending on the temperature reactions. For the second and third series, heated suspensions of the standard Shiga strain No. 114F are used. One cubic centimeter of suspension is usually given in each injection of the second series and in the first injection of the third. If there is little or no reaction, the dose may be slightly increased for the two following inoculations. The fourth series is started with 0.1 cc. of a suspension of living Flexner culture, and the dose slightly increased on the second and third days. For the fifth series, 0.1 cc. suspension of living Shiga F culture alone is given for the first inoculation and the dosage in the second and third is increased cautiously owing to the greater toxicity of strains belonging to this group. In later series, the other standard strains (Mt. Desert E and Shiga Pasteur)

are added and the dosage is increased. The increase should not be so rapid, however, as to impair markedly the general physical condition of the horse.

*Temperature Reactions. Trial and Whole Bleedings*

The procedures are those given in the "Production of Antimeningococcus Sera," p. 432, except that whole bleedings may be commenced after ten weeks' immunization if the potency of the serum is satisfactory.

### TREATMENT OF POLYVALENT ANTIDYSENTERY SERUM

The treatment of antidysentery serum is the same as that of antimeningococcus serum (see "Production and Standardization of Antimeningococcus Sera," p. 433), except that no slip giving the agglutinative titer of the serum is enclosed in the package for distribution.

### PRODUCTION OF MONOVALENT RABBIT SERA

For the production and standardization of monovalent, antidysentery rabbit serum see "Production and Standardization of Immune Sera," p. 495.

### STANDARDIZATION OF POLYVALENT SERUM

Polyvalent antidysentery serum is standardized by determining its agglutinative titer against the standard dysentery strains Shiga F, Flexner D, and Mt. Desert E; and its neutralizing action when combined with toxic filtrate from broth cultures of a Shiga strain, at present the Pasteur Shiga. The serum is also tested from time to time for its agglutinative action, against other and preferably recently isolated strains of the mannite-fermenting group. The agglutinative titer against all the strains tested should equal or exceed that of a control serum which is used in every test for purposes of comparison. No definite standard of antitoxic potency has as yet been established.[3]

### AGGLUTINATION TESTS

The procedures are those used in agglutination tests of antimeningococcus sera (see "Production and Standardization of Antimeningococcus Sera," p. 436), with the following modifications.

[3] Since in our experience of over eight years, but three strains of "Shiga" bacillus have been isolated by this laboratory from cases in New York State, serum with a high agglutinative titer for all the standard strains but somewhat deficient in neutralizing substances against "Shiga" toxin has at times been used. Serum with a high neutralizing value for the Shiga toxin, however, is always available for distribution wherever organisms of the nonmannite-fermenting group are isolated.

Dilutions of test sera of from 1:200 to 1:32,000 are generally used. The dilutions used for the normal horse serum control are 1:500 and 1:1000; for monovalent rabbit control sera, such dilutions as will give characteristic reactions with their homologous standard strains, generally 1:2000 and 1:4000, and will indicate any cross agglutination, generally 1:200.

While no definite standard of potency has been established, a control serum representing a satisfactory agglutinative titer is used. At the present time the titer of the control serum approximates 1:8000 against each of the standard strains.

TABLE 31

*Serum dilutions*

| VOLUME OF SERUM OR SERUM DILUTION | VOLUME OF SALT SOLUTION | FIRST DILUTION | FINAL DILUTION |
|---|---|---|---|
| *cc.* | *cc.* | | |
| 0.5 (undil.) | 4.5 | 1:10 | 1:20 |
| 1.0 (1:10 dil.) | 9.0 | 1:100 | 1:200 |
| 1.0 (1:100 dil.) | 1.5 | 1:250 | 1:500 |
| 1.0 (1:100 dil.) | 4.0 | 1:500 | 1:1,000 |
| 1.0 (1:100 dil.) | 9.0 | 1:1,000 | 1:2,000 |
| 0.5 (1:100 dil.) | 9.5 | 1:2,000 | 1:4,000 |
| 0.5 (1:100 dil.) | 14.5 | 1:3,000 | 1:6,000 |
| 0.1 (1:100 dil.) | 3.9 | 1:4,000 | 1:8,000 |
| 0.1 (1:100 dil.) | 4.9 | 1:5,000 | 1:10,000 |
| 0.1 (1:100 dil.) | 5.9 | 1:6,000 | 1:12,000 |
| 0.1 (1:100 dil.) | 7.9 | 1:8,000 | 1:16,000 |
| 0.1 (1:100 dil.) | 9.9 | 1:10,000 | 1:20,000 |
| 0.1 (1:100 dil.) | 11.9 | 1:12,000 | 1:24,000 |
| 0.1 (1:100 dil.) | 15.9 | 1:16,000 | 1:32,000 |
| 0.1 (1:100 dil.) | 19.9 | 1:20,000 | 1:40,000 |

The bacterial suspensions used are made from 18- to 24-hour cultures. Table 31 may be used in making serum dilutions.

The tubes containing the serum and suspension are incubated at 55°C. for two hours, and then placed in the cold room overnight. The racks are not shaken at the end of twenty-one hours.

### TESTS OF ANTITOXIC POTENCY

The antitoxic potency of the sera from different horses is tested from time to time by injecting rabbits intravenously with mixtures of a multiple of the lethal dose of a previously standardized toxic filtrate (*B. dysenteriae* Shiga) and varying volumes of the test serum. At the

same time, control rabbits are injected with toxin alone and with mixtures of the same dose of toxin and control serum. The method[4] as used at present does not fully meet the requirements for accurate standardization, owing largely to difficulty in obtaining a sufficiently potent and stable toxin. Different strains of the Shiga bacillus are being studied for the purpose of securing a more highly toxigenic culture, and the use of dried toxin is being tried. Control serum and dried toxin have been received from the U. S. Hygienic Laboratory. The use of mice as the test animals is under study.

### *Permanent Records*

The records for the preparation of the serum and agglutination standardization tests are similar in form to those described in "Production and Standardization of Antimeningococcus Serum," p. 440.

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[4] The procedures used have in general been based on the resolutions adopted at the International Serological Conference held in Paris in November, 1922, and on subsequent published reports of the League of Nations Health Organization.

## SECTION V

## CHAPTER 1

# PREPARATION OF DIPHTHERIA TOXIN-ANTITOXIN MIXTURE

Diphtheria toxin-antitoxin mixture is prepared for use in active immunization against diphtheria. The toxin and antitoxin are combined in such proportions as to give a slightly toxic mixture when diluted with physiological salt solution to contain 0.1 L+ dose of toxin per cubic centimeter.[1] The toxicity of each mixture is determined by tests on guinea pigs. Further tests for the purpose of gauging the antigenic activity of the material are made on the surviving animals. Preparation of mixture has been assigned to the diphtheria-toxin production group.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

### *Diphtheria Toxin*

Diphtheria toxin which has been held for at least one and a half years at cold-room temperature is used in the preparation of mixture. The potency of the toxin should be such that 5 L+ doses or more are contained in each cubic centimeter. In no case should a toxin containing less than 3 L+ doses be used. (The toxins actually used have contained from 5 to 7 L+ doses.) Toxin with an original M.F.D. of 0.003 to 0.002 cc. is generally selected, but material with a lower titer may be used provided the L+ dose comes within the requirements. The toxin is prepared as described in the chapter "Production and Standardization of Diphtheria Toxin," p. 308. It contains 0.5 per cent phenol. In order to insure that a lot of toxin provisionally selected for use in mixture does not contain protein substances which may in-

[1] Preparation of diphtheria toxin-antitoxin mixture was commenced in 1917. Mixture somewhat less toxic than that now prepared was made with undiluted toxin containing approximately 5 L+ doses per cubic centimeter. Later the mixture was diluted to contain 3 L+ doses. A mixture containing 0.1 L+ dose (152) was adopted when data obtained from clinical and animal tests of mixtures prepared in this laboratory indicated that it was equally, if not more, effective.

duce unusually marked reactions, a satisfactory report on the local reactions induced in a small number of persons by mixture containing the toxin, is required before the toxin is finally reserved for routine lots. Up to the present time, it has not been necessary to withdraw any lot of toxin provisionally selected.

### *Diphtheria Antitoxin*

An antitoxin with a titer of from 1200 to 1400 units is set aside for mixture. The antitoxin is restandardized very carefully against the standard toxin by the antitoxin-production group. The titer is based on the dose which protects a guinea pig weighing 250 grams against 1 L+ dose of the toxin for practically the same time as does one unit of standard antitoxin. In order to detect possible deterioration, retests are made about every six months. For procedure see "Production and Standardization of Diphtheria Antitoxin," p. 348.

### *Determination of the L+ Dose of Toxin*

The toxin is standardized by determining its L+ dose against the antitoxin with which it is to be combined. A preliminary test based on the original M.F.D. is usually made at intervals of about 0.02 cc. If from this test a fairly definite estimate of the probable titer can be made, a second test is made at intervals of 0.01 cc.; four doses are usually tested, four or five guinea pigs being used for each of the two or three doses close to the L+ dose, and one or two for each of the doses selected for the outside limits. That dose which kills all the animals inoculated with it in ninety-six hours or somewhat less, is chosen as the L+ dose. Certain variations are, however, permissible. Thus, one out of four guinea pigs may survive somewhat longer than ninety-six hours. Further tests may at times be necessary. For procedure, see "Production and Standardization of Diphtheria Antitoxin," p. 349.

### *Preparation of Mixture*

At present the toxin and the antitoxin, the latter in a 1:100-dilution, are combined in the proportion of about 104 to 107 per cent of the L+ dose of toxin to one unit of antitoxin. The mixture is diluted with phenolized salt solution so that 0.1 L+ dose of toxin is contained in each cubic centimeter. Approximately eight liters of material are prepared in one container, three or more containers, each comprising one lot, usually being prepared at one time. The toxin, antitoxin, and salt solution must be cold when they are combined. After

the mixture has been allowed to stand for a short time, it is filtered by pressure.

To obtain mixtures of the required toxicity, the percentage of the L+ dose of toxin combined with a unit of antitoxin must be varied somewhat for different lots of toxin and antitoxin. When a lot of toxin or of antitoxin is used for the first time, mixtures containing different percentages are made. From the results of tests of these mixtures, the optimum percentage is determined as closely as possible. Further modifications are made as necessary; usually a slight change in the value for the L+ dose is sufficient.[2] Since it has been found that the toxicity of the mixture first passed through a filter, especially a new filter, is usually somewhat lower, due to adsorption, the proportion of toxin in the mixture to be filtered first is slightly increased; usually by a change of 0.01 in the L+ dose. The volumes of toxin and antitoxin are calculated as in the following example.

Assume that 7800 cc. of mixture is to be made from toxin having an L+ dose of 0.165 cc. and antitoxin containing 1225 units per cubic centimeter.

$\frac{1}{10}$ L+ dose = 0.0165 cc.

0.0165:1 = x:7800

x = 128.7 cc. of toxin required.

$$\frac{128.7}{1.07 \times 0.165} \times \frac{1}{1225} = 0.595 \text{ cc. of antitoxin required}$$

= 59.5 cc. of a 1:100-dilution.

The computations are made on a working card (5 by 8 in.) on which are also entered at the time the mixture is prepared and filtered the required data and the initials of the workers performing the various steps. These records are kept for a year.

Special Apparatus and Supplies.—Sterile 8-liter white-glass bottles graduated at 100-cc. intervals from 7500 to 7800 cc.

2 100-cc. graduates (one reserved for toxin and one for antitoxin).

100-cc. calibrated, volumetric flasks with glass stoppers.

Sterile 1-liter and 500-cc. Erlenmeyer flasks.

Always use the same graduates and volumetric flasks for measuring and keep them for this purpose. After use, rinse with water; fill with cleaning solution and allow to stand half an hour or more; then rinse again with water followed by alcohol, and drain. On the day before the mixture is to be made, fill with 5-per-cent phenol for several hours and allow to drain overnight.

Salt solution: Freshly prepared salt solution (0.85 per cent), in 4-liter volumes; also two liters for diluting antitoxin and rinsing. Sterile distilled water to replace loss by evaporation.

A day or two before the mixture is to be prepared, replace the cotton plugs in bottles of salt solution with sterile rubber stoppers and add to

[2] Should more than a slight change be necessary, the percentage would be revised. In our experience, however, this has not as yet been necessary.

each enough 90-per-cent phenol solution to give 0.4-per-cent final concentration (16.6 cc. to 4000 cc.). Shake thoroughly. Then bring the level of the solution in each bottle to the original volume (indicated by the lower edge of a strip of adhesive tape) with sterile distilled water and shake thoroughly. Enter in red, on the label, "0.4 per cent phenol" and the date, and place in the cold room until required.

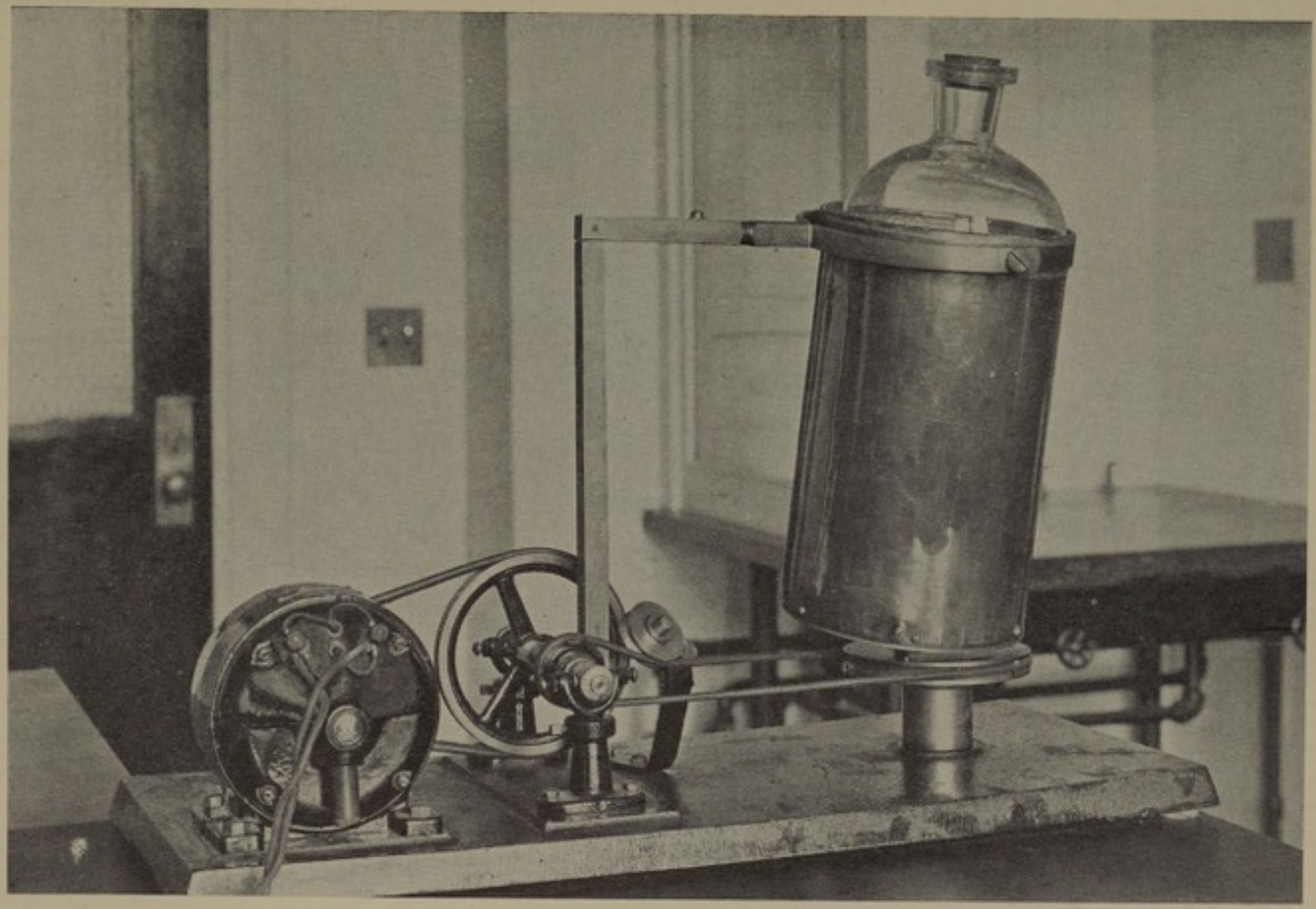
On the day the mixture is made, prepare somewhat more than the required volume of a 1:100-dilution of antitoxin. Use the 100-cc. calibrated flasks and a standard pipette. Mix each dilution thoroughly, then pool the diluted antitoxin in an Erlenmeyer flask.

Pipette or siphon, with aseptic precautions, the toxin for the day's work, into a small sterile bottle. (When more lots of mixture are to be prepared within a few days, the required amount of toxin for each day's work may be transferred to separate bottles, which are held in the cold room.) From the small bottle, pipette into the toxin graduate the required volume of toxin for the first bottle of mixture and pour it into a 1-liter Erlenmeyer flask. Transfer the toxin for the other bottles in the same way and protect the flasks with paper caps.

Pipette into the antitoxin graduate the diluted antitoxin for the first bottle and add it to the toxin in the flask. Mix thoroughly by rotating first in one and then in the other direction, taking care to include any drops on the sides of the flask. Rinse the graduate with salt solution, adding the rinsings to the mixture. Set the graduate aside to drain. Before using for the next lot, rinse with antitoxin dilution.

Carefully pour the mixture into an 8-liter bottle which has been chilled in the cold room. Rinse the flask twice, adding the rinsings to the mixture. Rotate a bottle of cold, phenolized salt solution to insure uniform solution and wipe the neck with 1-per-cent crude cresol. Pour somewhat less than the required volume of salt solution into the bottle of mixture. Siphon the remainder to bring the volume to the "7800-cc." graduation from a bottle of salt solution prepared for this purpose. Mix in the electric mixing apparatus for five minutes (See: plate VI). Then place in the cold room. Prepare the other bottles of mixture in the same way.

Allow the mixtures to stand in the cold room for at least one hour, then filter by pressure through a final filter candle. (For procedure, see"Filtration of Biologic Products," p. 517.) Hold the filtered mixture in the cold room for two days before making the usual sterility tests and removing a sample for the first animal test. See "Sterility Tests of Biologic Products," p. 543.



Mixing apparatus: designed to mix without unnecessary agitation. A copper container $7\frac{3}{4}$ inches by 12 inches (inside dimensions) held at the top by a hinged universal, and driven from below by a crank. The crank pin is a ball fitting in a socket attached to the center of the bottom of the container, and adjustable on the pulley to three different throws. The machine is driven at a speed of 140 R.P.M. by a $\frac{1}{8}$-horse-power motor. The belt is held taut by an idler and spring. The apparatus is mounted on a cast-iron base.

*Standardization of Mixture*

At least two series of tests of toxicity are made on material prepared at one time. The first test is made two days after the mixture is prepared when usually only the second bottle is tested. Three guinea pigs are injected with 5 cc. each. If this test is satisfactory, indicating that the toxicity of the mixture is slightly greater than, or equal to, the desired standard for material to be distributed, a second test is made at the end of about three weeks. At this time the material in each bottle is tested; at least three guinea pigs are injected with 5-cc. doses and at least two with 1-cc. doses from each bottle. Mixtures

Lot No.....

DIPHTHERIA TOXIN-ANTITOXIN MIXTURE

Animal Record Date.....Time.....

| | cc. | cc. | cc. | cc. | cc. |
|---|---|---|---|---|---|
| | No. Wt. | No. Wt. | No Wt. | No. Wt. | No. Wt. |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |

FIG. 47. RECORD CARD

which are too toxic are usually held in the cold room until later tests indicate that the toxicity has sufficiently diminished to permit their use. No toxin or antitoxin is added to a mixture for purposes of adjustment.

**Procedure.**—Follow in general the procedure in the chapter "Production and Standardization of Diphtheria Toxin," p. 313, except where specific directions are given in the following text. Use guinea pigs weighing between 230 and 280 grams. Clip a small area at the site of injection of the animals which are to receive 5 cc., so that collodion may be applied; shave the abdomen of those which are to receive 1 cc., so that the local reactions may be more readily observed. Enter

directly on the animal-record card (fig. 47), the date, mixture number, doses to be tested, with the number and weight of the guinea pig used for each. Allow the mixture to reach room temperature and inject it undiluted, measuring the volume from the syringe. Make the injections subcutaneously; in the case of 5-cc. doses in two parts, 3 cc. on one side of the abdominal region and 2 cc. on the other. Apply collodion to the sites of injection.

Weigh the guinea pigs before injection, and those surviving, on the fifth and tenth days after; or on the following day, if these days fall on Sundays or holidays. Inspect the guinea pigs twice daily. For the first four days, observe the general condition of those injected with 5 cc., handling them only if unusual symptoms are noted. At the morning inspection, examine each of the animals that received 1 cc., and after the fourth day, those that received 5 cc., and record on the animal-record card the kind and degree of local reaction, and the extent of the paralytic symptoms, as follows: the degree of redness, as "sl. rd.," "rd.," or "mkd. rd." (slight, definite, or marked redness); of oedema, induration, and necrosis as, "sl. edm.," "ind.," or "nec.," etc.; the degree of paralysis, as "v. sl. par.," "sl. par.," "par.," "mkd. par.," etc. or prostration, as "pros." (The worker making the examination should always report unusual symptoms at once, to the head of the group.) In the afternoon, inspect each cage for the general condition of the animals and record only unusual conditions or deaths. Omit the afternoon inspection on Saturdays, Sundays, and holidays. When examination for removal of dead animals by the night watchman is desired, attach a green tag to the cage. Chloroform animals which show complete prostration due to paralysis, recording the fact on the card. Autopsy all dead animals, entering the findings on the back of the record card. In the case of animals dying within ten days, note especially, lesions typical of diphtheria-toxin poisoning. Discharge surviving animals on the thirty-fifth day, if the test for antigenic activity is complete.

**Estimation of Toxicity.**—The toxicity desired is such that 5-cc. doses induce death in from four to eight days, with typical symptoms of diphtheria poisoning; 1-cc. doses, moderate local reaction and more or less definite paralysis, with death in from twenty to thirty-five days, or survival. Considerable variation is, however, allowed. Thus, if two-thirds of the guinea pigs receiving 5 cc. live over ninety-six hours, and the remaining third die between the eighty-fourth and ninety-sixth hour, the mixture may be used, provided the tests of the 1-cc. doses are satisfactory. On the other hand, mixtures which cause the death of

some of the guinea pigs in from seven to twenty days, or even longer, may be used, provided the tests of antigenic activity[3] are satisfactory. In the case of lots showing marked variation from the desired toxicity, the approval of the bacteriologist in charge of the department is required before the material is selected for distribution.

---

DIPHTHERIA TOXIN-ANTITOXIN MIXTURE

No.....

.....L+ doses per cc. Ratio.....% Date mixed.....Volume.....

Toxin No.....L+ dose.....cc. Volume.....cc. Antitoxin No.....

Units per cc.....Volume.....

Date filtered.....Number of filter.....Volume.....cc. Date prepared.....

Date filled.....Number bottles { 10 cc..... / 5 cc..... } Released.....

Outdated.....Exhausted { 10 cc..... / 5 cc..... }

Remarks:

---

*Animal Tests*

| DATE | DOSE: 5 cc. | DOSE: 1 cc. | ANTIGENIC VALUE | DATE | DOSE: 5 cc. | DOSE: 1 cc. | ANTIGENIC VALUE |
|---|---|---|---|---|---|---|---|
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |

FIG. 48. RECORD CARD

*Preparation of Mixture for Distribution*

Mixture is dispensed in 5- and 10-cc. volumes[4] (see "Preparation of Biologic Products for Distribution," p. 524). The guinea pig inoculated with 3 cc. in the test for harmlessness, acts as a further control of the toxicity of the mixture. Severe or unusual symptoms in the guinea pig, or in the mouse inoculated with 1 cc., are reported im-

[3] For purposes of comparison and study, all lots of mixture during the last two years have been tested for the "immunity index," by the intracutaneous method of Glenny (Brit. Jour. of Exper. Path., 1923, 4, 283 (153)). A uniform "immunity index" of 2 or slightly better has been obtained with few exceptions. At present the subcutaneous method for testing antigenic activity is under study.

[4] A smaller bottle, closed with a rubber stopper through which the syringe needle may be inserted, and containing 3.5 cc., is to be substituted for the bottles containing 5 cc.

mediately by the sterility-test group. The labels printed in red, to distinguish the product from diphtheria antitoxin, give the lot number and the return date.

### *Return Date and Retests*

The mixture, if kept under proper conditions, is considered satisfactory for use for six months from the date of the last satisfactory animal test. When mixtures, which tests have indicated approximate the upper limits of toxicity are held in bulk, an extension of three months may be made with the approval of the bacteriologist in charge of the department. In practice, since the supply of mixture maintained in stock is limited, comparatively fresh material is always distributed.

### *Permanent Records*

General record: A card (5 by 8 in.) is kept for each mixture on which are entered the lot number; number of L+ doses per cubic centimeter, per cent of one L+ dose per unit of antitoxin; date mixed; total volume; number, L+ dose, and volume of toxin; number, titer, and volume of antitoxin; date filtered, number of filter, and volume after filtration; dates prepared (first satisfactory test), and filled, and number of bottles filled; dates released, outdated, and exhausted; and summaries of animal tests of toxicity and antigenic value. A space is provided for remarks. (Fig. 48.)

Animal tests: The animal record cards described under "Procedure of Test," are filed as permanent records.

## CHAPTER 2

# PREPARATION OF STREPTOCOCCUS TOXIN FOR ACTIVE IMMUNIZATION AGAINST SCARLET FEVER

A limited supply of diluted toxin is prepared for the active immunization of persons whom the intracutaneous test has shown to be susceptible to "scarlet-fever" streptococcus toxin. The toxin used is prepared with the standard, scarlet-fever streptococcus strain and is standardized by preliminary tests on goats confirmed by tests on persons. The doses for immunization are at present 500, 2000, 5000, and 10,000 skin-test doses. Since the practical value of the toxin for active immunization is still to be determined, the material is distributed only on special request and with the understanding that complete reports will be submitted to the laboratory.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

### *Preparation of the Toxin*

A stable toxin of high titer which has been carefully standardized is used. (For preparation see chapter "Production and Standardization of Streptococcus Toxin (Scarlet Fever)," p. 326.) New toxins are selected as required. About a week before a fresh supply is to be dispensed for distribution, the required dilutions are prepared. At present three dilutions are made; the first containing 1000 skin-test doses per cubic centimeter, the second 4000, and the third 10,000. Approximately equal quantities of the first and second dilutions are prepared and three times this amount of the third. The dilutions are made in 0.85-per-cent sterile salt solution containing 0.4 per cent phenol.

To the exact amount of salt solution required, sterilized in bottles taped at the proper volume, add, with aseptic precautions, sufficient 90-per-cent phenol solution to give a final 0.4-per-cent concentration (16.6 cc. per 4000 cc.). Then add sterile distilled water to replace the loss by evaporation. Add the required volume of toxin to the salt solution.

When the volume of toxin to be added is 100 cc. or less, it may be added with a sterile pipette. When the amount of toxin is greater than 100 cc., the salt solution may be sterilized in a graduated bottle and the exact amount of toxin siphoned over from the large container.

Each lot of toxin prepared is given a separate number followed by a number in parentheses to indicate the dilution, (1) designating the 1000 skin-test dose; (2), the 4000; and (3), the 10,000 [7(1), 7(2), 7(3)]. The sterility of each bottle is tested by inoculating three aerobic and three anaerobic tubes of sterility-test broth with a total of 2 cc., and two 160-cc. bottles of Hitchens' medium with 3 cc. each.

*Standardization Tests*

Before the diluted toxin is filled, a sample from each bulk container is tested intracutaneously on goats to determine its titer. Dilutions of each sample are prepared to contain one-half, one, and two skin-test doses in the amount injected (0.1 cc.). For procedure, see chapter "Production and Standardization of Streptococcus Toxin (Scarlet Fever)," p. 330. Retests are made every two or three months to determine deterioration. If there is evidence of deterioration, fresh dilutions are prepared.

*Preparation for Distribution*

As soon as the sterility tests are completed, the diluted toxin is filled in 10-cc. amounts by the filling and boxing group. The diluted toxin is prepared for distribution in two sets, consisting of three and two 10-cc. bottles respectively. The first set contains one bottle each of dilutions (1), (2), and (3), for the first, second, and third injections (individual doses 0.5 cc., given at 5- to 7-day intervals); the second, two bottles of dilution (3) for the fourth injection (individual dose 1 cc.). The material for the fourth injection is not sent out until the third week, the amount shipped depending on the report received of the actual number of immunizations in progress.

The period during which the material is considered satisfactory is at present limited to three months from the date of testing. If retests indicate that the toxin has not deteriorated, the return date may be extended two months. A second or even a third retest may be made at two months' intervals and the date extended if the supply has not been exhausted. The return date is entered on the package and on the label of the bottle. Material is not sent out, however, as a stock supply, but for immediate use only.

*Permanent Records*

On a card (4 by 6 in.) are recorded: product number; number of toxin used, date of its preparation, and potency; date toxin diluted, dilutions prepared, volumes of toxin and phenolized salt solutions used, and results of sterility and of tests of the diluted material; date finished product filled, number of bottles filled, results of sterility tests of the filled material, and date released for distribution.

## SECTION VI

## CHAPTER 1

# PREPARATION AND STANDARDIZATION OF TYPHOID AND TYPHOID-PARATYPHOID VACCINES

This chapter describes procedures connected with the preparation of monovalent typhoid and combined typhoid and paratyphoid A and B vaccines.[1] The vaccines are standardized by determining the number of organisms in each cubic centimeter by the Helber counting-chamber method. The immunity response induced in rabbits is used as an index of the antigenic value of the vaccine. It is measured by the agglutinative activity of the rabbit serum as compared with that of serum from other rabbits immunized with the standard vaccine obtained from the U. S. Hygienic Laboratory.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

### *Standard Strains Used in Preparation of Vaccines*

The following standard strains are used.

*B. typhosus*, "Rawlings." Collection No. 270B. English army strain isolated from a fatal case in 1905 during the Boer War. Received February, 1918 from the Army Medical School.

*B. paratyphosus* A, "Kessel." Collection No. 235S. Isolated from a case in the New York militia troops on the Mexican border in 1916. Received in October, 1923 from the Army Medical School.

*B. paratyphosus* B, "Rowland." Collection No. 236D. English army strain isolated from a case in Flanders in 1915. Received in January, 1918 from the Army Medical School.

**Maintenance of Strains.**—The strains are maintained on beef-infusion agar by monthly transfer. Each strain is transferred separately and incubated and stored in a separate labeled container. Select one

[1] The preparation of typhoid vaccine for distribution was begun by the state laboratory in July, 1914. Paratyphoid A and B vaccine and combined typhoid-paratyphoid vaccine were added in June, 1917, but the former was discontinued in March, 1920. In February, 1918, the bacterial content of the combined vaccine was increased from 500 million typhoid and 250 million each of paratyphoid A and B bacilli per cubic centimeter to the present standard.

previously unopened culture of each strain from the previous transfers and inoculate from it three agar slants and one triple-sugar Andrade tube (slant and butt). Incubate the cultures from eighteen to twenty-four hours at from 35 to 37°C. Examine the agar slants, note the reactions in the triple-sugar tube and make a slide preparation from it stained by Gram's method. If there is no evidence of contamination, seal the agar slants with paraffin and store in the cold room. Always retain the unopened cultures of the preceding month. The identity and purity of the cultures are sufficiently controlled by the preliminary tests made in connection with each lot of vaccine.

## PREPARATION AND STANDARDIZATION OF VACCINES

Vaccine is prepared from 18- to 24-hour beef-infusion agar cultures. The growth is suspended in salt solution, the number of bacilli per cubic centimeter determined by means of the Helber counting chamber, and the suspension heated at 53°C. for one hour, after which cresol to give a 0.3-per-cent solution, is added. The concentrated suspension is diluted in cresolized salt solution to the desired concentration. For the monovalent typhoid vaccine, this is 1000 million bacilli per cubic centimeter; for the triple vaccine, 1000 million typhoid and 750 million each of paratyphoid A and B bacilli.

Five days are required for making the monovalent vaccine. The combined vaccine requires from seven to nine days as strains belonging to different groups (in order to guard against mixing) are always started on different, though usually successive days. It is advisable to plan the work so that all the concentrated suspensions can be completed during one week and the dilutions made as early as possible the following week. Vaccines should be commenced from four to five weeks before they will be needed for distribution in order to allow for preparation, potency and sterility tests, filling and boxing.

The cultures are grown on beef-infusion agar which has been allowed to harden in 500-cc. Blake bottles laid on their sides. Usually about thirty-five bottles are inoculated with *B. typhosus* and fifteen with each of the paratyphoid strains. The bottles are incubated in an inverted position for forty-eight hours before inoculation to insure sterility and to permit drawing off the "water of condensation" should more than a few drops remain.

Requisitions for supplies: Place orders with the media department at least a week in advance, giving the media, number of bottles and swabs, volume of salt solution, etc., and the dates required. The card should be initialed by the head of the group.

Media: Beef-infusion agar in 65-cc. volumes in 500-cc. Blake bottles with neck and cotton plug protected by a paper cap.

Swabs: Swabs of nonabsorbent cotton on 12-inch wire rods are used for inoculating the bottles. On the day preceding inoculation the swabs are sterilized in copper pipette containers which are not opened until required.

Pipettes: Graduated and bulb pipettes of different capacities are sterilized in separate containers.

Bottles: A 1-liter Blake bottle, graduated in 50 cc. with cotton plug and paper cap for the pooled suspension is sterilized the day before each growth is harvested.

Salt solution: Eighty-five-hundredths-per-cent salt solution is dispensed in 200-cc. bottles in 150-cc. volumes for use in washing off the growth; and in 4-liter bottles (green glass) in the volumes required for diluting the vaccines.

### *Preliminary Transfers. Identification and Purity Tests*

1st day. Select a previously unopened tube from the stock cultures; transfer a loop of the growth to a tube of beef-infusion broth.

TABLE 32

*Serum dilutions of paratyphoid A serum No. 1, titer 1:14,000*

| VOLUME OF SERUM OR SERUM DILUTION | VOLUME OF SALT SOLUTION | FIRST DILUTION | FINAL DILUTION |
|---|---|---|---|
| *cc.* | *cc.* | | |
| 0.1 (undil.) | 9.9 | 1:100* | |
| 1.0 (1:100 dil.) | 9.0 | 1:1,000 | 1:2,000 |
| 0.5 (1:100 dil.) | 9.5 | 1:2,000 | 1:4,000 |
| 0.2 (1:100 dil.) | 7.8 | 1:4,000 | 1:8,000 |
| 0.1 (1:100 dil.) | 6.9 | 1:7,000 | 1:14,000 |
| 0.1 (1:100 dil.) | 8.9 | 1:9,000 | 1:18,000 |

* The lowest dilution (1:100) is not used in the test but is made because of the convenience of using it for further dilutions.

Mix thoroughly. Streak one loop of this suspension on two Endo plates. Incubate at from 35 to 37°C. for twenty-four hours.

2d day. Examine growth and mark three typical, distinct colonies. From each inoculate two broth tubes and make a culture (slant and butt) on triple-sugar Andrade medium. Keep each set distinct. Incubate twenty-four hours.

3d day. Examine growth and if typical, take a broth culture from two of the sets for macroscopic agglutination tests with the homologous serum. (For preparation of sera see the chapter "Production and Standardization of Monovalent Immune Sera," p. 495.)

Test the cultures in serum dilutions chosen according to the known titer of the serum; as in table 32.

Set up six tubes (11 by 75 mm.) for each culture. Pipette 0.3 cc.

of serum dilutions and 0.3 cc. of broth culture into the first five; into the sixth, 0.3 cc. of salt solution and 0.3 cc. of culture as a control. Shake, and incubate for two hours at 37°C. Make readings and record.

From this point, during all operations requiring sterile conditions the following additional precautions are taken to avoid contamination. Before work is commenced all flat surfaces in the draft-free room are wiped with a cloth dampened in 1-per-cent crude cresol. Bottles and other supplies are also wiped before being taken into the room. The workers wear sterile caps and gowns, and wash the hands thoroughly with soap and water, followed by glycerin solution if the skin is dry or rough. Only pipettes from previously unopened containers are used.

From the second broth culture of the set giving the best agglutination reaction and most typical appearance and reaction on triple-sugar Andrade medium, inoculate two more than half as many agar slants as there are bottles to be inoculated. Incubate twenty-four hours.

4th day. Examine the cultures. Pipette 0.75 cc. of beef-infusion broth into each tube; loosen the growth by allowing the broth to flow over the surface of the agar or by means of a loop if necessary. Make slide preparations from each culture, stain by Gram's method, and examine. Discard any contaminated or doubtful cultures and proceed at once to the inoculation of the bottles.

*Inoculation and Incubation of Bottles*

Remove the bottles from the incubator to the draft-free room. Examine without turning and discard any that appear unsatisfactory. If necessary pipette off the excess "water of condensation." Turn over the inverted bottles and stack with agar on the lower surface.

Inoculate two bottles in succession from the suspension in one tube. After the assistant has removed the cap and plug of the first bottle and flamed the neck, pass a swab soaked in the suspension quickly over the entire agar surface. While the assistant again flames the neck and replaces the plug and cap, recharge the swab. Proceed in like manner to the inoculation of the second bottle. Take a fresh swab for every two bottles. Place the contaminated swabs and pipettes in a jar containing 1-per-cent crude cresol. Record on a tag on the first bottle of the series the kind of vaccine, date, hour, and number of the strain; and on each of the others record, with a red wax pencil, the date and identifying initial ("T," "A," or "B"). Incubate the bottles in wire baskets, containing ten each, for twenty-four hours at from 35 to 37°C.

*Removal of Growth*

5th day. Take the bottles from the incubator directly to the draft-free room. Examine them carefully and discard any showing definite contamination. Should suspicious colonies be observed, make slide preparations directly from them, stain by Gram's method, and examine.

Add 20 cc. of salt solution to each bottle, taking a fresh pipette (20 cc.) for each four or five bottles. Let the salt solution cover the surface of the agar for five minutes, then turn the bottle gently from side to side so that the entire surface growth on the agar is washed off. Make a slide preparation from each bottle, stain by Gram's method and examine. Discard bottles in which contaminations are found or suspected.

Transfer the suspensions from the bottles into a 1-liter graduated bottle, taking a fresh pipette for each four or five suspensions. When the suspensions from all the bottles have been pooled, replace the cotton plug with a sterile rubber stopper. Shake the bottle vigorously until a uniform suspension has been obtained. Remove immediately about 1 cc. for the bacterial count, then cap the bottle with four layers of fishskin wet with 1-per-cent crude cresol and fastened down tightly with rubber bands. Attach a tag to the bottle giving the kind of vaccine and date.

*Heating of Suspension*

Remove the tag temporarily and completely submerge the bottle in a water-bath containing a false bottom. Stir the water to insure a uniform temperature throughout. If the bath is not equipped with an electric stirrer below the false bottom, stir frequently with a glass rod. Suspend a thermometer in the water and another in a control bottle containing water, submerged in the same manner as that containing the bacterial suspension. Heat the water until the thermometer in the control bottle registers 53°C. Hold at this temperature for one hour, then remove the vaccine bottle from the bath and replace the tag.

*Bacterial Count*

The count should be made promptly after the sample has been removed. It is usually convenient for the worker in charge, or a trained and responsible assistant designated by him, to make the bacterial count while the suspension is being heated; the latter procedure meanwhile being carefully controlled by an assistant. The vaccine is counted in a Helber counting chamber.

Preparation for count: The following arrangement has been found convenient: After wiping the table with 1-per-cent crude cresol place on it a rack with several 150-by-19-millimeter tubes; in front of them, four clean watch glasses (one for stain and three for vaccine dilutions) and dilution pipettes and connections; to right, the bottle of stain, bottles containing alcohol, ether, and distilled water, evaporating dish with 2-per-cent cresol compound for contaminated counting chambers, etc., and a jar with 1-per-cent crude cresol for discarded pipettes; beyond, containers with 1-, 5-, and 10-cc. graduated pipettes; to left, pieces of filter paper for wiping the tips of the dilution pipettes, and a bottle of salt solution for dilutions. A microscope with a mechanical stage attachment and an electric lamp are also arranged. After counts have been made, place the counting chambers, watch glasses, and pipettes in cresol compound for at least five minutes, then clean as described below. Place the filter paper in the same solution. Thoroughly wash the table with 1-per-cent crude cresol.

Dilute the vaccine 1:20, 1:30, 1:40, or 1:50, depending upon the density of the suspension. (The most usual dilution is 1:40.) Mix thoroughly. In a white blood cell, diluting pipette draw up the bacterial suspension exactly to the 0.05-mark, then freshly filtered staining solution (a 1:200-dilution of carbolfuchsin stain in 5-per-cent phenol) to the 1.1-mark (this makes a 1:20 dilution). Slip a wide rubber band over the ends of the pipette and mix by shaking for several minutes. Do not allow the dilution to stand in the pipette. Discard a few drops, then place one drop in the center of the Helber counting chamber. Put on a cover slip so that there are no bubbles; when it is in apposition, Newton's rings are visible at some point of contact of the cover slip and the slide. The drop must just fill the center area and not run over into the safety trench nor under the cover slip. Let the slide stand without disturbing for fifteen minutes.

Use a No. 6 objective and No. 4 eyepiece for counting. In order to include in the count all the bacilli in a given field, focus on different levels; otherwise the count will be too low. Count at least twenty squares. Beginning with the first completely ruled square at the upper left-hand corner, count diagonally across the counting chamber; then count a few additional squares from sections of the chamber not already included. Determine the average number in a square. Make at least one count from each of three different dilutions of the same strength. If these counts agree well (within about 10 per cent), take their average as the count of the vaccine; otherwise make more counts and take the average of all. Multiply the number of bacilli in one square by 400 million[2] to obtain the number in one cubic centimeter of dilution.

[2] The figure, 400 million, is derived as follows: cubic contents of each small square in counting chamber;

$$\tfrac{1}{20}\text{ mm.} \times \tfrac{1}{20}\text{ mm.} \times \tfrac{1}{50}\text{ mm.} = \tfrac{1}{20{,}000}\text{ cmm.} = \tfrac{1}{20{,}000{,}000}\text{ cc.}$$

$$\tfrac{1}{20{,}000{,}000} \times \tfrac{1}{20}\text{ (dilution in pipette)} = \tfrac{1}{400{,}000{,}000}.$$

Multiply the result by 40 (if an original 1:40 dilution was used) to obtain the number of bacilli per cubic centimeter of suspension.

After the count has been made, place the counting chambers and cover slips in 2-per-cent cresol compound for five minutes, rinse thoroughly in tap water and dry with a piece of linen. Clean pipettes by first drawing up and discharging 2-per-cent cresol compound and rinsing thoroughly in water, then alcohol and finally ether. The pipettes must be absolutely clean and dry, and the bead must roll freely in the bulb.

*Purity Test on Heated Suspension*

Just before adding the preservative, streak an agar plate with the heated suspension, using a pipette. A few typhoid (or paratyphoid A or B) colonies may develop but there should be no contaminating organisms.

*Addition of Preservative to Concentrated Suspension*

A 1.5-per-cent solution of cresol, freshly prepared in 0.85-per-cent salt solution, is added to the suspension to give 0.3-per-cent concentration.

Ascertain the volume of vaccine to be treated by the graduations on the bottle, calculate the volume of cresol solution to be added, pipette the required volume into the bottle, and mix thoroughly.

The formula used for calculating is:

$$P = V \frac{C_2}{(C_1 - C_2)}$$ in which: P = volume of preservative to be added.

V = volume of vaccine to be treated.

$C_1$ = concentration of cresol solution used.

$C_2$ = concentration of cresol desired in treating material.

In this case: $C_1 = 0.015$ and $C_2 = 0.003$, and

$$\frac{C_2}{C_1 - C_2} = \frac{0.003}{0.015 - 0.003} = \frac{0.003}{0.012} = 0.25$$ = a constant as long as 1.5-per-cent cresol is added to give 0.3-per-cent solution.

Then: $P = V \times 0.25$

Given 600 cc. of concentrated vaccine:

$P = 0.25 \times 600$ cc. = 150 cc. = volume of 1.5-per-cent cresol to be added.

The addition of preservative necessitates correction in the bacterial count as is shown in the following example:

Volume of vaccine = 600 cc.

Count = 41000 million per cc.

Volume of cresol added = 150 cc.

41000 million × 600 = total number of organisms.

600 + 150 = 750 new volume.

$$\frac{41000 \times 600}{750} = 32800 \text{ million per cc.} = \text{revised count.}$$

*Sterility Tests of Concentrated Suspensions*

Before storing the cresolized suspension on the "Vaccine" shelf in the cold room, pipette about 4 cc. into a sterile bottle. Keep the sample at room temperature for about eighteen hours so that any bacilli remaining alive in the heated suspension may be killed by the cresol before the tests are made.

Pipette 0.5 cc. of the concentrated vaccine into one aerobic and one anaerobic sterility-broth tube and prepare a deep dextrose-agar tube and pour plate, using five drops for each. (For technic see "General Bacteriological Technic," pp. 5, 8.) Inoculate a guinea pig subcutaneously with 1.5 cc. of a 1:20-dilution of the concentrated vaccine. Refer the inoculated media and animal to the sterility-test group (see "Sterility Tests," p. 543). If the tests show contamination, retest. If contaminating organisms are again found, discard the material.

*Dilution of Concentrated Suspension*

Dilute the bacterial suspension to the final concentration required for distribution without unnecessary delay, using cresolized salt solution. Dilutions may be made three days after the last sterility and animal tests on the concentrated material have been made, provided the tests are satisfactory up to that time.

Make the necessary calculations on the back of the dilution card (p. 470), as in the following examples.

Assuming that it is desired to make 3200 cc. of suspension containing 1000 million organisms per cubic centimeter from a vaccine with a count of 25000 million.

No. of cc. of vaccine required =

$$\frac{\text{No. of organisms per cc. desired} \times \text{no. of cc. desired}}{\text{count of vaccine}}$$

or

$$X = \frac{1000 \times 3200}{25000} = 128 \text{ cc. volume of vaccine to be used.}$$

Then: 3200 cc. − 128 cc. = 3072 cc. = volume of cresolized salt solution required.

3072 × 0.003 = 9.216 = 9.2 cc. of cresol to be added to give a final concentration of 0.3 per cent.

and 3072 cc. − 9.2 cc. = 3062.8 cc. = volume of salt solution required.

It is frequently more convenient to start with a known volume of concentrated vaccine. Assuming this to be 125 cc. of the above vaccine, the substituted formula becomes

$$125 = \frac{1000 \times Y}{25000} \text{ or } Y = 3125 \text{ cc.} = \text{volume of diluted material.}$$

Assuming that it is desired to make 3430 cc. of a suspension containing 1000 million typhoid bacilli per cubic centimeter, and 750 million each of paratyphoid A and B, from vaccines with the counts:

Typhoid = 34,300 million
Para A = 37,600 million
Para B = 38,900 million

$$X_T = \frac{1000 \times 3430}{34300} = 100 \text{ cc.} = \text{volume of typhoid vaccine to be used.}$$

$$X_A = \frac{750 \times 3430}{37600} = 68.4 \text{ cc.} = \text{volume of Para A vaccine to be used.}$$

$$X_B = \frac{750 \times 3430}{38900} = 66.1 \text{ cc.} = \text{volume of Para B vaccine to be used.}$$

Then: 100 cc. + 68.4 cc. + 66.1 cc. = 234.5 cc. = total vaccine added,

and 3430 cc. − 234.5 cc. = 3195.5 cc. = volume of cresolized salt solution required.

3195.5 × 0.003 = 9.5865 = 9.6 = cresol to be added to give 0.3-per-cent solution in salt solution.

3195.5 cc. − 9.6 cc. = 3185.9 cc. = volume of salt solution required.

Requisition for supplies: After the bacterial count has been made and the amount of vaccine and the required volume of salt solution determined, send the media department an order form giving the number of 4500-cc. bottles (green glass) and amount of 0.85-per-cent salt solution (usually about three liters) required in each. The bottles when filled are capped with paper and tagged with the volume of salt solution, date, and initials of the worker. The level of the salt solution is marked by the lower edge of a small strip of adhesive tape to gauge the loss from evaporation during sterilization. Order at the same time sterile distilled water dispensed in 250-cc. amounts in 500-cc. flasks to be used in making up the volume.

On receipt of the bottles from the media department, check the volume of salt solution in each by placing beside it a similar bottle, graduated and kept as a standard.

Assemble the required supplies in the draft-free room, observing special precautions previously described. From the original card giving amounts and dilutions required, verify the amount on the tag of each bottle. Enter on the tag the kind of vaccine. With a pipette add enough sterile water to each bottle to bring the level of the solution to the lower edge of the adhesive tape; then add the preservative. Replace the cotton plug with a sterile rubber stopper. Wrap around the stopper a cotton wad dampened in 1-per-cent crude cresol, and shake the bottle vigorously. Make sure that the cresol is all in solution before the vaccine is added. (If the salt solution is warm, the cresol dissolves more rapidly.) Attach to each bottle a tag giving the kind of vaccine, vaccine number, and bottle letter, number of organisms per cubic centimeter, total volume of diluted vaccine, date of heating, and initials of the worker.

First, add the required volume of typhoid suspension to each of the bottles prepared for monovalent typhoid vaccine (using as large pipettes as possible in order to avoid opening the bottle unnecessarily). After these bottles have been set aside, add the typhoid suspension to the bottles labeled "Typhoid-paratyphoid vaccine" and then add in turn the paratyphoid A and B suspensions. Always finish with one suspension and set it aside before starting with the next. The kind and amount of vaccine to be added to each bottle is read aloud by the assistant and repeated by the worker. After the vaccine has been added, rotate the bottles to mix the contents thoroughly.

### *Sterility Tests of Diluted Material*

Before making sterility tests, remove about 12 cc. of vaccine from each of two or three bottles, and place approximately 7 cc. in a bottle for potency tests, and the remainder in the dilution-test tube. Transfer 5 cc. samples from the other bottles to dilution-test tubes. For sterility tests remove 10 cc. from each of the large bottles, and inoculate two aerobic and two anaerobic tubes of sterility-test broth with 0.5 cc. each, and one 180-cc. container of Hitchens' medium with 8 cc. Use a fresh pipette every time a bottle is opened. Wrap a cotton wad dampened with 1-per-cent crude cresol around the stoppers of the stock bottles and cover with tinfoil. Store the bottles immediately in the cold room apart from other material, keeping each kind in separate rows.

Should the report from the sterility test group indicate slight contamination in one bottle only, repeat the test on that bottle; if in two bottles, repeat the test on all bottles or consult the bacteriologist in charge.

### *Control Tests of the Final Dilution*

As a control of the dilution of the vaccine, samples from every bottle are compared, by the worker in charge of sterility tests, for turbidity, with each other, and also with two control samples from the previous lot of vaccine, one undiluted and one diluted one-third. (Since vaccines become somewhat more turbid on standing, the required dilution has been found to lie between these limits with vaccines between five and eighteen months old.) If the readings do not agree, a count of the diluted vaccine is made by the Helber counting-chamber method. Previously standardized tubes (150 by 15 mm.) are used.

Dilute one control sample from the previous lot, two to one with 0.85-per-cent salt solution and compare it and an undiluted sample with a sample of the fresh vaccine, making the readings against a dark background. Then compare the samples from the other bottles with the tube of fresh vaccine. Seal two tubes with sterile rubber stoppers and paraffin, and store in the cold room for later use.

### *Preparation for Distribution*

The vaccines are filled by the filling and boxing group on written order from the worker in charge of production who should be kept closely informed by the shipping group as to the rate of distribution. The vaccine may be released for filling as soon as the sterility tests are satisfactorily completed, but before the potency tests have been finished. Immediately before it is bottled, the vaccine in the stock bottle is examined and passed upon by the head of the production group or by a specially trained assistant.

The vaccines are prepared for distribution in the following amounts:

I. Typhoid. Individual outfits: Sets for immunization of one person consisting of three vials each containing a single dose. The first dose is 500 million bacilli in 0.5 cc.; the second and third, 1000 million in 1 cc. each.

The sets are prepared by dispensing 0.7 cc. into a 1-cc. vial and 1.2 cc. into each of two 2-cc. vials. The additional 0.2 cc. in each vial is added to allow for loss. It is usually convenient to fill 300 outfits, or three hundred 1-cc. and six hundred 2-cc. vials at one time.

II. Typhoid. Bottles: 10 cc. of vaccine containing 1000 million bacilli per cubic centimeter dispensed in 10-cc. bottles. About 300 bottles are usually filled at one time.

III. Typhoid-paratyphoid. Individual outfits: The same vials and amounts

are used as in the typhoid sets but the vaccine contains 1000 million typhoid bacilli and 750 million each of para A and B per cubic centimeter.

IV. Typhoid-paratyphoid. Bottles: 10 cc. of vaccine containing 1000 million typhoid bacilli and 750 million each of para A and B dispensed in 10-cc. bottles. About 300 bottles are usually filled at one time.

Except in special emergencies, the vaccines are not released for distribution within less than three weeks from the date of preparation, which is the date the first suspension is heated. The return date, stamped on the labels, is nine months from the date of preparation. Except in special emergencies, however, no vaccine is sent out from the laboratory which is more than seven months old. Since vaccines are distributed only on special orders or in limited amounts to supply stations for stock, most of the material is used within seven months. Vaccines returned before the expiration date are not redistributed.

### *Potency Test*

The antigenic value of a vaccine is estimated by inoculating a series of three rabbits with it, and then determining the agglutination reaction of their sera with the homologous culture or cultures as a measure of the immunity response induced. A serum prepared by immunizing rabbits against a standard vaccine obtained from the U. S. Hygienic Laboratory is always included in the agglutination tests for purposes of comparison.

Samples taken from two stock bottles of the vaccine to be tested are pooled and each rabbit inoculated with doses of the pooled vaccine. (If a batch consists of more than six large bottles, samples are taken from three bottles and pooled.)

When the same suspension of the typhoid strain is used in preparing typhoid-paratyphoid and typhoid vaccines, separate rabbit tests on the typhoid vaccine may be omitted at the discretion of the head of the group, provided the potency tests on the triple vaccine have given a satisfactory titer for the typhoid strain.

**Inoculation of Rabbits.**—Select previously unused rabbits weighing not less than 1600 and preferably not more than 2200 grams. Give each rabbit three subcutaneous inoculations of 0.5, 1.0, and 1.0 cc. respectively at intervals of three days. Five days after the last inoculation draw about 10 cc. of blood from the ear vein into a sterile tube (160 by 19 mm.) (see "The Use of Experimental and Test Animals," p. 38). Allow it to stand, if necessary rim the clot with a pipette, and place in the cold room overnight.

**Agglutination Tests.**—The technic used is that described in the

chapter "Production and Standardization of Antimeningococcus Sera," p. 436, with the following differences.

Standard serum: The standard serum is produced by immunizing three rabbits with the standard vaccine, which contains 1000 million typhoid bacilli per cubic centimeter and 750 million each of para A and B. The method used is that previously described. After the agglutinative titer of the serum from each rabbit has been determined for the three standard vaccine strains, the sera are pooled. Standard serum is produced with fresh vaccine about once a year. (If, before pooling, the agglutinative titer of serum from any of the control rabbits should be below 1:500, another rabbit should be immunized and its serum substituted.)

TABLE 33

*Serum dilutions**

| VOLUME OF SERUM OR SERUM DILUTION | VOLUME OF SALT SOLUTION | FIRST DILUTION | FINAL DILUTION |
|---|---|---|---|
| *cc.* | *cc.* | | |
| 0.1 (undil.) | 9.90 | 1:100 | 1:200 |
| 1.0 (1:100 dil.) | 1.50 | 1:250 | 1:500 |
| 1.0 (1:100 dil.) | 2.75 | 1:375 | 1:750 |
| 1.0 (1:100 dil.) | 4.00 | 1:500 | 1:1,000 |
| 1.0 (1:100 dil.) | 6.50 | 1:750 | 1:1,500 |
| 0.5 (1:100 dil.) | 4.50 | 1:1,000 | 1:2,000 |
| 0.5 (1:100 dil.) | 5.75 | 1:1,250 | 1:2,500 |
| 0.5 (1:100 dil.) | 7.00 | 1:1,500 | 1:3,000 |

* It may be necessary to repeat the test, using additional dilutions to obtain a more exact titer of the serum.

Bacterial suspensions: Suspend in 0.85-per-cent salt solution the growth from 18-hour beef-infusion agar cultures of the standard strains and dilute to the turbidity of the barium sulfate suspension, standard No. 3.

Serum dilutions: Use the serum dilutions given in table 33.

The test: Pipette 0.3 cc. of serum dilution and 0.3 cc. of bacterial suspension into the agglutination tubes. Use a salt-solution control, and a normal-serum control in a dilution of 1:200. Incubate for two hours at 37°C., place in the cold room overnight and read the following morning.

**Estimation of Potency.**—The average titer of the sera produced should equal or exceed that of the control serum. Differences in individual titers must be attributed to differences in the response of the animals used, but if the sera on the whole do not approximate the standard

serum, at least two additional rabbits should be immunized with the vaccine. If their sera also fail to approximate the control serum, the batch of vaccine is discarded. (This, however, has never been necessary.)

### *Permanent Records*

Standard strains: A separate card (4 by 6 in.) is kept for each strain on which are entered: name and number, source, date received, and any other information of interest.

Preparation of bacterial suspension: A card (5 by 8 in.) is kept for each suspension prepared giving: at the top, kind of vaccine, date prepared, revised count, final volume, and initials of worker; and in chronological order with dates, each step in the preparation. The entries are culture number, preliminary tests and results of each, appearance of growth and presence or absence of contamination in bottle cultures, number of bottles used, temperature and time of heating suspension, bacterial count, volume of undiluted suspension, result of purity tests on heated suspension, volume and kind of preservative added, volume of suspension plus preservative, revised bacterial count, results of sterility tests of concentrated suspensions. The kinds and lot numbers of vaccines in which the suspension is used are also noted. On the back of the card are entered: details of the bacterial count, calculations of the volume of preservative, and those to obtain revised count, with the initials of the worker making each, and of the worker checking them. Each card is given a serial number.

Vaccine record: On a card (5 by 8 in.) are entered the following data in regard to each bottle of completed vaccine: lot number and bottle letter, kind of vaccine, number of preparation card for concentrated suspension used, date killed, bacilli per cubic centimeter in the completed vaccine, date diluted, volume of salt solution, of preservative, and of concentrated vaccine, total volume diluted vaccine, result of nephelometric control test, results of sterility tests of diluted vaccine, date filled, results of sterility tests of filled material, number of potency test card, results of potency tests, and date released. On the reverse of this card are made the calculations for diluting the suspensions. Cards are arranged so that the lot numbers follow in sequence.

Potency tests: On a card (4 by 6 in.) are recorded the following data in regard to the potency test for each vaccine: kind and lot number of the vaccine and letters of the bottles from which samples are taken, date pooled, bacilli per cubic centimeter of the vaccine; in regard to each serum, rabbit number, dates and methods of injections, doses, and date of bleeding. On the back of this card are recorded the agglutination reactions of each serum, dates tested and read, and the initials of the worker making the test. Each card is given a serial number.

## CHAPTER 2

## PREPARATION OF PERTUSSIS VACCINE

Pertussis vaccine is prepared for prophylactic and therapeutic use.[1] It is standardized by the Helber counting-chamber method. The value of the vaccine has not been demonstrated by laboratory tests nor are satisfactory methods at present available for determining the potency of the material distributed. Clinically, however, there appears to be evidence that the vaccine may be effective both as a preventive and curative agent. In preparing pertussis vaccine, the procedures given in "Preparation and Standardization of Typhoid and Typhoid-Paratyphoid Vaccines," p. 457, are followed except where indicated in the following directions.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

### *Standard Strains*

The following standard strains received from the New York City Research Laboratory as representing groups A and B (154) are used.

*B. pertussis* A, Collection No. 237E. Received in January, 1918.
*B. pertussis* B, Collection No. 237G. Received in June, 1923.

**Maintenance of Strains.**—The cultures on coagulated-blood agar (150-by-19-mm. tubes) are maintained in the incubator at from 36 to 37°C. Transfers are made twice a week (Wednesday and Saturday). Should difficulty in obtaining satisfactory growth be experienced, a series of daily transfers is advisable. Each strain is kept in a separate clearly labeled container. To guard against loss, the cultures of the two preceding transfers are always retained and two cultures of each strain are carried on in different incubators.

[1] Pertussis vaccine was first prepared in October, 1915. It was distributed in immunizing sets of three vials containing 1000, 2000, and 3000 million bacilli respectively, and in 10-cc. bottles containing 1000 million bacilli per cubic centimeter. In July, 1918, the suspension in the 10-cc. bottles was increased to 2000 million and in April, 1920, to 4000 million for special lots. In December, 1921, the 4000 million suspension was adopted for routine distribution and the number of bacilli in the immunizing doses was doubled.

**Identification and Purity Tests.**—At every second transfer a slide preparation is made from the growth remaining on the slant, stained by Gram's method, and examined. Once each month, or oftener if indicated, the cultures are streaked on blood-agar plates, and colonies fished and stained by Gram's method. Fishings, however, are not used for standard cultures unless the preceding transfers held in reserve fail to give satisfactory transplants. The identity and the purity of the cultures are further controlled by the preliminary tests made in connection with each lot of vaccine. Serologic tests have not as yet proved sufficiently satisfactory to warrant their adoption as routine procedure.

## PREPARATION AND STANDARDIZATION OF VACCINE

Pertussis vaccine is prepared from 48-hour 5-per-cent glycerin, beef-infusion-agar cultures. The concentration of the completed vaccine is 4000 million bacilli per cubic centimeter, made up of 2000 million each of the two strains used. Work with the two strains is commenced on different days. The vaccine should be started about a month before it is required. Thirteen to fifteen days are needed for the actual preparation. Distribution may be commenced as soon as the final tests for sterility and harmlessness are satisfactorily completed.

### *Preliminary Transfers and Identification and Purity Tests*

1st day. Select the most recent transfer of one of the strains used for vaccine. Make a slide preparation and stain by Gram's method. If satisfactory, inoculate from it three coagulated-blood agar slants and incubate them for forty-eight hours. Also streak a blood-agar plate and examine it for purity after twenty-four and forty-eight hours' incubation.

3d day. Examine each culture carefully and make slide preparations from suspicious ones. Then make four transfers from each slant and incubate them forty-eight hours.

5th day. Examine each culture carefully and make slide preparations from any that appear suspicious. From the twelve transfers inoculate two more slants than there are bottles to be inoculated. Incubate them forty-eight hours.

7th day. Remove the cultures to the draft-free room, examine them carefully and make slide preparations from any in which contamination is suspected.

*Inoculation and Incubation of Bottles*

Pass the sterile swab over the surface of a slant, removing all the the growth possible and with it inoculate a bottle quickly and thoroughly. Repeat the procedure until all the bottles are inoculated using a fresh culture and a fresh swab for each. Incubate forty-eight hours at from 36 to 37°C.

*Removal of Growth and Heating of Suspension*

The procedures are the same as for typhoid vaccine, except that the concentrated suspension is pooled in a bottle containing glass beads (40 grams added in the media department before the bottle is sterilized) and shaken very vigorously before heating, before and after adding preservative, and again before the suspension is made up to the final dilution. This is to insure the breaking up of the stringy clumps which are always present. To obtain the correct volume of the concentrated suspension, 15 cc. (the volume occupied by the beads) is subtracted from the volume as read from the graduations. The suspension is heated for one hour at 56°C. The blood plate, streaked with two loopfuls of the heated suspension, should remain sterile. No sample of vaccine for potency tests is required.

*Preparation for Distribution*

Pertussis vaccine may be dispensed five days after the sterility tests on the diluted material are made, provided the tests are satisfactory up to that time. Pertussis vaccine is prepared for distribution in the following amounts:

Individual outfits: Sets for the immunization of one person, consisting of three vials, each containing a single dose. The first dose contains 2000 million bacilli in 0.5 cc.; the second, 4000 million bacilli in 1 cc.; and the third, 6000 million bacilli in 1.5 cc.

Bottles: 10 cc. of vaccine containing 4000 million bacilli per cubic centimeter dispensed in 10-cc. bottles.

Pertussis vaccine may be distributed as soon as the tests for sterility and harmlessness are satisfactorily completed.

*Permanent Records*

The records are similar in form to those described in "Preparation of Typhoid and Typhoid-Paratyphoid Vaccines," p. 470.

## CHAPTER 3

## PREPARATION OF PNEUMOCOCCUS VACCINE

Pneumococcus vaccine is prepared for prophylactic treatment, only as required and after approval by the bacteriologist in charge of the department. The vaccine contains types-I, -II, and -III pneumococci. Potency tests on the finished product are not made as a routine procedure. In preparing pneumococcus vaccine, the procedures given under "Preparation of Typhoid and Typhoid-Paratyphoid Vaccines," p. 457, should be followed except as indicated below.

### *Maintenance of Strains Used in Preparation of Vaccine*

The strains used are:

Type I, "N" strain: Collection No. 5.
Type II, "D" strain: Collection No. 5C.
Type III: Collection No. 5A.

For sources and maintenance of the cultures, see "Production and Standardization of Antipneumococcus Sera," p. 409.

### PREPARATION OF VACCINE

Pneumococci of each type are grown in pneumococcus broth containing 0.1-per-cent dextrose (200 cc. in a 500-cc. Erlenmeyer flask) for from eighteen to twenty-four hours. Each culture is centrifugalized, and the sediment, suspended in salt solution, transferred to a 200-cc. graduated bottle. The suspensions are heated at 55°C. for one-half hour, the bacterial count is determined, and preservative added. To guard against error, work with each culture is commenced on a different day. When the three suspensions have been completed, they are diluted in salt solution to give a vaccine containing 3000 million organisms per cubic centimeter (1000 million of each type). About three weeks should be allowed for preparing the vaccine.

### *Preliminary Identification and Purity Tests. Inoculation and Incubation of Flasks*

1st day. Select a recent semisolid culture and transfer from 0.1 to 0.2 cc. to a tube of broth. Seal the original culture and return it to the cold room. Incubate the broth culture overnight.

2d day. Make a slide preparation, stained by Gram's method, of the broth culture, and streak a blood-agar plate. Test for bile solubility and agglutinative reactions, using the homologous and heterologous sera. If the tests are satisfactory, inoculate one or more broth tubes from the original semisolid culture.

3d day. Examine the plate culture. Make slide preparations stained by Gram's method from the broth cultures and inoculate one or more flasks of broth with 2 cc. of culture. Incubate from eighteen to twenty-four hours.

*Removal of Organisms*

4th day. Make a stained preparation from each flask and streak a blood-agar plate. Under aseptic precautions centrifugalize the culture until the supernatant fluid is clear. Draw off the supernatant broth and add about 25 cc. of salt solution to the sediment in each centrifuge bottle. Transfer the suspension to a 200-cc. graduated bottle.

*Heating of Suspension and Bacterial Count*

The procedures from this point on are those given in the chapter "Preparation of Typhoid and Typhoid-Paratyphoid Vaccines," p. 461, with the following exceptions. The suspension is heated at 55°C. for one-half hour. No potency test is made as a routine procedure.

*Preparation for Distribution*

The vaccine is prepared for distribution in 5-cc. volumes. Each bottle is labeled with the kind of vaccine, pneumococcus types, number of organisms, volume, product number, and return date. Requests for pneumococcus vaccine must be approved by the bacteriologist in charge of the department.

*Permanent Records*

The records are similar in form to those described in the "Preparation of Typhoid and Typhoid-Paratyphoid Vaccines," p. 470, except that no potency test is recorded.

# CHAPTER 4

## PREPARATION OF AUTOGENOUS VACCINES

Autogenous vaccines are prepared for the treatment of subacute or chronic infections on special request when approved by the bacteriologist in charge of the department.[1] The vaccines are made only from pure cultures of the organism considered to be the incitant of the infection. Vaccines are generally prepared from staphylococcus, less frequently from streptococcus and pneumococcus strains, and occasionally from other organisms. The strains are isolated by the diagnostic department from specimens received for examination and recovery of the predominating organisms.

The organism isolated is given the miscellaneous examination number of the specimen. Several transplants, representing fishings of the predominating organism from a plate culture, are received from the diagnostic department with a miscellaneous history blank giving the necessary data. The method used in preparing the vaccine is very similar to that given in "Preparation of Typhoid and Typhoid-Paratyphoid Vaccines," p. 457, which should be followed closely except as indicated in the following text. From nine to twelve days should be allowed for making an autogenous vaccine. The vaccine is generally diluted so that 1000 million organisms are contained in each cubic centimeter of suspension.

### *Preparation of Staphylococcus Vaccine*

1st day. Inoculate from each of two cultures at least one beef-infusion-agar slant. Make a slide preparation, stained by Gram's method, and streak a blood-agar plate from each of the cultures used. Incubate about eighteen hours.

2d day. Examine the plates. Make slide preparations from the cultures and examine. Add 3 cc. of salt solution to each slant, loosen the growth, and pool the suspension, adding enough salt solution if necessary to bring the volume to at least 10 cc.

[1] Since the etiologic relationship of a particular organism to a subacute or chronic infection is often difficult to establish from morphologic study alone, the preparation of autogenous vaccines should, when facilities are available, be carried on in the local laboratory.

Should any suspicious colonies be present on a plate, make slide preparations. If any contaminating organisms are found, use only the agar slants from the other set, provided that all tests indicate that that set is pure. If both plates show only slight contamination, fish three or four discrete colonies of the predominating organism to agar slants. Incubate overnight. Make slide preparations and examine. If satisfactory, inoculate at least two agar slants, and proceed as above.

After removing 2 cc. of the pooled suspension for the count, heat the suspension for one hour at 60°C. (The thermometer in the water-bath is sufficient control.) Proceed at once to the final dilution of the suspension, adding enough preservative to the salt solution to allow for the uncresolized suspension. In case dilution of the suspension is delayed, add the necessary preservative to it and place in the cold room.

To prepare the diluted vaccine, pipette into a sterile bottle the volume of salt solution needed and add the required volume of preservative. After shaking the bottle of concentrated suspension, inoculate one aerobic and one anaerobic tube, each with 0.5 cc. of the suspension, and streak a blood-agar plate. Then add the required volume of suspension to the cresolized salt solution, mix thoroughly, and dispense immediately. Hold the remainder of the concentrated suspension at least until the sterility tests on the filled material are completed.

*Preparation of Streptococcus and Pneumococcus Vaccines*

1st day. Inoculate from each of two cultures at least one tube of pneumococcus broth. Make a slide preparation, stained by Gram's method, and streak a blood-agar plate from each of the cultures used. Incubate from sixteen to twenty hours, depending on the growth.

2d day. Examine the plates. Make slide preparations from one culture of each set, stain by Gram's method and examine. In the case of pneumococcus, test also the bile solubility and agglutinative reactions. Inoculate from each set 100 cc. of pneumococcus broth with about 1 cc. of culture and incubate from sixteen to twenty-four hours.

Should any contaminating organisms be found on a plate, use only the broth culture from the other set, provided that all tests indicate that that culture is pure. If both plates show contamination, inoculate one broth tube with fishings from two or three discrete colonies and proceed as previously described.

From this point on proceed as described in "Preparation of Polyvalent Pneumococcus Vaccine," p. 474, pooling the suspension in a smaller bottle, and heating the streptococcus for one hour at 60°C.

instead of 55°C. Make the dilution as described in the preparation of staphylococcus vaccine.

*Preparation of Other Vaccines*

One of the above methods is generally used in preparing other vaccines. The bacteriologist in charge of the department should be consulted as to which method is to be used, and what variations may be necessary.

*Preparation of Vaccines for Distribution*

The vaccines are dispensed by the preparation group. After shaking the bottle of diluted vaccine thoroughly, dispense from a pipette in approximately 5-cc. volumes in sterile 5-cc. bottles. (Throughout this procedure special precautions against contamination must be rigidly observed.) Flame and cover the stoppers of the bottles with fishskin, wet with 1-per-cent crude cresol and fastened down with rubber bands. Mark the vaccine number on each bottle and place the bottles in the cold room in a separate, labeled, wire basket. When sterility tests are satisfactorily completed, label each bottle with the kind of vaccine, serial number, name of the patient, date prepared (killed), and the number of organisms per cubic centimeter and the volume. Stamp in red on each label, "Shake Well Before Using." Send the vaccine with a "Memorandum of Shipment" to the shipping group. Hold at least one bottle in reserve for three months. Usually about five bottles are forwarded to the physician requesting the vaccine.

**Final Sterility and Animal Tests.**—If there are ten bottles or less, inoculate from one bottle two aerobic- and two anaerobic-broth tubes with 0.5 cc. each and one 40-cc. bottle of Hitchens' medium with 1 cc.; if there are more than ten bottles, make tests from two bottles. Inject 1 cc. subcutaneously into a mouse.

*Permanent Records*

Cards (4 by 6 in.) are kept for each vaccine, on which are recorded: serial number, date culture received, miscellaneous number, kind of organism, details of preparation of vaccine, and mathematical calculations.

## CHAPTER 5

## DYSENTERY VACCINE

A small supply of polyvalent dysentery vaccine is prepared each spring to meet possible demands during the summer months. The vaccine may be used for preventive injections or for treatment of certain selected subacute or chronic cases. The procedure is that given under "Preparation of Typhoid and Typhoid-Paratyphoid Vaccines," p. 457. The vaccine is diluted to contain 12 million *B. dysenteriae* Shiga, and 100 million each of Flexner and Mt. Desert. The standard strains used are "Shiga F," Collection No. 114 F, "Flexner D," No. 114 D and "Mt. Desert E," No. 114 E.

## SECTION VII

## CHAPTER 1

## PREPARATION OF OLD TUBERCULIN (KOCH'S O.T.)

Tuberculin is prepared for diagnostic use. It consists of the filtrate of a heated 5-per-cent glycerin broth culture of the tubercle bacillus concentrated to $\frac{1}{10}$ of its original volume, and contains the products of growth of the bacillus together with the elements of the culture medium. The finished product is tested clinically before it is released for distribution.[1] About four months should be allowed from the time preparation is commenced until the finished product is ready for distribution.

The tuberculin test may be made on the scarified skin by the cutaneous method of Von Pirquet, intracutaneously (Mantoux's method), and subcutaneously. The methods of performing the test are given in the circular of directions distributed with each package.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

### *Standard Strain Used*

A human strain is used at present in the preparation of tuberculin.

*B. tuberculosis.* Collection No. 161, received in 1915 from the New York City Research Laboratory.

The strain is maintained in the bacterial collection by transfers every two months on Petroff's egg medium without dye. A transplant is usually maintained on glycerin broth in the research department. If available, a subculture from glycerin broth is preferred.

[1] In addition to the tests on persons, each lot of tuberculin is tested for potency in guinea pigs by the modified Koch method, the Frankfurt standard at present being used for comparison, until further data as to the most desirable method of standardization are available. (The modified Koch method is described by Otto, R., and Hetsch, H., Arb. a. d. Staatsinst. f. Exper. Therapie, u. d. Georg Speyer-Hause zu Frankfurt, A. M., 1921, No. 13, p. 107 (155).) The intracutaneous test on guinea pigs is under investigation.

*Preliminary Culture. Identification and Purity Tests*

Use a 3- to 4-week culture on glycerin broth. The growth should cover the surface of the medium and be sufficiently heavy to permit the transfer of pellicle with a large nichrome wire loop. If no broth culture is available, inoculate glycerin broth (120 to 130 cc. in a 450-cc. Blake bottle) with several loopfuls of growth from the egg medium, floating the particles carefully on the surface. Incubate at from 36 to 38°C. until the desired growth has been obtained. Remove the culture from the incubator, taking care that the pellicle does not sink to the bottom or become moist on the surface. Examine for evidence of contaminating organisms; the culture should show the characteristic pellicle with clear, sparkling fluid below. Place a loopful of growth on a slide and with a second slide spread it in a thin film. Stain with acid fuchsin and methylene blue and examine. Inoculate one aerobic and one anaerobic tube of sterility-test broth and streak a 0.2-per-cent dextrose beef-infusion-agar plate.

*Inoculation and Incubation of Bottles. Purity Tests. Heating of Culture*

The cultures are grown in 450-cc. Blake bottles containing from 120 to 130 cc. of 5-per-cent glycerin broth previously incubated for forty-eight hours to insure sterility. Between 20 and 40 bottles are usually inoculated.

Remove the bottles to a draft-free room and examine each for evidence of contamination, then place the bottle on its side. Inoculate by floating upon the surface of the broth small portions of the pellicle from the seed culture. To prevent evaporation, cap the bottle with sterile tinfoil fastened by a rubber band. Transfer the inoculated bottles very carefully to the incubator where they should remain undisturbed during growth. Incubate at from 36 to 38°C. for from six to nine weeks, depending upon the rate of growth. Examine each bottle as it is removed from the incubator, for contamination, indicated by clouding of the broth or atypical appearance of the pellicle. Make preparations, stained by Gram's method, from any in which contamination is suspected and from at least two which show characteristic growth. Discard any bottles that appear questionable.

Shake each bottle until the pellicle is suspended uniformly throughout the broth, then kill the cultures by heating in steam at 100°C. for one hour.

### *Concentration of Heated Culture*

The following day pool the heated cultures and concentrate to approximately one-tenth of the original volume.[2] Pour the heated culture into one or more weighed porcelain evaporating dishes. Reweigh and place over a steam bath. Hold the culture at approximately 90°C., stirring slowly at frequent intervals. When the weight of the concentrated culture has reached one-tenth the original weight, allow the material to cool under a shield, and transfer to a sterile, stoppered bottle. Filter at once or place in the cold room until the following day.

### *Filtration, Addition of Preservative, and Sterility Tests*

Filter the material through paper pulp on a Buchner funnel (see "Filtration of Biologic Products," p. 515). Then add slowly, while stirring, cresol to give a 0.3-per-cent solution. Return the tuberculin immediately to the cold room. On the following day, or as soon after as possible, filter through a filter candle by suction. Store the material in the cold room. After filtration, inoculate one aerobic and one anaerobic sterility-broth tube with 0.5 cc. each, and one 80-cc. container of Hitchens' medium with 2 cc. Inject 0.5 cc. into a guinea pig. After six weeks kill and autopsy the animal to rule out the presence of lesions of tuberculosis.

### *Preparation for Distribution*

Tuberculin is filled in 0.5 cc. amounts in 1-cc. amber vials by the filling and boxing group (see "Preparation of Biologic and Chemical Products for Distribution," p. 524). The bottles are labeled with the kind of product, volume, lot number, and date of concentration. Tuberculin retains its activity for an indefinite period. No time limit has, therefore, been placed on the material.

### *Tests of Potency*

Before the finished product is finally released for distribution, its activity is tested clinically by the cutaneous method or by the intracutaneous method, on a series of cases. Before the material is released for general distribution, the approval of the bacteriologist in charge of the department is required. For further tests see footnote 1, p. 480.

### *Permanent Records*

A card (5 by 8 in.) is kept for each lot of tuberculin prepared giving the complete record of preparation. The results of the clinical tests are given on the reverse side of this card.

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[2] The application of the pervaporation method to the concentration of tuberculin is at present under study.

## CHAPTER 2

## PREPARATION OF OUTFITS FOR THE SCHICK TEST

A special outfit[1] is prepared for use in performing the Schick test. It consists of a small vial containing undiluted diphtheria toxin, two standardized capillary pipettes for measuring the toxin, a bottle containing the exact amount of salt solution necessary to give the required dilution when a drop of toxin from one of the pipettes is added, and a small sterile tube for use in preparing the control test. The outfits are prepared under the supervision of the worker in charge of the toxin-production group.

By use of this outfit, a very accurately measured quantity of undiluted toxin is added to a known volume of salt solution, giving the desired final dilution of $\frac{1}{50}$ minimum fatal dose in 0.1 cc., the dosage employed in the intracutaneous test. A circular giving the method of performing the test and the proper interpretation of the reactions induced is distributed with each outfit.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

### *Preparation of Capillary Pipettes*

Capillary pipettes with a very small bore are prepared. Each pipette is standardized by titrating the drop of 4N HCl it delivers against approximately N/100 NaOH, standardized in terms of the HCl. From this titration and the M.F.D. of the toxin is calculated the volume of diluent to give the required dilution. The titrations are made only when the light is favorable, since they depend upon a delicate color reaction.

**Drawing out of Pipettes.**—Use from 4- to 5-mm. glass tubing with a rather thick wall. Break the tubing into 10-cm. lengths and anneal the ends slightly. Place the pieces in cleaning solution overnight. Rinse them well first in tap water, then in distilled water, and dry in a hot-air oven. Using a fish-tail burner, heat the tubing at the center until it is flexible, turning it constantly so that the glass will heat

[1] The problem of devising an outfit for the Schick test sufficiently accurate and reliable for state-wide use was undertaken by Miss Tula Lake Harkey in 1916. The outfit which she devised, with but slight modification, has now been in satisfactory use for ten years. See Wadsworth, A. B., Jour. Lab. & Clin. Med., 1925, 10, 495 (156).

evenly. Remove from the flame and draw it out about ½ cm. so that the tubing is slightly constricted at the center. Cool slightly, then reheat at the center until flexible; remove from the flame and, holding the tubing horizontally, draw it out quickly and steadily to form two capillary pipettes with a very small bore. Break, so as to leave the length of each about 7½ to 8½ cm. If a pipette is unsatisfactory, weld a small piece of tubing to the tapering end, heat and proceed as before.

**Standardization of Sodium-Hydroxide Solution.**—The hydrochloric acid (4N) and the sodium hydroxide (approximately N/100) are prepared by the analytical chemist. The sodium hydroxide is placed in a paraffin-lined bottle fitted with a siphon, lined and coated with paraffin. The sodium hydroxide is then standardized in terms of the acid with alizarine sodium sulfonate (1-per-cent aqueous solution) as an indicator.

Alizarine-sodium sulfonate gives a deep violet color with alkalies, a yellow green with acids, and a pale amber at the neutral point. Since it is an extremely sensitive indicator, it is necessary to determine the end-point in a titration by comparison with a control flask prepared at the same time as the titration flask. This prevents errors due to the reaction of the water used, the absorption of $CO_2$ from the air, or possible fading of the indicator.

Burette and siphon: Allow the burette used for the sodium-hydroxide solution to stand in cleaning solution, then rinse it with tap water and finally with distilled water. Rinse the siphon thoroughly in tap and in distilled water. Clamp the burette into position on an iron stand and attach the siphon connection to the siphon previously inserted in the sodium-hydroxide bottle. Smear the stopcock with a mixture of beeswax and vaseline and wire it into position. Plug the top of the burette lightly with cotton. Start the siphon and fill and empty the burette several times before using any of the alkali. Keep the burette full when not in use, and clean it whenever the stopcocks begin to stick.

To obtain accurate results dilute the 4N HCl to N/10 HCl by adding 2.5 cc. from a certified pipette to 97.5 cc. of distilled water measured from a burette. Make duplicate dilutions, and titrate three 2-cc. samples from each with sodium hydroxide as follows: Place about 70 cc. of distilled water in each of four 300-cc. Erlenmeyer flasks and to each add one drop of indicator. Rotate and compare the resulting color in the four flasks. If all are not the same light amber, empty any flask in which the color is different. Rinse thoroughly and prepare a fresh solution. Mark one flask "C" and set it aside as a control. To the three others, add 2 cc. each from one of the N/10 HCl dilutions. Titrate with the NaOH solution, determining the end-point by comparison with the control flask. Repeat with samples from the other N/10 HCl dilution and a fresh control flask. The six titra-

tions should not vary by more than 0.1 cc. If the variation is 0.15 or 0.2 cc., make two additional titrations of each flask. If it is greater, prepare new dilutions. Take the average and calculate the value of 1 cc. 4N HCl in terms of NaOH as follows:

Average reading—2 cc. N/10 HCl = 19.925 cc. NaOH
1 cc. N/10 HCl = 9.962 cc. NaOH
1 cc. 4N HCl = 40.00 cc. N/10 HCl
1 cc. 4N HCl = 398.48 cc. NaOH

Do not use a solution over two months old without restandardizing it.

**Standardization of Pipettes.**—To determine the size of the drop from a pipette, prepare four flasks, following the same procedure as above. Draw up 4N HCl into the pipette until it is about half full. Holding it in a vertical position, press gently upon the upper end with the index finger until a drop begins to form, then remove the pressure and allow the drop to fall unaided. Discard the first drop; start a second in the same way and allow it to fall into one of the flasks. In a similar manner, add a drop to each of two other flasks. Rotate the flasks. Place the pipette at the back of the desk in a small paper pocket numbered with the serial number of the pipette. Titrate the contents of each flask with NaOH, determining the end-point by comparison with the control. Record the number of the pipette and each titration. If the readings vary by more than 0.05 cc., titrate two additional drops. If the variation is greater than 0.1 cc., disregard all previous titrations and begin again. In case of repeated discrepancies, discard the pipette.

When the titrations have been finished, take one pipette at a time in its numbered pocket from the back of the desk. Place a rubber bulb (4½ by 3 cm.) on the end of the pipette, and after discharging the acid, rinse with dilute NaOH followed by distilled water, return the pipette to its pocket, and place at one side. When all are rinsed, take each pipette in turn, and after pasting a label with its serial number on the upper end, put the pipette in a swab tube with a cotton guard at the bottom, and plug the tube with cotton. Pack these tubes in large test tubes (180 by 25 mm.) for hot-air sterilization. After sterilization the pipettes are paired as described under "Determination of Required Volume of Diluent," p. 486, each pair fastened by a rubber band, and stored.

Check on pipettes: From time to time, with a certified pipette or burette, measure out into each of four dilution flasks one-half the amount of salt solution required to dilute the drop from the pipette to be checked. Add a drop of toxin from the pipette to each. Mix thoroughly. With a certified "to deliver"

pipette transfer 2.5 cc. from each to amber bottles; bring the volume up to 3 cc. with salt solution, and proceed as in the tests for the M.F.D. of toxin (see page 316), using guinea pigs weighing between 250 and 280 grams.

Requirements for pipettes: A pipette satisfactory for use with the outfit must have a bore large enough to allow the drop to fall easily and unaided, gentle pressure being used only to start its formation. On the other hand, the drop should not fall too easily or rapidly. The volume of salt solution which will be required should also be kept in mind. When toxin with an M.F.D. of 0.003 cc. is used, it is desirable to keep the volume between 11 and 17 cc., which corresponds approximately to titrations of 2.6 and 4.1 cc.

An ample supply of standardized pipettes is always kept on hand. Most of the pipettes are prepared and standardized during the summer when there are more hours of satisfactory light.

### *Determination of Required Volume of Diluent*

The volume of 0.85-per-cent salt solution used for diluting a drop of toxin delivered by the pipette, so that a final dilution of 1/50 M.F.D. in 0.1 cc. is obtained, is computed as follows:

Assume that the drop of 4N HCl delivered by the pipette is neutralized by 3.5 cc. of the NaOH solution standardized above, and that the M.F.D. of the toxin to be used is 0.003 cc.

Vol. drop: 1 cc. = T (titration of drop by NaOH): 398.48

$$\text{Vol. drop} = T \times \frac{1}{398.48}$$

To obtain the desired dilution (1/50 M.F.D. in 0.1 cc.)

$$\frac{1}{10} \times \frac{1}{\text{vol. salt sol.}} \times \text{vol. drop} = 1/50 \times 0.003$$

$$\text{or: Vol. salt sol.} = \frac{5 \times \text{vol. drop}}{0.003} = \frac{5}{0.003} \times \frac{1}{398.48} \times T$$

$$= 4.182 \times T$$

$$= 4.182 \times 3.5$$

$$= 14.63 \text{ cc.}$$

The volume of salt solution required to dilute the drop from this pipette is then 14.63 cc. and the factor for this solution of NaOH, if toxin with an M.F.D. of 0.003 is to be used, is 4.182.

The calculations may be expressed in the general formula:

$$\text{Vol. salt solution} = \frac{5}{\text{M.F.D.} \times S} \times T$$

in which: S = value of 1 cc. of 4N HCl in terms of NaOH

T = value of drop of 4N HCl in terms of NaOH

When a number of pipettes have been titrated, average the titrations for each and multiply the results by the factor. (The calculations must

be checked by a second person.) Select for a pair two pipettes requiring volumes of salt solution which are equal or which vary by not more than 0.05 cc.

The data from the original sheets, which contain titrations of the pipette and the calculations for determining the volume of diluent, are copied on the permanent record pipette cards (p. 491), and the sheets are filed one year for reference.

### *Selection and Preparation of Toxin*

A well-stabilized diphtheria toxin with an accurately determined M.F.D. must be used for the Schick test. In order that toxin of the required potency may be available when needed, it is advisable to set aside portions of lots of toxin with a high titer soon after the potency has been determined. When the toxin is to be used, the M.F.D. is determined with great accuracy and the reactions induced by intracutaneous injections in guinea pigs and finally in persons are ascertained. Toxin with an M.F.D. of slightly less than 0.003 cc. is selected, when possible, as giving a convenient and uniform standard (1/50 of 0.003 cc.) on which to base the volume of salt solution, and to a certain extent, the bore of the pipettes. When toxin is to be filled, the desired volume is siphoned from the stock bottle into a smaller bottle, which receives the number of the stock bottle followed by the serial letter of the filling (406A-a).

**Standardization of Toxin.**—The M.F.D. of the toxin is determined by the procedure given in "Standardization of Diphtheria Toxin," p. 313. At least four guinea pigs weighing between 250 and 280 grams are injected with each volume tested. Seventy-five per cent of the guinea pigs injected with a given dose must die in somewhat less than ninety-six hours with typical lesions, in order satisfactorily to establish it as the M.F.D. When the M.F.D. has been determined, at least two guinea pigs are injected intracutaneously with 1/50, 1/250 and 1/500 of an M.F.D., each contained in 0.1 cc. The 1/50 dose should induce a very marked reaction, usually heavy necrosis; the 1/500 dose, a reaction corresponding to a definite Schick reaction in a person. At the same time, each animal is injected with 0.1 cc. of the 1/50 dilution which has been heated at 100°C. for three minutes. No reaction should develop from this injection. After the animal tests are satisfactorily completed, the toxin is used in Schick tests upon several individuals. Laboratory workers who have previously shown a definite reaction, a weak reaction and no reaction may be retested. Should the reactions be unsatisfactory the toxin is rejected.

**Bottling of Toxin.**—When standardization tests have been completed, the toxin is dispensed in approximately 0.75-cc. amounts in 1-cc. amber vials by the filling and boxing group (see "Preparation of Biologic and Chemical Products for Distribution," p. 524). The sealed vials in boxes clearly labeled "Schick Toxin, No. (—)," are placed in the cold room. The labels which are later attached to the vials are printed in red and read "Poison—Undiluted Diphtheria Toxin No. —. Read directions before using."

*Assembling of Outfits Including Bottling of Salt Solution*

Each outfit consists of:
A 1-cc. amber vial containing about 0.75 cc. undiluted toxin.
A pair of capillary pipettes.
One or three bottles (at present 15 or 20 cc. sizes)[2] containing sterile 0.85-per-cent salt solution.[3]
A sterile plugged tube (100 by 15 mm.).
A circular giving directions for the use of the outfit.
A wooden box with a sliding cover (7 by 1¼ by 1¼ in. for the "one-bottle" outfit; 7 by 2½ by 1¼ in. for the "three-bottle" outfit).

Workers from the filling and boxing group assist in the assembling of the outfits.

It has been found convenient to prepare the outfits as far as possible before the salt solution is dispensed. A cotton pad, and a sterile plugged tube (100 by 15 mm.) wrapped in tissue paper, are placed in each box and a label is pasted on the cover. The pipettes are selected, and their numbers, together with the volume of salt solution required (the smaller if the pipettes vary), are recorded on the bottom of the box. A second worker checks the numbers on the pipettes with those on the box, wraps the two pipettes in tissue paper, and checks the record on the box with the original cards. The volume of salt solution required and the numbers of the pipettes in the outfit are entered on labels for the salt-solution bottles. The labels are checked by a second worker. Each outfit is given a serial number which is entered on the cover and on the bottom of the box. The required data are then entered on the outfit card (p. 491). The boxes are arranged in the draft-free room in the order for filling.

[2] Results of a few tests suggest that the diluted toxin when transported before injection, may undergo some deterioration, if there is considerable air space in the bottle.

[3] Each bottle contains sufficient diluent for from 30 to 60 tests. A limited number of outfits containing eight bottles of salt solution are prepared.

The salt solution is dispensed from a sterile, certified 50-cc. burette. Sterile precautions are observed throughout, and the routine, sterility tests are made.

Orders are placed with the media department for specially prepared 0.85-per-cent salt solution dispensed in a 4-liter bottle; and for bottles (15 and 20 cc.) selected with uniform necks to fit exactly the stoppers used. The stoppers are boiled in 0.5-per-cent crude cresol and scrubbed to remove talc. Just before use they are again boiled in 0.5-per-cent crude cresol, in which they are kept.

The burette is filled with cleaning solution, allowed to stand, rinsed with distilled water, and filled with 80-per-cent alcohol, which is allowed to remain in it overnight, care being taken that the alcohol penetrates between the stoppers and the sides of the sockets. Before use, the burette is drained; and the stoppers are dried with sterile filter paper, the sockets with an absorbent cotton swab; the stoppers and sockets are then smeared with a sterile mixture of beeswax and vaseline, fitted together, and wired. A siphon with connections is prepared and sterilized in the usual manner.

The siphon is inserted into the bottle of freshly prepared sterile 0.85-per-cent salt solution and the tubing connected with the burette. The burette is filled with salt solution and emptied several times, the contents being discarded each time, until there is no indication of alcohol remaining. The usual number of broth tubes are inoculated from the burette for sterility tests, and the filling commenced. In measuring the salt solution, the reading is always made from "0" or, if later, drops adhere to that part of the burette, from "10." An assistant takes one box at a time and places it upside down beside the filler, who fills a bottle or bottles with the exact volume indicated on the bottom of the box. As she hands the bottle to the assistant the filler reads the volume from the burette, and is checked by the assistant who reads it from the box. The assistant closes the bottle with a rubber stopper dried with a flame, and places it immediately in the box, or, in the case of outfits containing three or more bottles, sets it at one side until the others are filled. It is essential that the bottles belonging to each outfit be kept at all times by themselves in or near the outfit.

The worker in charge of sterility tests withdraws from the unfinished outfits the bottles to be tested, which are then replaced by other bottles.

The stoppers are tightened and sealed with fishskin dipped in 0.5-per-cent crude cresol, the bottles are examined for defects, and the labels attached. All the bottles from a given outfit are placed on a level surface, and a mark about $\frac{1}{2}$ cm. long made with a diamond pencil on each, at the level of the meniscus. The graduation is checked by a second person. The bottles are returned to the boxes and a layer of cotton and the circular are placed on top. The outfits are then

arranged in serial order, the toxin added, and the outfits assigned at once to the filling and boxing group who place them in the cold room. Cards (5 by 8 in.) on which are entered the numbers of the outfits and volume of salt solution per bottle in each, take the place of the record forms used for other products. These are signed by the worker in charge of the toxin-production group when the material is ready for distribution. The toxin-production group should be kept informed in regard to the supply of outfits on hand and rate of distribution.

### *Final Preparation for Distribution*

After the sterility tests are completed, the bottles of salt solution in each outfit are examined for possible leakage. They are then wrapped in tissue paper and returned to the outfits.

Check on volume of diluent: The accuracy of the volumes dispensed is checked occasionally.

Check the level of the meniscus, and empty, rinse, and dry the bottle. Add the required volume from a certified burette, and again check the level with the graduation mark on the bottle.

Check on complete outfit: From time to time an outfit is withdrawn from the stock released for distribution and tested.

Add two drops of toxin from one of the pipettes to each bottle of salt solution in the outfit, and mix thoroughly. From these solutions, transfer 2.5 cc. to each of four amber bottles, and proceed as in the test for the M.F.D. of toxin (p. 316), using guinea pigs weighing between 250 and 280 grams.

### *Return Date and Retests*

When an outfit is sent out, the "Return date" is entered on the label of the box. In the case of toxin standardized for the first time for use in the Schick test, this date is three months later than that of the standardization test. About a month before the date of expiration the toxin in the small bottles is retested. If no deterioration is indicated, the time limit on the outfits is extended two months, and the new date sent to the filling and boxing group to be used on the boxes distributed during that period. The toxin is tested at bimonthly intervals and if there is no evidence of deterioration the time may be further extended. Should the toxin have deteriorated, other vials containing a toxin of the original titer may be substituted in the outfits.

### *Permanent Records*

Standardization of NaOH solution: On a card (5 by 8 in.) are recorded in connection with each standardization: date, volumes of 4N HCl and distilled water and final dilution of HCl; titrations of HCl dilutions with NaOH solution

and the average; number of cubic centimeters of NaOH equivalent to 1 cc. 4N HCl; M.F.D. of toxin to be used; factor of NaOH solution; and initials of workers.

Pipette card: On a card (5 by 8 in.) are recorded the following data in regard to each pipette: date standardized, initials of worker, serial number, individual titrations and average, volume of dilution required for a stated M.F.D., and the number of the pipette with which it is paired. The factor for the NaOH solution is entered at the top of the card; a new factor, in red at the top of the card and just above the first pipette standardized with the new solution.

Outfit card: On a card (5 by 8 in.) are recorded the following data in regard to each outfit: serial number of the outfit, numbers of pipettes, volume of salt solution required in each bottle, number of bottles, date filled, initials of filler, and result of sterility tests; lot and bottle numbers, and M.F.D. of the toxin, date filled, and date placed in outfit.

## CHAPTER 3

# PREPARATION OF OUTFITS FOR THE INTRACUTANEOUS TEST OF SUSCEPTIBILITY TO STREPTOCOCCUS TOXIN (SCARLET FEVER)

A limited number of outfits, similar to the one used for the Schick test, are prepared for use in performing the intracutaneous test for susceptibility to "scarlet-fever" streptococcus toxin. The procedures described in the chapter "Preparation of Outfits for the Schick Test," p. 483, should be followed except as indicated below. The outfits are distributed only on special request and with the understanding that complete reports will be submitted to the laboratory.

The outfit consists of a small vial containing partially diluted streptococcus toxin, two standardized, capillary pipettes for measuring the toxin, a bottle containing the exact amount of salt solution necessary to give the required dilution when one drop of toxin from one of the pipettes is added, and a bottle of diluted toxin, already heated, for the control test. The drop of toxin added to a known volume of salt solution gives the desired skin-test dose in 0.1 cc. Each outfit contains a report form to be returned to the laboratory so that information may be collected.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

### *Determination of Required Volume of Diluent*

The partially diluted toxin (at present 1:3) distributed in the outfits for the intracutaneous test, has a skin-test dose of 1/10,000 cc. The volume of 0.85-per-cent salt solution used for diluting one drop of the toxin delivered by the pipette so that the skin-test dose is contained in 0.1 cc. is computed as follows:

$$\frac{1}{10} \times \frac{1}{\text{Vol. Salt Sol.}} \times \frac{\text{Vol. of drop}}{1} = \frac{1}{10{,}000}$$

Vol. Salt Solution = 1000 × vol. of drop.

The calculation may be expressed in terms of the general formula:

$$\text{Vol. Salt Solution} = \frac{1000}{1} \times \frac{1}{S} \times \frac{T}{1}$$

in which S = value of 1 cc. of 4N HCl in terms of NaOH
T = value of drop of 4N HCl in terms of NaOH

### *Selection and Preparation of Toxin*

A stable toxin of high titer, which induces a reaction persisting for at least twenty-four hours, is selected for use in the intracutaneous test.

While the streptococcus toxins are apparently much more stable than diphtheria toxin, there is evidence that some are more stable than others. Until it can be determined, therefore, how rapidly deterioration takes place and whether the toxins reach an equilibrium where the toxicity remains stable for a long period of time, toxins released for distribution are tested at frequent intervals, at least every six weeks, to determine whether they are satisfactory for use. Three lots of partially diluted toxin were still satisfactory for use after from eight to ten months; in another, however, rapid deterioration took place within a few weeks.

The skin-test dose is determined according to the procedure given in "Production and Standardization of Streptococcus Toxin (Scarlet Fever)," p. 330. Since the skin-test dose of the toxin is usually so high that the volume of salt solution required with the capillary pipettes used in the outfits would be from 30 to 50 cc., the toxin is diluted with sterile 0.85-per-cent salt solution containing 0.45-per-cent phenol, so that 1 cc. contains 10,000 skin-test doses. (The toxin may be diluted from three to five times according to the titer of the toxin.) Usually from 150 to 300 cc. of diluted toxin are prepared at one time. The pipettes used are standardized and reserved for this purpose.

With rigid aseptic precautions, pipette the exact volume of phenolized salt solution into a bottle and then, with a second pipette, add the required volume of toxin. Remove a sample of the diluted material for standardization tests and make sterility tests by inoculating two aerobic and two anaerobic tubes of sterility-test broth with 0.25 cc. each and one 160-cc. bottle of Hitchens' medium with 2.0 cc.

### *Heated Toxin for Control Tests*

The heated toxin for the control test is distributed already prepared. Usually about 800 cc. of material are prepared at one time.

Dilute the toxin with sterile 0.85-per-cent salt solution so that the skin-test dose is contained in 0.1 cc. With the usual aseptic precautions, siphon the exact amount of salt solution required into an accurately graduated bottle. With a sterile graduated pipette add the required amount of toxin. Completely immerse the bottle in a water-bath except for the neck and the cotton plug, which is kept dry by a covering of tinfoil held in place with a rubber band. Bring the water in the bath to the boiling point (100°C.) and hold at this temperature for one and one-half hours. When the bottle is removed from the bath,

take a sample for standardization tests, and make sterility tests as for the unheated toxin. Close the bottle with a sterile rubber stopper.

*Confirmatory Tests on Persons*

After the sterility tests are completed, make control tests of the diluted toxin on at least three persons known to be susceptible. Use two outfits for the intracutaneous test in preparing the dilutions. Inject each person with 0.1 cc. of each dilution and with 0.1 cc. of the heated control. For further details, see the circular giving directions for the use of the outfit and interpretation of results. If the tests are unsatisfactory, prepare other dilutions or select and standardize a fresh toxin.

*Bottling of Toxin*

The partially diluted toxin for the test is dispensed in approximately 0.75-cc. amounts in 1.0-cc. amber vials. The labels, which are orange-colored to distinguish the toxin from diphtheria toxin for the Schick test, read "Scarlet Fever—Streptococcus Toxin No. —. Use diluted. Read directions before diluting."

The diluted, heated toxin for the control test is bottled in approximately 5-cc. amounts in 5-cc. bottles. The labels, which are also orange colored, read, "Diluted Toxin—Heated Control No. —. Read directions before using."

*Assembling of Outfits*

Each outfit contains, in addition to the material previously described, a circular giving directions for the use of the outfit and a report form for recording the results of the test.

Check on completed outfit: From time to time an outfit is withdrawn from the stock released for distribution and tested, intracutaneous tests being made on two or three persons known to be susceptible to the toxin.

*Return Date and Retests*

When an outfit is sent out, the expiration date is entered on the label (also orange colored) on the box, "Not reliable after....." At present the date is one month from that on which the toxin was tested. If retests show no deterioration the time limit may be extended.

*Permanent Records*

The permanent records are similar to those described in the "Preparation of Outfits for the Schick Test," p. 491.

# CHAPTER 4

## PRODUCTION AND STANDARDIZATION OF MONOVALENT IMMUNE SERA (FOR DIAGNOSTIC USE)

Monovalent agglutinating sera for use as diagnostic agents are produced by the immunization of horses or rabbits[1] against the specific standard group or type strains. Methods for the production of these sera with the exception of those produced against the pneumococcus and meningococcus groups are given in this section. A list of the monovalent[2] immune sera used at present in this laboratory follows.

Typhoid, and paratyphoid A, and B, immune sera produced by the immunization of horses or rabbits.

Antidysentery sera, produced by the immunization of rabbits against standard strains representative of the Shiga, Flexner and Mt. Desert types.

Antipneumococcus sera, types I, II, and III, produced by the immunization of horses as described in the chapter "Production and Standardization of Antipneumococcus Sera," p. 409.

Antimeningococcus sera, groups I, II, and III, produced by the immunization of rabbits as described in the chapter "Production and Standardization of Antimeningococcus Sera," p. 428.

*B. melitensis* and *B. abortus* sera produced by the immunization of rabbits.

Sera are also produced in limited amounts against *B. enteritidis*, *B. morganii*, *B. suipestifer*, *B. pestis caviae*, and *B. proteus* X19.

Besides the monovalent sera for routine diagnostic work, special sera are produced as required against organisms of other species or atypical strains of the same species.

In addition to the immune sera listed, a supply of normal serum of every species of animal used in the production of immune sera is maintained for control purposes.

Enter on the permanent record forms provided for the purpose, each step in the procedure. Make each entry as soon as the data become available.

## PRODUCTION OF IMMUNE SERA (TYPHOID, PARATYPHOID, AND DYSENTERY GROUPS)

### *Strains Used*

The following strains have been selected for use in the production of immune sera.

[1] The immunization of goats has been undertaken. If these animals prove satisfactory they may be substituted for rabbits in certain instances.

[2] Polyvalent antimeningococcus and antidysentery sera are also used for diagnostic purposes.

*B. typhosus* Rawlings: Collection No. 270B
*B. typhosus* Bender: Collection No. 270C
*B. paratyphosus* A: Collection No. 235A
*B. paratyphosus* B: Collection No. 236B
*B. dysenteriae* Shiga: Collection No. 114F
*B. dysenteriae* Flexner: Collection No. 114D
*B. dysenteriae* Mt. Desert: Collection No. 114E

These strains were received in 1915 from the New York City Research Laboratory, with the exception of *B. typhosus* Bender, received in 1915 from the Bender Laboratory, Albany; *B. typhosus* Rawlings, received in 1918 from the Army Medical School.

### *Maintenance of Strains*

Cultures of *B. typhosus* Bender and *B. paratyphosus* A, and B, and the dysentery strains are maintained on beef-infusion agar by monthly transfer following the procedure given in "Preparation of Typhoid and Typhoid-Paratyphoid Vaccines," p. 457. Transplants of *B. typhosus* Rawlings are obtained, when required, from the standard culture carried on for the preparation of vaccine.

## IMMUNIZATION OF RABBITS

Normal rabbits weighing preferably from 1800 to 2200 grams are used. Before immunization is commenced, 5 cc. of blood are taken from the ear vein and the serum is held for comparison if required. Usually from four to eight rabbits are immunized at one time, depending upon the amount of serum required. Two methods of immunization, requiring sixteen and twenty-eight days respectively, are used. The more rapid method gives serum of high titer and is usually preferred except in the case of *B. dysenteriae* Shiga. In both methods, preliminary injections of heated suspension are given followed by injections of living organisms.

Method I.[3]—
1st day: 1/10 of a slant, killed culture.
2nd day: 1/10 of a slant, killed culture.
5th, 7th, and 9th days: 1/10 of a slant, living culture.
14th day: Take a trial bleeding.
15th day: Take a whole bleeding if trial bleeding is satisfactory.

The first injection is given intraperitoneally, the others intravenously. With the paratyphoid-B strain it may be advisable to reduce the first two doses to 1/20 of a slant, since this strain appears to be slightly more toxic than the others of the typhoid-paratyphoid group.

[3] The method is similar to that described by Bull, C. G., Jour. Exp. Med., 1916, 23, 419 (157).

Method II.—
1st day: 1/200 of a slant, killed culture.
4th day: 1/100 of a slant, killed culture.
7th day: 1/50 of a slant, killed culture.
15th day: 1/200 of a slant, living culture.
18th day: 1/100 of a slant, living culture.
21st day: 1/50 of a slant, living culture.
26th day: Take a trial bleeding.
27th day: Take a whole bleeding if trial bleeding is satisfactory.

All injections are given intravenously.

Before beginning immunization, determine the identity and purity of the cultures to be used by the method given in the chapter "Preparation and Standardization of Typhoid and Typhoid-Paratyphoid Vaccines," p. 459. Select two instead of three colonies for the test, and on the third day inoculate two agar slants from the broth culture. After eighteen hours' incubation, examine one agar culture microscopically. If pure, add 4 cc. of salt solution (0.85 per cent) and loosen the growth with a loop. Filter 2 cc. of suspension through a cotton filter (see "General Instructions," p. 624), previously moistened with 1 cc. of salt solution. Wash the filter with 2 cc. of salt solution. If killed organisms are to be given, heat the suspension at 60°C. for thirty minutes (see "Production and Standardization of Antipneumococcus Sera," p. 413). Dilute the filtered suspension (corresponding to the entire slant in 10 cc.), if necessary, so that the required dose is contained in 1 cc. (for 1/10 of a slant, no dilution; 1/20, 1 + 1; 1/50, 1 + 4; 1/100, 1 + 9; 1/200, 1 + 19). Inject 1 cc. of the dilution. Should injections with different strains fall on the same day, complete the dilutions of one strain and set them aside before beginning work with the next; complete the injections of one strain before taking the next into the operating room. Sixteen to eighteen hours before the next dose is to be prepared, inoculate two agar slants from the second culture, one for the rabbit dose and one as a seed for further transplants.

Take a trial bleeding of about 4 cc. from the ear vein, and test immediately. If the titer is sufficiently high (1:5,000 to 1:10,000), and agglutinative reactions with heterologous type or group strains are not excessive, bleed out the rabbit or, should it be desirable to keep the animal, take a smaller volume of blood from the heart, or preferably from the ear vein. See that all food is removed from the animal's cage on the night before the bleeding. Allow the clot to form, if

necessary rim the clot with a sterile pipette, and place the bottle in the cold room overnight. If the titer of the trial bleeding is not sufficiently high, continue immunization, basing the interval and dosage on the condition of the animal.

### IMMUNIZATION OF HORSES

The method of immunization is the same as that for the production of polyvalent antidysentery serum (see "Production and Standardization of Antidysentery Sera," p. 442), except that each horse is immunized against a single strain and the size of the dose depends upon the organisms used. Living cultures however, are substituted for killed organisms in the later series of injections only if the killed cultures fail to produce a satisfactory serum. Before immunization is commenced the serum of the horse is tested for agglutinative reaction. If high nonspecific agglutinative reactions are obtained with the type or group strains, the horse is not used.

Whole bleedings are taken as soon as the serum reaches a sufficiently high titer against the homologous strain (usually 1:10,000) as the agglutinative titer against the heterologous groups usually becomes higher as immunization progresses. (Horse sera are higher in group agglutinative activity than rabbit sera.)

### IMMUNIZATION OF GOATS

Immunization of a limited number of goats has recently been carried on by the method used for horses. Agglutinating sera of the Mt. Desert and Flexner types have been produced. These sera were satisfactory when produced. Considerable deterioration has, however, been noted at the end of a year.

### TREATMENT OF IMMUNE SERA

Rabbit serum is drawn off from the clot and centrifugalized, if necessary. Sera from several rabbits immunized with the same organism[4] are pooled and given a serial number. To this pooled serum an equal volume of glycerin (Merck's "T.P. Reagent" sterilized in the autoclave at 15 pounds pressure for twenty minutes) is added. Part of the serum may be reserved to be dispensed without glycerin. Horse serum is treated as described in "Care and Treatment of Animals—

[4] A polyvalent agglutinating serum for tests for *B. morganii*, *B. enteritidis*, *B. suipestifer*, *B. pestis caviae*, *B. paratyphosus* A and *B. paratyphosus* B has been prepared by pooling in suitable proportions monovalent sera produced with each organism.

Large Animals," p. 595, and "Production and Standardization of Antimeningococcus Sera," p. 433, except that glycerin may be added before the serum is dispensed. When outdated therapeutic sera are to be used as diagnostic sera, glycerin is added (to give final 50-per-cent concentration) in addition to the cresol already contained.

Monovalent sera are dispensed in 2.5-cc. volumes; glycerinated polyvalent sera in 5 cc. and unglycerinated polyvalent sera in 5 and 10 cc. Pooled sera are dispensed by the filling and boxing group, routine sterility tests being made. (See "Preparation of Biologic Products for Distribution," p. 524.) The stoppers are sealed and covered with tinfoil. Small lots may be dispensed by the production group by pipetting the material into the final containers under rigid aseptic precautions. If less than 10 bottles are filled, one bottle is tested for sterility by inoculating one aerobic and one anaerobic tube, and one 20-cc. tube of Hitchens' medium with 0.5 cc. On the labels are entered "Diagnostic serum," species of animal from which obtained, organism against which produced, titer and lot number; if absorbed, "Absorbed"; if glycerinated "50% glycerin" and "For agglutination tests only." For typhoid and paratyphoid A, and B sera both the macroscopic titer and the dilution to be used in microscopic tests are included. A small supply of serum without glycerin is maintained for use in precipitation tests and special research work.

Each kind of serum is stored in the cold room in a separate, clearly marked container. Each bottle is examined carefully before being sent out. Horse serum is retested at least every six months. Rabbit serum is usually distributed promptly but, if over six months old, it is retested before being distributed.

Glycerinated sera are always standardized after the addition of glycerin. Workers in the diagnostic department are expected to report immediately to the production group any irregularities in the tests or deterioration in the titer of a serum.

## STANDARDIZATION OF SERA

The sera are standardized by macroscopic agglutination tests. The microscopic titer of typhoid and paratyphoid sera to be used as controls in the examination of blood by the microscopic Widal test is also determined. A serum is tested against its homologous[5] strain and against related strains to determine the limit of group agglutination.

[5] In the case of typhoid agglutinating serum the standard "Bender" strain of *B. typhosus*, which for years has been used in the diagnostic laboratories as a control culture, is used.

It is advisable also to test the serum against recently isolated strains of its own type. The agglutinative titer of the serum is determined promptly after bleeding. Tests of trial bleedings are usually made with the cultures maintained by the production group; the final standardization tests of sera to be distributed with the cultures maintained and distributed by the bacterial collection group.

### *Macroscopic Tests*

The procedures for macroscopic agglutination tests are those given for polyvalent antidysentery serum (see "Production and Standardization of Antidysentery Sera," p. 444) with the following differences.

TABLE 34

*Serum dilutions**

| VOLUME OF SERUM OR SERUM DILUTION | VOLUME OF SALT SOLUTION | FIRST DILUTION | FINAL DILUTION |
|---|---|---|---|
| *cc.* | *cc.* | | |
| 0.1 (undil.) | 9.9 | 1:100 | 1:200 |
| 1.0 (1:100 dil.) | 1.5 | 1:250 | 1:500 |
| 1.0 (1:100 dil.) | 4.0 | 1:500 | 1:1,000 |
| 1.0 (1:100 dil.) | 9.0 | 1:1,000 | 1:2,000 |
| 0.1 (1:100 dil.) | 1.4 | 1:1,500 | 1:3,000 |
| 0.1 (1:100 dil.) | 1.9 | 1:2,000 | 1:4,000 |
| 0.1 (1:100 dil.) | 2.4 | 1:2,500 | 1:5,000 |
| 0.1 (1:100 dil.) | 2.9 | 1:3,000 | 1:6,000 |
| 0.1 (1:100 dil.) | 3.4 | 1:3,500 | 1:7,000 |
| 0.1 (1:100 dil.) | 3.9 | 1:4,000 | 1:8,000 |
| 0.1 (1:100 dil.) | 4.9 | 1:5,000 | 1:10,000 |
| 0.1 (1:100 dil.) | 5.9 | 1:6,000 | 1:12,000 |

* It may be necessary to make higher or intermediate dilutions according to the titer of the sera.

The tubes are incubated at 37°C. instead of 55°C.

Table 34 may be used in making serum dilutions.

The dilution in which clumping is easily visible to the unaided eye is recorded on the bottles of serum to be distributed for diagnostic use. In selecting the titer, allowance is made for differences in technic and for possible deterioration. No serum is distributed in which agglutination with heterologous group or type cultures is sufficiently high to confuse the results of the test when the dilution indicated is used.

It may be advisable to treat by the absorption method, sera in which the agglutinative reactions with heterologous group or type cultures is marked.

Absorbed sera are prepared by saturating the serum with organisms of the heterologous type strains. The following method has given satisfactory results.

Suspend the growth from eighteen- to twenty-four-hour beef-infusion agar cultures (for 80 cc. of serum use 30 to 40 Blake bottles, see "Preparation of Typhoid and Typhoid-Paratyphoid Vaccines," p. 459), in 0.85-per-cent salt solution and centrifugalize the suspension until the organisms are packed to a constant volume. Draw off the supernatant fluid and ascertain the volume of the sedimented organisms. Add the serum to be absorbed to the packed organisms, usually in the proportion of nine parts of serum to one part of organisms. (The use of a higher or lower dilution is indicated in the case of serum giving a very high or very low group-agglutination reaction.) Mix the serum and organisms thoroughly and incubate in a water-bath at 45°C. for three hours, shaking the mixture every half hour. Then centrifugalize the mixture and filter the supernatant serum through a filter candle.

TABLE 35

*Serum dilutions made with 0.85-per-cent salt solution**

| VOLUME OF SERUM OR SERUM DILUTION | VOLUME OF SALT SOLUTION | FIRST DILUTION | FINAL DILUTION |
|---|---|---|---|
| *cc.* | *cc.* | | |
| 0.1 (undil.) | 9.9 | 1:100 | 1:200 |
| 1.0 (1:100 dil.) | 1.5 | 1:250 | 1:500 |
| 1.0 (1:100 dil.) | 4.0 | 1:500 | 1:1,000 |
| 1.0 (1:100 dil.) | 9.0 | 1:1,000 | 1:2,000 |
| 0.5 (1:100 dil.) | 9.5 | 1:2,000 | 1:4,000 |
| 0.2 (1:100 dil.) | 5.8 | 1:3,000 | 1:6,000 |
| 0.2 (1:100 dil.) | 7.8 | 1:4,000 | 1:8,000 |

* When testing absorbed sera the lower dilutions only need be used; unabsorbed, only the higher.

### *Microscopic Tests*

The technic is that used in the examination of blood for agglutination with typhoid bacilli in the diagnostic laboratories (see "Standardization of Immune Sera," p. 165). The bacterial suspension is made from a beef-infusion broth culture grown overnight on top of a 37°C.-incubator. Serum dilutions made with 0.85-per-cent salt solution may be used, as in table 35.

The lowest serum dilution which shows complete or almost complete agglutination of the homologous strain and no or very slight agglutination with the heterologous type strains is selected as the microscopic titer to be entered on the labels. No serum is distributed in which reactions are not sufficiently specific to give satisfactory differentiation.

## PRODUCTION OF OTHER IMMUNE SERA

Monovalent immune sera for use in the diagnosis of undulant fever are produced with strains of *B. abortus* and *B. melitensis* by the immunization of rabbits. The strains are obtained as required from the bacterial collection.

*B. abortus* (Collection No. 97C), *B. melitensis* (Collection No. 48E). Intervals and mode of injections same as method I. Killed culture is used throughout. Usually doses of from one-half to one slant have been given.

A limited supply of immune sera is maintained for use in the diagnosis of other diseases. Because of the highly toxic properties of some of the cultures and the wide range of agglutination with type or group strains frequently encountered, special care may be necessary in the selection of strains to be used for immunizing purposes, and in the dosage given. Before the serum is distributed for diagnostic use the range of group agglutination and the interval between it and the upper limits of the specific reaction must be ascertained. Serum failing to differentiate clearly between the two is not distributed.

The following methods for the immunization of rabbits have so far given the most satisfactory results.

*B. enteritidis* (Collection No. 190E): intervals and mode of injection according to method I. Thirteen injections given. Doses of killed culture reduced to $\frac{1}{5}\frac{1}{0}$ of a slant; of living cultures to $\frac{1}{2}\frac{1}{0}\frac{1}{0}$, $\frac{1}{1}\frac{1}{0}\frac{1}{0}$, $\frac{1}{5}\frac{1}{0}$, up to $\frac{1}{1}\frac{1}{0}$ of a slant. Serum with fair agglutinative action with homologous type strains but considerable group agglutination obtained.

*B. morganii* (Collection No. 271B): produced by method I except that doses of living organisms on the 5th, 9th, and 11th days were reduced to $\frac{1}{2}\frac{1}{0}$ of a slant. Serum with high titer and practically no group reactions obtained.

*B. suipestifer* (Collection No. 261B): injections intravenously every third or fourth day. Ten injections of killed culture (heated at 60°C. for one-half hour) given. Initial dose of $\frac{1}{4}\frac{1}{0}$ of a slant, gradually increased to $\frac{1}{1}\frac{1}{0}$. (Living organisms even in small doses were not tolerated.) Serum with high specific titer and low group agglutination obtained.

*B. pestis caviae* (Collection No. 142): intervals and mode of injection same as method I. Nine injections given. Doses of killed culture reduced to $\frac{1}{2}\frac{1}{0}$ of a slant; of living culture to $\frac{1}{2}\frac{1}{0}\frac{1}{0}$ for initial dose, gradually increased to $\frac{1}{1}\frac{1}{0}$ for the last injection. Serum with fairly high specific agglutinative titer but with marked agglutinative action for *B. paratyphosus* B, and *B. suipestifer* obtained.

*B. proteus* X19 (Collection No. 242C): intervals and mode of injection according to method I. Five injections given. Doses of killed culture and first two doses of living culture reduced to $\frac{1}{2}\frac{1}{0}$ of a slant. Serum with a titer of 1:8000 obtained.

### *Permanent Records*

Immunization of rabbits: A card (4 by 6 in.) is kept for each rabbit, giving rabbit number, weight, kind and number of culture used, date and method of each injection and dose, and dates of trial and whole bleedings.

Immunization of horses: The records are similar in form to those described under "Permanent Records" in "Production and Standardization of Anti-pneumococcus Sera," p. 427.

Standardization tests: The records are similar to those for standardization tests of polyvalent antidysentery serum (p. 446), except that no standard serum is used and the test for each serum is put on a separate card which, in the case of rabbit sera, is filed with the immunization card.

## SECTION VIII

## CHAPTER 1

# PREPARATION OF CONVALESCENT MEASLES SERUM AND SERUM FROM RECOVERED CASES OF POLIOMYELITIS

Small supplies of convalescent measles serum for prophylactic use, and of serum from recovered cases of poliomyelitis for therapeutic use, are maintained for distribution upon special request. The procedures are similar in the preparation of both sera. The preliminary treatment of the serum, its removal from the clot, centrifugalization, and the addition of preservative, may be carried on at the state laboratory in Albany or by local laboratories. The final preparation of the serum is always carried on at the state laboratory. A sample of every serum is tested by the complement-fixation test for syphilis. No laboratory procedures are available by which to gauge the prophylactic or therapeutic value of the sera.

Bleedings for convalescent measles serum may be taken from ten to fifteen days, preferably about the fifteenth day, after the subsidence of fever, up to one month, provided the physical condition of the donor is satisfactory; for poliomyelitis serum, from four weeks to as long as two years from the subsidence of fever, though the serum from the more recent cases is preferred. The method of obtaining the blood is given in the circular of directions for the collection of human blood.

Enter on the permanent record forms provided for the purpose each step in the procedure. Make each entry as soon as the data become available.

### *Preliminary Preparation of Serum*

Observe strictly aseptic precautions in carrying on all procedures connected with the preparation of the serum. Upon receipt of the blood at the laboratory, rim the clot with a glass rod or pipette to loosen it from the sides of the container and place in the cold room overnight. The following day, with a sterile pipette, remove the serum very carefully from the clot and transfer to centrifuge tubes, centrifugalize to free the serum from any red blood cells which may have been carried over, and pipette the clear serum into a sterile bottle. (Poliomyelitis serum, which may be injected intraspinously,[1] should not contain more

[1] Since, except in rare instances, the serum when used is at least a month and usually several months old, its inactivation has not been considered necessary, or advisable, as this treatment might affect its value.

than a very faint trace of hemoglobin; slightly more hemoglobin is permissible in convalescent measles serum, which is injected intramuscularly.) Label with the kind of serum, name of donor, and date of bleeding. Before the addition of preservative, remove 3 cc. of serum to a tube (100 by 16 mm.) labeled with the same data, for a complement-fixation test for syphilis, and inoculate Hitchens' medium with 0.5 cc. of serum for a test of sterility. Enter on a syphilis history blank the kind of serum (convalescent measles, etc.) and other necessary information. Refer at once to the diagnostic laboratories for testing. After accurately measuring with a pipette, or in a sterile graduate, the amount of serum remaining, proceed to the addition of preservative.

**Addition of Preservative.**—Add to convalescent measles serum, phenol to give a final concentration of 0.5 per cent, in the proportion of 1 part of a 5-per-cent phenol solution to 9 parts of serum; to poliomyelitis serum, add cresol to give a final concentration of 0.2 per cent, in the proportion of 0.8 cc. of a 25-per-cent cresol solution to 100 cc. of serum. Add the preservative slowly to the serum, rotating the latter after each addition to insure thorough mixing.

Enter under "Preliminary Laboratory Report," on the form received with the blood, the date and hour received, date serum removed from clot, volume of serum, and of preservative added, with additional information as required.

When the preliminary preparation is carried on by a local laboratory the procedure is the same, except that the sterility test is not made. The serum is pipetted from the clot into a bottle included in the outfit for bleeding provided by the state laboratory. After the sample for the complement-fixation test, which is forwarded separately in a regular syphilis outfit, has been removed, and the preservative added, the bottle is securely stoppered and sent with the report, at once, to the state laboratory.

When serum is received from a local laboratory, examine it to determine whether it meets the requirements as to the absence of excessive hemoglobin, contamination, etc. Inoculate Hitchens' medium with 0.5 cc. of serum. Verify the recorded volume by measuring in a graduate or pipette.

### *Final Preparation of Serum*

Upon receipt of a report that no reaction was obtained in the complement-fixation test for syphilis, pool the serum with similar lots and filter immediately; or, provided the sterility tests are satisfactory, hold until other lots are received, then pool and filter. Filter serum through infusorial earth and a final filter candle, and test for sterility. (For procedure, see, "Filtration of Biologic Products," p. 511.) If required, filter and dispense a single lot immediately, and distribute as

soon as satisfactory reports of complement-fixation and sterility tests are received.

The sera are dispensed by the filling and boxing group, convalescent measles serum in 5-cc. volumes, poliomyelitis serum in 15-cc. (For procedure, see "Preparation of Biologic Products for Distribution," and "Sterility Tests of Biologic Products," pp. 524 and 543.) On the label of each bottle is given the kind, volume, and lot number of the serum.

The sera are considered satisfactory for use for one year from the date of bleeding. Since, however, the supply of material maintained in stock is limited, relatively fresh serum is generally used. The sera are distributed only on special request for immediate use, any material not used being returned for redistribution. The return date is not given on the outside of the package.

### *Permanent Records*

The forms (11 by 8 in., mimeographed), "Human Convalescent Measles Serum" and "Serum from Recovered Cases of Poliomyelitis," giving the report of blood collection and preliminary preparation of serum, are filed as permanent records.

Preparation of serum: A record similar to that for "Antipneumococcus Sera," p. 427, is kept.

## CHAPTER 2

## PREPARATION OF NORMAL HORSE SERUM

Normal horse serum is used chiefly in the therapeutic treatment of hemophilia. The serum is obtained from a healthy normal horse which has previously received the customary prophylactic injections of tetanus antitoxin and has satisfactorily passed the mallein test. No horse is used which has not been under observation for at least one month. The temperature must be normal at the time the whole bleeding is taken. The procedures connected with drawing off the serum from the clot, addition of preservative, filtration, and cultural and animal tests of sterility and harmlessness are the same as those described under "Production and Standardization of Antipneumococcus Sera," p. 417. The serum is distributed in bottles containing 50 cc., and is considered satisfactory for use for eighteen months from the date of bleeding.

The serum, without preservative, is also distributed in 5- and 10-cc. volumes for use as a control in certain diagnostic tests with immune sera.

### *Permanent Records*

The record kept is the same as that described under "Sera" in the chapter "Production and Standardization of Antipneumococcus Sera," p. 427.

# CHAPTER 3

## PREPARATION OF ARSPHENAMINE

The preparation of arsenical derivatives, the distribution[1] of which was undertaken during the war period, has always been limited to that of salvarsan or arsphenamine. For special purposes, the other derivatives, such as neo- or sulf-arsphenamine have been purchased from the manufacturer for distribution. Since these have now practically supplanted arsphenamine, and their manufacture is upon a satisfactory commercial basis, the preparation of arsphenamine by the laboratory may be discontinued. The method which has been used in the preparation of arsphenamine up to this time has given very satisfactory results. It is briefly as follows:

The preparation of arsphenamine dihydrochloride includes five major operations: (1) the synthesis of the P-arsanilic acid from anilin and arsenic acid; (2) the synthesis of the Phenol-p-arsonic acid from P-arsanilic by diazotization and heating; (3) nitration of the Phenol-p-arsonic acid to produce 3-Nitro-4-hydroxyphenylarsonic acid; (4) reduction of the 3-Nitro-4-hydroxyphenylarsonic acid to 3:3′-Diamino-4:4′-dihydroxyarsenobenzene (arsphenamine base) with sodium hydrosulfite; (5) preparation of the dihydrochloride of 3:3′-Diamino-4:4′-dihydroxyarsenobenzene (arsphenamine) from the arsphenamine base by precipitation from aqueous hydrochloric acid (158).

The preparation of the intermediates by synthesis with phenol and arsenic acid, however, has been tested and found to be an economical substitution.

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[1] Arsenicals have in general been distributed by the laboratory for use in state institutions and in clinics approved by the commissioner of health, which would not otherwise be able to procure the remedy.

## CHAPTER 4

## BIOLOGIC AND CHEMICAL PRODUCTS DISTRIBUTED BUT NOT PREPARED BY THE LABORATORY[1]

In addition to the various products prepared by the laboratory and which have previously been described, the following preparations are distributed.

### *Silver Nitrate Solution*

Outfits containing 1-per-cent solution of silver nitrate for the prevention of ophthalmia neonatorum are purchased and distributed. The outfits consist of two wax ampules, each containing two or three drops of the solution and a needle for piercing the wax. Each ampule contains a sufficient quantity for one case.

### *Rabies Vaccine*

Rabies vaccine prepared by the laboratories of the New York City Department of Health, is available to physicians in the state outside of New York City under special arrangement. If the patient is unable to pay for the vaccine and it is not furnished at municipal expense, it is provided by the state. The vaccine is prepared by the Semple method. Material for the entire treatment, consisting of fourteen doses to be given at 24-hour intervals, is sent at one time.

### *Antianthrax Serum*

A small supply of antianthrax serum for therapeutic treatment is purchased and held in stock for emergency use. It is distributed in 50-cc. volumes.

[1] Vaccine virus for smallpox is not distributed. Since a reliable product at a relatively small cost may readily be obtained from commercial laboratories, the vaccine when required, is purchased by the local boards of health. A study of the preparation of pure virus by the method of Noguchi of the Rockefeller Institute was commenced in 1916. While an effective bacteria-free product was obtained, the vaccine was not sufficiently uniform or stable to permit of its general distribution. The strain is, however, still maintained by passage through the testicles of rabbits.

### *Erysipelas Antistreptococcus Serum*

A limited supply of erysipelas antistreptococcus serum (159) is held in stock for emergency use.

### *Tetanus-Perfringens Antitoxin*

A small supply of tetanus-perfringens antitoxin, for use in cases of gas gangrene in which *B. welchii* is the incitant, is purchased and held in stock for emergency use. It is distributed in 100-cc. volumes.

## SECTION IX

## CHAPTER 1

# FILTRATION OF BIOLOGIC PRODUCTS

### *General Procedure*

Biologic products are sterilized by passage through a filter candle of diatomaceous earth which effectively holds back the organisms present. Preliminary clarification is necessary for many products. This is usually accomplished by passage through paper pulp or infusorial earth; or by centrifugalization. Certain products are also passed through a coarse candle (preliminary filter).

Sera and antitoxins for prophylactic and therapeutic use, diagnostic sera (horse), and serum and ascitic fluid for use in the preparation of culture media are filtered by the filtration group in a room reserved for the purpose and equipped with apparatus for suction and pressure filtration. The worker in charge of filtration is responsible for the proper performance of the various procedures connected with filtration; for the maintenance of order and cleanliness in the room; and for planning the work to conform with the requirements of the production groups. An assistant bacteriologist, designated by the head of the department, has general supervision of the work, acting in an advisory capacity.

Complete physical separation of products and apparatus connected with the filtration of sera, antitoxins, etc. and of infectious material is maintained. Bacterial toxins are filtered by the production groups in separate draft-free rooms. For special precautions to be observed in filtering infectious material (broth cultures, etc.) see "Production and Standardization of Diphtheria Toxin," p. 311. Diphtheria toxin-antitoxin mixture is filtered by the production group. Small or experimental lots of sera, toxins, etc. are usually filtered by the groups preparing them.

Only one kind of product is allowed in a filtration room at one time. For any modification of this rule the approval of the bacteriologist in charge of the department is necessary.

Two workers are required to carry out all procedures, such as setting up apparatus, changing bottles, inoculating culture media, etc., when aseptic precautions are necessary. The flame method is used through-

out. The worker in charge of serum and antitoxin filtration is assisted by a member of the production group for which the material is filtered.

*Treatment of Products*

When the material to be filtered contains precipitate, cells, or other matter which would quickly clog the pores of a candle, it is clarified by passage through infusorial earth or paper pulp in a Buchner funnel.[1]

Viscous products or those containing a heavy precipitate have been found to filter more satisfactorily through infusorial earth. This method has the added advantage that no dilution of the material takes place. Some sera which contain little or no precipitate are rendered sufficiently clear by passage through cotton. Certain products require preliminary filtration through a coarse filter candle before being passed through the final candle. Material is drawn through the candles by suction, or forced through by positive air pressure. The pressure method is used principally in filtering sera and antitoxins, while suction is employed when less viscous fluids, such as broth cultures or small lots of any material, are to be filtered. The process of filtration should not be a long one, as microörganisms may grow or may be forced through the pores of the candle. When no preservative is present preliminary and final filtration should be completed on the same day. Different lots of the same kind of product may be filtered in succession through the same candle; that having the highest titer being put through first. The various products are usually treated in the following manner.

**Cultures (Toxins).**—Broth cultures from which filtrates are prepared are usually clarified by passage through paper pulp or filter paper and cotton. Diphtheria, dysentery, and streptococcus toxin-broth cultures are then passed directly through the final filter candle; tetanus and botulinus, through a preliminary and then through a final candle. Suction is used in filtering these products. When small amounts of filtrate are required the preliminary clarification is usually unnecessary.

**Sera.**—Sera to which cresol has been added are usually allowed to stand for several weeks so that most of the precipitation which occurs may take place before filtration. All sera containing more than a slight precipitate are passed through infusorial earth or paper pulp. Sera (other than antitoxins) are then usually passed directly through the final filter candle. It may be desirable with serum in which an unusual amount of precipitate has settled to the bottom of the bottle,

[1] For certain experimental work asbestos wool has been used for the filtration of broth cultures, either for complete or partial removal of the organisms.

to filter the final portion through cotton before filtration through infusorial earth or paper pulp. Pressure is used for filtering large lots of serum; suction for small. Ascitic fluid is treated similarly.

**Antitoxins (Concentrated Sera).**—Antitoxins are usually allowed to stand three months or more before preliminary filtration to permit the maximum precipitation to take place. They are then filtered through infusorial earth and a preliminary filter candle, and returned to the cold room for several weeks to allow for further possible precipitation before final filtration. Pressure is used for filtering antitoxins except when very small volumes are filtered.

**Toxin-Antitoxin Mixture (Diphtheria).**—Toxin-antitoxin mixture after preparation is filtered directly through a filter candle by positive pressure.

**Equipment and Special Supplies Required.**—Buchner funnels: Standard size, 8 inch (20 cm.) for large volumes; smaller sizes as required.

Flasks: Heavy-walled Pyrex suction flasks. Standard sizes 4- and 2-liter for large volumes; smaller sizes as required.

Paper pulp: Prepared from a soft filter paper as follows: Tear 90 grams of paper (Eaton and Dykeman Co. No. 615) into small pieces and cover with boiling water. Allow to stand for several minutes, pour off the water, add lukewarm water and work with the hand. Pour off the water, transfer the pulp to an 8-liter bottle, and add 5 liters of 0.5-per-cent phenol solution (4500 cc. water and 500 cc. 5-per-cent phenol solution). Place in the bottle a 2-hole stopper fitted with a short outlet tube, and an inlet tube extending nearly to the bottom of the bottle. Connect the outlet tube to the suction intake through a guard bottle. Agitate the mixture by drawing air through it, until a smooth pulp is obtained.

Infusorial earth: A special high-grade earth is used.

Asbestos wool: Fiber, acid washed, for filtering—Eimer and Amend.

Filter candles: 10-by-2-inch Mandler filters for filtration of large volumes, small candles of standard sizes (as $2\frac{1}{2}$ by $\frac{5}{8}$, $1\frac{3}{8}$ by $\frac{5}{8}$, and $\frac{3}{4}$ by $\frac{5}{8}$), Berkefeld and Mandler, for smaller volumes. Filters are graded according to permeability as follows: Mandler, 10-by-2-inch preliminary filters, to withstand air pressure of from 4 to 6 pounds; 10-by-2-inch final filters, from 10 to 16 pounds; smaller size final filters, from 6 to 12 pounds; Berkefeld preliminary filters, grade V (very porous); final filters, grade N (medium), and grade W (very slightly porous). All candles are provided with washers and a lock nut. Upon receipt, a tag is attached to the stem of each candle giving its serial number, and later the kind of product for which it is reserved is entered. The tag is removed from a candle only when the latter is in use.

Glass mantles: Ordered in sizes corresponding to filter candles used.

Pressure apparatus: As described under "Filtration by Pressure," p. 517.

Bottles: Two-, four- and eight-liter green-glass bottles are generally used for the filtrate; 200- and 500-cc. bottles for "Residues." Sterile bottles are requisitioned a day or two in advance from the media department. (Bottles are sterilized at 15-pounds pressure for forty minutes. No bottles which have stood more than one week after sterilization are used.) A measuring tape is prepared

for each kind of bottle used by means of which the approximate volume contained can be determined.

Cleaning solutions: See "Cleaning of Filter Candles," p. 521.

### *Receipt of Orders. Record Forms*

Orders for filtration of sera and antitoxins are received from the production groups by the worker in charge of filtration. The orders are made out in duplicate on "Serum and antitoxin record" forms, which give the kind of product, lot or order number, and total volume. Orders for the following week are received on Saturday morning, so that the work may be planned in advance. Emergency orders are complied with, however. If adjustments cannot be made or the volume of work is too great, the bacteriologist in charge of the department should be consulted.

### *Preparation for Filtration*

**Sterilization of Apparatus.**—Apparatus used in the preliminary clarification of products must be clean but need not necessarily be sterile. The preliminary candles with connections and all bottles are sterilized, however. All equipment which comes in contact with the final filtrate must be sterile. Apparatus is sterilized by steam at 15-pounds pressure for forty minutes. The candles and connections are placed in covered, copper boxes with a protecting cotton pad on the bottom. During sterilization the cover of the box is raised slightly on one side. It is closed when the autoclave is opened. The apparatus is assembled and sterilized on the day before filtration. Should a Sunday or a holiday intervene, it may be sterilized on the previous day, and the closed box placed in the filtration room.

**Sterilization of Gowns and Caps.**—Sterile gowns and caps are worn by workers during the final filtration of all products intended for human use. These garments are placed in muslin bags and sterilized at the same time as the apparatus.

**Care of Filtration Rooms.**—The special rooms in which products are filtered must be kept clean and free from dust. After each day's work they are thoroughly cleaned, including pipes, etc. If the room has not been used for two or more days, it is given a thorough preliminary cleaning on the day preceding filtration. Immediately before a filtration, the table tops, apparatus, and other flat surfaces where dust may collect are wiped off with a cloth dampened in 1-percent crude cresol.

*Preliminary Filtration*

**Through Infusorial Earth.**—Set up one or more Buchner funnels in suction flasks and connect the side arms of the flasks with the vacuum system. If several flasks are connected with one vacuum intake by means of a branched connection, use Hoffman clamps to control the suction on every flask. Place in the funnel two layers of coarse soft filter paper (such as American Standard low ash No. 5160) slightly smaller than the bottom of the funnel but covering the holes. Mix infusorial earth with enough of the product to be filtered[2] to form a thin paste, and pour the mixture into the funnel. (In general, the thinnest layer that is still effective should be used; for most products, about 50 grams of earth in an 8-inch funnel; for others, from 50 to 100 grams.) Apply suction until a well-packed layer is formed. Then place a small piece of hard filter paper on top, and add more of the material to be filtered, letting the stream strike the paper guard. Examine the first 100 or 200 cc. of filtrate. If it appears cloudy, refilter. Keep the funnel well filled to prevent clogging. When a filter shows evidence of clogging, it is sometimes advisable to scrape off the film from the surface after allowing the funnel to become empty. As the suction flasks are filled, pour the filtrate into bottles to which the tags from the original bottles have been transferred. Use a new filter for each separate lot of material. Enter on the tag the date of filtration, and volume recovered, and in the case of antitoxins the time required for filtration.

Recovery of antitoxin. "Washings:" Rinse all bottles and suction flasks which have contained antitoxin with sterile 0.85-per-cent salt solution and combine the rinsings. Collect all washings in a sterile bottle, and attach a tag marked "Washings" on which are recorded the kind of antitoxin, lot number, and date. Place in the space reserved for such material in the cold room.

**Through Pulp.**—Set up the funnels as for filtration through earth. Place in a funnel a piece of hard filter paper (Whatman No. 50). To about 500 cc. of pulp (for an 8-inch funnel) add an equal volume of water and after shaking thoroughly, pour into the funnel and turn on the suction. Repeat with the other funnels. Allow practically all the water to drain from the pulp. (A well-packed uniform layer from 3 to 4 mm. thick is necessary to secure the best filtration.) Disconnect the suction, and after emptying the flask, rinse it with distilled water, and drain thoroughly. Reassemble the filter and place a small piece

[2] In the case of a very viscous lot of antitoxin, it may be advisable to mix the earth with physiological salt solution and empty or exchange the flask after the bed is formed.

of hard filter paper on the pulp. Turn on the suction, and pour in the liquid to be filtered. Examine the first 100 or 200 cc. of filtrate. If it appears to be diluted with water, discard it, or in the case of antitoxins combine it with the washings. If it appears cloudy, refilter. Proceed as in filtration through earth. If the filtrate runs too slowly make a new pulp filter.

**Through Asbestos Wool.**—Suspend the wool in water and proceed as in filtering through paper pulp.

**Through Cotton.**—Place a small disk of coarse wire in a ribbed-glass funnel, and line the funnel with two thin layers of nonabsorbent cotton arranged so that the fibers cross. Pour in the material to be filtered carefully. Refilter the first material which comes through.

**Through Soft Filter Paper and Cotton.**—Place a piece of soft filter paper (Eaton and Dykeman No. 615) in a glass funnel, and line the filter paper with two thin layers of nonabsorbent cotton arranged so that the fibers cross. Pour in the material to be filtered carefully. Refilter the first material which comes through, if not clear.

**Through a Preliminary Filter Candle.**—Prepare and set up the apparatus and proceed as described for final filtration by suction or pressure. The rubber stopper and connections may, however, be sterilized by boiling in 0.5-per-cent crude cresol for from ten to fifteen minutes, immediately before the filter is set up. Transfer the tags from the original to the new bottles. Enter on them the date of filtration and in the case of antitoxin, the time required for filtration.

*Final Filtration by Suction*

**Preparation of Apparatus.**—The apparatus required is a filter candle and mantle, bottles for the filtrate, and a 2-hole rubber stopper with connections. The stopper is prepared by passing through one hole a straight glass tube to which is attached a short piece of heavy, rubber tubing to connect with the nipple of the candle; through the second, a right-angled glass tube with a cotton guard to connect with the suction tubing. (The outlet tube may conveniently be made from a discarded bulb pipette.)

Examine the outlet tube to see that the guard is satisfactory. Boil the stopper and straight connection in 0.5-per-cent crude cresol, and assemble while wet in such a way that the opening of the straight inlet tube extends below that of the outlet tube in the bottle. Wire the rubber tubing on the inlet tube. Protect the stopper and end of the rubber tubing with cotton and cheesecloth guards, and wrap tinfoil around the nipple of the filter candle. Place in a copper box for sterilization.

When a small candle is to be used, the filter, mantle, and stopper are usually assembled before sterilization, and sterilized in a wire basket covered with paper. The lock nut, however, must not be screwed tight until after sterilization.

**Procedure.**—Slip a rubber washer over the nipple of the sterile candle, and pass the nipple through the mantle. Then slip a second rubber washer, a metal washer, and a lock nut over the nipple, and screw firmly in place. Using aseptic precautions remove the tinfoil from the nipple and attach the rubber tubing from the stopper, wiring the joint if necessary. Support the filter and mantle on a ring stand. Invert a closed cylinder, slightly larger in diameter than the candle, over the candle, to aid in keeping it covered. Insert the stopper in a sterile bottle and wrap a strip of cotton, dampened in 0.5-per-cent crude cresol, over the stopper. Place a tag on the bottle, giving the kind of product, number, and a letter if more than one bottle is to be used, (166 A). Record on the back of the tag the number of the filter candle. After connecting the suction intake to the inlet tube of the bottle, turn on the suction gradually and pour the material to be filtered into the mantle.[3] Discard the filtrate if it appears to be diluted with water, or refilter if cloudy. Keep the candle well covered. When the bottle is full, transfer the stopper to a fresh bottle, at the same time closing the full bottle with a rubber stopper previously boiled in 0.5-per-cent crude cresol and dried in the flame. These steps must be performed rapidly. Tag each fresh bottle when connected with the filter.

### *Final Filtration by Pressure*

**Preparation of Apparatus.**—The following apparatus is required: Pressure tank and connections; filter candle; sterile bottles for the filtrate; and a stopper and connections as described in "Final Filtration by Suction," except that the tubing is of soft rubber and from 4 to 5 feet long. The air outlet tube when in use is connected with a Woulff bottle (see "Preparation of Biologic Products for Distribution," p. 529).

The pressure tank consists of a cast-iron bowl lined with enamel, equipped with air pressure and exhaust valves (besides the control valve, the inlet tube is equipped with a check valve), and a pressure gauge, and connected at the bottom with a detachable nickel-plated cylinder which holds the filter candle. The

[3] It has been found from experience that in the routine filtration of diphtheria and tetanus toxins this method is satisfactory. In principle, room air should not be drawn through a candle, especially before the pores have been filled by the product to be filtered.

tank has an air-tight cover held down by wing nuts. A stopcock controls the flow of liquid into the cylinder. The bottom of the cylinder through which the nipple of the filter candle passes, is detachable. All joints are protected by rubber washers. The tanks in use are of two sizes, 4-liter bowl equipped with connection for one cylinder; and 8-liter bowl with connections for two cylinders. After each filtration the tank and outlet pipes are cleaned with warm water and Ivory soap or a fine-grained high quality pumice soap, with a long-handled brush, and very thoroughly rinsed and dried. The cover is always kept on when the tank is not in use.

Boil and assemble the stopper and connections as in "Final Filtration by Suction." Pass the nipple of the candle through a rubber washer, then through the detached bottom of the cylinder and finally through a metal washer and a lock nut. Screw the lock nut loosely in place. Attach the rubber tubing connecting the nipple with the stopper, wiring the connections. Place in the copper box for sterilization.

**Procedure.**—Tighten the lock nut on the sterile candle, screw the base into the cylinder and attach the latter to the tank. Using aseptic precautions, connect the sterile bottle as in "Final Filtration by Suction." Make sure that all joints are tight. Place a clean pan under the cylinder to catch possible drippings, which may later be returned to the tank for filtration. Pour the material into the tank (never more than three liters when the small tank is used; seven when the large). Make sure the exhaust valve is closed, and the stopcock into the cylinder is open. Turn on the pressure gradually. Watch the gauge constantly and do not exceed the prescribed pressure.[4] Open the exhaust valve to relieve mounting pressure if necessary. Should the first filtrate appear diluted with water, add it to the residues or washings or discard; should it contain tiny particles from the candle, refilter. Do not allow the foam to rise sufficiently to touch the stopper. Be on the alert to turn off the main valve instantly and to open the exhaust to prevent forcing air into the bottle when the tank becomes empty. When a bottle is full, shut off the pressure, open the exhaust valve, and allow pressure equilibrium to become established. Avoid breaking off the pressure suddenly. Replace the full bottle by an empty one and close the full one as in "Final Filtration by Suction."

When the material runs through very slowly, showing that the filter is clogged, shut off the pressure, open the exhaust valve slightly, and close the valve between the tank and the cylinder. Unscrew the cylinder and pour the product into the drip pan. Unscrew the metal base and draw out the filter carefully. Hold the candle over an enamel

[4] In filtering antitoxins do not permit the pressure to exceed 80 pounds. For sera use pressure under 25 pounds if possible, never more than 60 pounds.

pail containing about a liter of 0.85-per-cent salt solution. Brush the candle thoroughly and very gently with a medium soft brush wet repeatedly with the salt solution. In the case of antitoxins save the salt solution and pool with the "washings." Then hold the filter under the tap and brush in a stream of luke-warm water. Replace the filter in the cylinder and after screwing the latter into position, continue filtration. Return the antitoxin in the pan to the tank, when the tank is next filled. This procedure may have to be repeated, but it is preferable to substitute a second filter for the first instead of washing frequently.

When filtration is completed, unscrew the cylinder from the tank and pour the product remaining in it into a small sterile bottle, attach a tag marked "Residues" on which are recorded the kind of product, lot number, volume, and date, and refer to the production group, or in the case of antitoxins, to the concentration group. After preliminary filtration, record the volume on the tag of the first bottle of material filtered; after final filtration, the total volume (residue from preliminary and final) on the "Serum and antitoxin record" form.

When antitoxin has been filtered rinse all bottles and apparatus that have contained antitoxin with 0.85-per-cent salt solution, and pool as "washings." (See "Recovery of antitoxin," p. 515.) Brush the candle with salt solution as when the filter clogs. Rinse the tank with about one liter of 0.85-per-cent salt solution, force this through the candle and add to "washings."

Antitoxin residues and "washings" are placed in the cold room and held for reconcentration. Serum residues are placed immediately in the cold room, and if in the judgment of the worker in charge of production a residue is in good condition and of suitable titer, it may be added to the next lot of the same kind of serum filtered. Diphtheria toxin-antitoxin mixture residues are discarded.

### *Sterility Tests*

Make routine sterility tests of all filtrates after passage through final filters. For details as to the technic of testing, procedures in case of contamination, etc. see "Sterility Tests of Biologic Products," p. 543. (The sterility of sera and ascitic fluid to be used in media is tested by the media department.)

Withdraw from one bottle of each lot of antitoxin (and of other products as directed) 5 cc. for potency tests, and place in a sterile bottle labeled with the kind of antitoxin, lot number, and date.

### *Care of Materials and Room. Record Entries*

Cover the stopper of each bottle with cotton dampened in 1-per-cent crude cresol, tie down securely and cover with tinfoil and a paper cap.

Enter the required information on the tags and daily record; and after final filtration enter on the "Serum and antitoxin record" form the volumes bottled, used for tests, recovered as residue, and unaccounted for; number of bottles; date; initials of the worker; and the volume in the first bottle. If there is more than one bottle, make out a separate form for each additional bottle of a lot, entering only the kind of product, lot and bottle number, and volume. Store the material in the cold room. (The worker in charge of filtration of antitoxins and sera returns material to the production groups.) After cleaning the apparatus and removing from the room or placing in wall cupboards all unnecessary equipment, clean the room thoroughly.

**Sterilization of Apparatus Used for Bacterial Filtrates.**—All equipment which comes in contact with material containing pathogenic organisms during the process of filtration must be sterilized; Buchner funnels, flasks, candles, mantles, bottles, connections, pipettes used in sterility tests, "rinsings," etc. Draw water through the candles before sterilization. Have all equipment except the candles and connections used for filtering nonspore-bearing organisms other than *B. mallei*, *B. pestis*, and *Sp. cholerae* sterilized in the autoclave at 15-pounds pressure (121°C.). Loosen the lock nut on filters before sterilizing in the autoclave, to avoid breaking the mantle. Personally supervise placing in the autoclave all material contaminated by pathogenic spore-bearing organisms (*B. tetani*, *B. botulinus*, etc.) or *B. mallei*, *B. pestis*, or *Sp. cholerae*.

In the case of the nonspore-bearing organisms with the exception noted, do not take down the filter, but wipe off the outside of the mantle with crude cresol and proceed at once to clean the candle by the permanganate method.

It may, in certain instances, be more convenient to clean candles used for bacterial filtrate by the sodium-carbonate method. The filtering apparatus is then first sterilized in the autoclave, or boiled for fifteen minutes; the lock nut in either case being first loosened.

### *Refiltration*

Refiltration is required when the sterility tests indicate the presence of contaminating organisms in the filtrate, or when excessive precipitation occurs on standing. Contaminated material is passed through a final filter only. The preliminary treatment of cloudy material depends on the degree of precipitation. "Refiltered" and the date are entered on the tag. Instructions for refiltration of antitoxins and sera are received from the production groups by the worker in charge of filtration.

*Cleaning of Filter Candles*

The candles may be cleaned by either of two methods. Candles used for the filtration of antitoxins and sera are cleaned by the first method, those for cultures usually by the second.

**First Method.**—1. Wash by brushing gently the surface of the candle with a moderately soft brush using lukewarm 0.85-per-cent salt solution. If little or no film has formed on the candle this step may be omitted.

2. Pass 3-per-cent salt solution through the candle (about 4 liters for a 10-by-2-in. candle) unless 0.85-per-cent salt solution has already been used.[5]

3. Attach the candle to the faucet by rubber tubing and force water through from the inside for at least one-half hour, beginning with gradual pressure and increasing to not more than from 20 to 25 pounds. Brush the candle gently.

4. Boil the candle in 2-per-cent washing soda for thirty minutes.

5. Boil in tap water for thirty minutes, changing the water several times.

6. Allow to cool, then hold under the tap and brush gently.

7. Place the filter in the nickel cylinder, screw the latter on to the faucet and force water through for at least five minutes; or for the smaller candles, set up in a suction apparatus and draw water through.

8. Dry thoroughly. See "Drying of Candles," p. 522.

**Second Method—Potassium Permanganate.**—Solutions for cleaning: Potassium permanganate (0.5 per cent); sodium sulfite (5 per cent); and hydrochloric acid (5 per cent). The permanganate solution may be used over until the color changes to a brownish tint but should be refiltered each time shortly before use. The sulfite solution may be used two or three times.

Procedure: 1. Draw water through the candle.[6]

2. Fill the mantle with permanganate solution and draw through by suction until the water has been colored by the permanganate.

3. Replace the bottle by a fresh one and draw through the remainder of the permanganate solution. Then substitute another bottle.

4. Pour over the candle a volume of hydrochloric-acid solution, followed immediately by an equal volume of sulfite solution. For a 10-by-2-inch candle, use 150 cc. of each. (When the sulfite solution is used a second or third time, the relative volume of acid should be somewhat less.)

5. Place a cylinder over the candle and turn on the suction.

6. Add sufficient sulfite solution to fill the mantle and draw through.

7. Should brown areas remain on the candle repeat the treatment with sulfite solution.

8. Pass tap water through the candle until the filtrate is free from acid as shown by litmus paper.

9. Fill the mantle with distilled water and draw through.

10. Dry thoroughly. See "Drying of Candles."

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[5] If necessary the remainder of the cleaning process may be postponed until the next day, the filter meanwhile being immersed in water.

[6] Before starting the cleaning process brush with water candles which have been sterilized by heat. When the cleaning process is also used for sterilization, brush the candle before rinsing with the tap water.

**Drying of Candles.**—Clamp the clean candle to a ring stand, connect the nipple with the vacuum inlet, using a guard bottle. Turn on the suction for about ten minutes. Dry thoroughly in the sunshine, over a radiator, or in an oven at 100°C. If the candle is set up in a mantle, the air may be drawn through before taking the filter apart. Make sure the candles are perfectly dry. Store in wire baskets or on racks which permit the circulation of air.

### *Testing of New Candles*

All new candles are cleaned before being used. Large filter candles (10 by 2 in.) are tested by the filtration group when received, to confirm the guaranteed air pressure; large final filters, to determine their sterilizing efficiency also.

**Cleaning of Candles.**—After examining each filter carefully for obvious defects, immerse the entire candle except the end of the nipple in tap water and soak for from eight to eighteen hours. Then remove extraneous particles by passing water through the candle in both directions at a pressure of about twenty pounds. To force water through from the outside, place the candle in the metal cylinder of the pressure apparatus and screw on the faucet. To reverse the flow, attach the nipple to the faucet by rubber tubing.

**Air-Pressure Test.**—Remove air from the candle by forcing water through from the inside. Connect the filter by rubber tubing to a positive-pressure outlet provided with a gauge. Submerge the entire candle in a tall glass container filled with clear water and apply air pressure gradually. Mark any imperfections (indicated by the escape of air bubbles at a low pressure) and enter on the tag "Defective at . . . . lbs. pressure." If the resistance is equal to or higher than the guaranteed pressure enter "Good at . . . . lbs. pressure." It is unwise to test much beyond the guaranteed pressure.

**Filtration Test.**—To assure further the efficiency of the candle, pass through it a lot of serum for use in media. Note the rate of filtration and determine the efficiency by sterility tests on the filtrate.

A suspension of organisms drawn through by suction may be used to test the efficiency of filter candles. The filter is assembled as usual and a diluted broth culture of *B. pyocyaneous* filtered through it.

**Records and Tags.**—Enter on a card (5 by 8 in.) the serial number of each filter tested, date received, date, and results of tests. Attach a tag to the stem of each candle, giving the number, "good" or "defective," and return to the purchasing group.

### *Permanent Records*

Filtration report (monthly): A report (8 by 11 in., mimeographed) gives in regard to each filtration, date, kind and lot number of product, volumes filtered through Buchner funnel, preliminary filter candle, and final filter candle, and serial number of final filter candle used.

The entries on the "Serum and antitoxin record" forms (p. 520) serve as further permanent records.

## CHAPTER 2

# PREPARATION OF BIOLOGIC AND CHEMICAL PRODUCTS FOR DISTRIBUTION

### *General Procedure*

The preparation for distribution of biologic and chemical products for prophylactic and therapeutic use, and of certain sera and reagents for diagnostic purposes, is carried on by the filling and boxing group. The duties of the group include the bottling, labeling, boxing, storage and transfer to the shipping group of these products. Members of the group also assist in testing the products for sterility and harmlessness. A specially trained worker is responsible for the work of the group. This worker, besides performing technical procedures, plans and supervises the work and keeps the records. At least three shifts of two workers each are fully trained in filling-room procedure, and at least two workers are competent to use the printing apparatus. Three members of the group, of whom one is always on duty during regular hours, are authorized to remove boxed material from the cold room for shipping. An assistant bacteriologist designated by the head of the department has general supervision, acting in an advisory capacity.

The worker in charge trains each assistant to arrange her materials so that there will be as little lost motion as possible. Since the worker's whole attention is required if the work is to be properly done, there should be no unnecessary conversation. The highest standards of work must be maintained in every detail.

Two workers, designated as head fillers, take special responsibility in connection with the procedures of filling. Filling-room workers are trained both to fill and to stopper the bottles, so that they may alternate. Work is so planned that one pair works only half a day in the filling room except in emergencies. In very hot weather 2-hour shifts with at least two hours between are arranged if possible.

The filling of biologic products is carried on in two draft-free rooms reserved for the purpose in which rigid aseptic precautions are observed. The products are labeled and boxed in a work room. Adequate space is provided for storing the necessary equipment and supplies, such as circulars, cartons, parts of filling apparatus, etc. Each drawer or box must be clearly labeled, and a high degree of order must be maintained throughout. When there is any possibility of mixing

TABLE 36

*Products and containers in which dispensed*

| PRODUCT | AMOUNT FILLED* | BOTTLES |
|---|---|---|
| Antidysentery serum | 20 cc. | 20 cc. |
| Antimeningococcus serum | 20 cc. | 20 cc. |
| Antipneumococcus serum (therapeutic) type I | 50 cc. | 50 cc. |
| Antipneumococcus serum (diagnostic) types I, II, III | 10 cc. | 14 cc. |
| | 20 cc. | 20 cc. |
| Botulinus antitoxic serum type A | 20 cc. | 20 cc. |
| Botulinus antitoxic serum type B | 20 cc. | 20 cc. |
| Diphtheria antitoxin | 1,000 units* | 2,3 cc. |
| Diphtheria antitoxin | 3,000 units* | 3,5,10 cc. |
| Diphtheria antitoxin | 6,000 units* | 5,10 cc. |
| Diphtheria toxin-antitoxin mixture | 5 cc. | 5 cc. |
| Diphtheria toxin-antitoxin mixture | 10 cc. | 10 cc. |
| Diphtheria toxin for Schick test | 0.75 cc. | 1 cc. |
| Normal horse serum | 50 cc. | 50 cc. |
| Ox bile, for diagnostic use | 10 cc. | 10 cc. |
| Pertussis vaccine | 10 cc. | 10 cc. |
| Pertussis vaccine for "sets" | 0.5 cc.* | 1 cc. |
| Pertussis vaccine for "sets" | 1 cc.* | 2 cc. |
| Pertussis vaccine for "sets" | 1.5 cc.* | 2 cc. |
| Scarlet fever—antistreptococcus serum and globulin: | | |
| Therapeutic | 3,000 units* | 10,14 cc. |
| Prophylactic | 1,500 units* | 3,5,10 cc. |
| Blanching test | 1 cc. | 2 cc. |
| Scarlet fever—streptococcus toxin: | | |
| For active immunization | 10 cc. | 10 cc. |
| For intracutaneous test | 0.75 cc. | 1 cc. |
| Tetanus antitoxin | 1,500 units* | 2,3 cc. |
| Tetanus antitoxin | 10,000 units* | 10,14,20 cc. |
| Tetanus antitoxin | 20,000 units* | 14,20,30 cc. |
| Tuberculin | 0.5 cc. | 1 cc. |
| Typhoid vaccine | 10 cc. | 10 cc. |
| Typhoid vaccine for "sets" | 0.5 cc.* | 1 cc. |
| Typhoid vaccine for "sets" | 1 cc.* | 2 cc. |
| Typhoid-paratyphoid vaccine | 10 cc. | 10 cc. |
| Typhoid-paratyphoid vaccine for "sets" | 0.5 cc.* | *1 cc. |
| Typhoid-paratyphoid vaccine for "sets" | 1 cc.* | 2 cc. |
| Convalescent measles serum | 5 cc. | 5 cc. |
| Poliomyelitis serum (human) | 15 cc. | 20 cc. |

* The number of units is that given on the label. For actual units contained, add 20 per cent. In bacterial vaccine sets add 0.2 cc. to each dose.

products, such as in handling similar material in the same type of containers, there must never be more than one product nor one lot of that product on the work table in the boxing room at one time.

Biologic products which have been dispensed are stored in special cold rooms. Material in final containers is kept until boxed in open, wooden trays which can be stacked; boxed material, in large metal boxes or drawers. The trays are plainly marked and those containing different kinds of products are kept in different sections of the cold room, each lot separate. Boxed products of different kinds and lots are kept in separate storage boxes or drawers.

Biologic products are kept at room temperature only as long as is necessary for the procedures of filling, labeling and boxing. They are never permitted to stand in the sunlight.

**Products and Containers in which Dispensed.**—A list of routine products, together with the amounts usually filled as given on the label, and the sizes of containers used, is given in table 36.

Bottles of 1-, 2-, 3-, and 20-cc. capacity are "homeopathic vials;" other sizes, "prescription rounds." The vials used for tuberculin and for Schick toxin are of amber glass, the others of clear glass. All have been tested for soluble alkali.

### *Arranging for Fillings. Record Forms*

The fillings for a week are arranged in advance by the head of the filling and boxing group. Written instructions for the fillings for the following week are received not later than Saturday, from the production groups. Special requests for emergency fillings should be complied with, however. If adjustments cannot be made or the volume of work is too great, the bacteriologist in charge of the department is consulted.

Orders are made out in duplicate by the production group on "Serum and antitoxin record" or "Bacterial vaccine record" forms, which give the kind of product, lot and bottle number, total volume, volume to be dispensed, and data for the labels. Entries relating to filling, labeling, boxing, etc. are made by the filling and boxing group at the completion of each step. When the material is ready for distribution, its release is signed by the worker in charge of production, and the form is sent to the shipping group. On the reverse side are entered records of individual shipments (date, number of bottles sent, and to whom shipped). (For "Serum and antitoxin record" form, see figs. 49 and 50.)

When material is to be dispensed in two volumes, a separate form is made out for each, except when vials containing different volumes are to be combined in

## Serum and Antitoxin Record

Product..... Volume..... Bottle No.....

RECORD OF FILTRATION

| Order Number | Total volume received | Total volume filtered | Residue | Unaccounted | Tests | Number bottles | Date filtered | Filtered by | Date Refiltered |
|---|---|---|---|---|---|---|---|---|---|
| Vol. in cc. | | | | | | | | | |

DIRECTIONS FOR FILLING AND LABELING

| Actual Quantities in Container | Labeled | |
|---|---|---|
| No. of cc..... | Total Units.....Method..... | Signed |
| Units per cc..... | Units per cc..... | |
| Total Units..... | Prepared, Tested..... | |
| ..... | Date (Bled).....Date Ret'd..... | |
| Special Directions: ..... | | |

RECORD OF PRODUCTS FILLED, BOXED AND SHIPPED

| | Containers | cc. | cc. | Total in cc. | Workers and Dates | Released |
|---|---|---|---|---|---|---|
| Filled | Bottles..... | ..... | ..... | ..... | Filled by..... | |
| | Residue..... | ..... | ..... | ..... | Filled by..... | |
| | Lab. Tests.. | ..... | ..... | ..... | Checked by..... | |
| | Unacct'd.... | ..... | ..... | ..... | Date..... | |
| Boxed | Bottles..... | ..... | ..... | ..... | Boxed by..... | Date Signed |
| | Defective... | ..... | ..... | ..... | Checked by..... | |
| | Reserved.... | ..... | ..... | ..... | Date Finished..... | |
| Shipped | Bottles..... | | | | Date Shipment Begun ..... | |
| | | | | | Date Supply Exhausted..... | |

Additional information:.....

.....

Returned to stock.....

Approved by.....Date.....Record Clerk.....Date.....

Fig. 49. Record Form (Front)

sets. When a lot in bulk consists of more than one bottle a letter follows the lot number to identify each bottle. When the lot is to be dispensed in two volumes a number is placed after this letter, or if the lot consists of one bottle, after the lot number (66A-1, 66A-2, 67-1, 67-2). In the rare instance when only part of a bottle is to be filled; "-a," "-b," etc. are used to designate the filling.

### *Ordering Supplies*

The head of the filling and boxing group is responsible for maintaining a sufficient supply of labels, circulars, etc. Other supplies are requisitioned as needed.

Printed matter: Order all printed matter in amounts sufficient for at least six months, six weeks before the supply on hand will be exhausted.

| Lot No. ..... | | | | NO. PACKAGES | | | |
|---|---|---|---|---|---|---|---|
| DATE | NAME AND ADDRESS | WITHDRAWN | NO. SENT | DATE | NAME AND ADDRESS | WITHDRAWN | NO. SENT |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |

FIG.. 50. RECORD FORM (BACK)

Send for approval two samples of the circular or label with the requisition to the bacteriologist in charge of the department. When the new circular or label is received, again submit two samples for approval. Keep each kind of label in a separate tin box with a sample pasted on each end.

Glassware, etc.: When the orders for filling are received, plan the work for the week and estimate the number of bottles and stoppers required for each filling, allowing an ample margin. Fill out separate "Glassware order" forms for each day's supply, stating the number and sizes required, and place with the media department. If it is necessary to place an order later than 4:00 p.m. on the day before the materials are to be prepared and sterilized, mark the requisition "Emergency filling." Order other glassware as needed.

Bottles and corks, packed in pails, are sterilized by dry heat on the work-day preceding the filling. For detailed methods see "Preparation of Glassware," p. 51. The materials are removed from the sterilizer by workers of the filling group, who take them immediately to the filling room.

Animals: Place on Monday requisitions covering as far as possible the week's supply of guinea pigs and mice for use in tests of harmlessness.

Culture media: Order sterility-test broth, and Hitchens' medium on the day preceding that on which they are to be used, stating whether Hitchens' medium is to be used in the morning or afternoon. (See "Sterility Tests of Biologic Products," p. 544.)

## FILLING OF BIOLOGIC PRODUCTS

### *Preparation for Filling*

**Preparation and Sterilization of Filling Apparatus.**—When possible all the siphons and burettes to be used during a week are prepared for sterilization in advance. The apparatus for each filling is sterilized on the day before it is to be used. Should a Sunday or a holiday intervene, it is sterilized on the previous day and left in the sterilizer.

The filling apparatus[1] consists of: (1) A 2-hole rubber stopper having a glass siphon and an air inlet tube for the bottle of material to be filled. (2) A burette (25 or 50 cc.) for use with a pinchcock, with a T-tube inserted between the burette and the delivery tip. (A cotton plug is inserted in the top of the burette.) (3) Rubber tubing connecting the siphon with the T-tube. (4) A filling bell wired to a stopper through which the delivery tip passes. (5) A Woulff bottle partly filled with phenol solution, connected to the air intake of (1).

The Woulff bottle is fitted with two 1-hole rubber stoppers; through one passes a bent glass tube (air inlet) with outer end plugged with cotton and inner extending to the bottom of the bottle; through the other, a small right-angled tube extending just below the stopper. The outer end of this tube is protected when not in use by sterile rubber tubing closed with a pinchcock. To prepare, fill the bottle three-fourths full with 5-per-cent phenol solution. Close the openings with cotton plugs and sterilize in the autoclave the areas not covered by the solution. Boil the rubber stoppers and glass connections in 1-per-cent crude cresol for ten minutes, insert in place of the cotton plugs and seal the joints with paraffin. Replace the phenol solution every six months.

The siphon is inserted into the bottle of material to be filled, and the air intake connected with the Woulff bottle. The burette is clamped on a ring stand, the bottles elevated, and the flow started by applying suction at the top of the burette. The flow is controlled by two pinchcocks, one between the siphon and burette, used in filling the latter; and one on the tubing just above the delivery tip, used in dispensing the material.

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[1] Various automatic filling devices have been considered, but as yet none has been found sufficiently satisfactory to adopt as a standard.

Place the rubber tubing and stoppers in 0.5-per-cent crude cresol and allow to stand overnight. Rinse in hot, recently boiled tap water, then boil for a few minutes in distilled water. Place the glass connections in strong cleaning solution for at least half an hour. Rinse thoroughly in tap water, and then in distilled water. Pass the glass delivery tip through a No. 2 rubber stopper, and insert the latter in a filling bell. Wind pliable copper wire around the neck of the bell and the stopper and tube to hold the bell securely in place. Connect the T-tube to the burette and to the upper end of the delivery tip by means of short pieces of rubber tubing. Plug the burette with nonabsorbent cotton and protect with a paper cap. Slip a small cotton bag over the filling bell and tie in place.

Insert the glass siphon and inlet tube into the 2-hole rubber stopper, plug the air inlet lightly with cotton, and attach a short rubber tube for connecting the Woulff bottle. Protect the end of this tubing by a test tube (150 by 19 mm.) with a cotton plug. Connect the delivery outlet of the siphon and the side arm of the T-tube with about four feet of rubber tubing. Wire all rubber and glass connections. When the filling apparatus is prepared for dispensing vaccines, tie a double layer of cheesecloth, previously boiled, over the intake of the siphon.

Protect the part of the siphon which is to be inserted in the bottle with a light-weight paper "envelope," folding a 6-inch flap over the stopper, and fastening in place with pins. Wrap the entire filling apparatus in heavy manila paper, and fasten with paper clips and cord. Mark with the product to be filled, and attach a tag. Before taking to the media department for sterilization, enter on the tag the date on which to be sterilized, and on which to be used.

**Sterilization of Gowns and Caps.**—Place gowns and caps to be worn in the filling room in heavy muslin bags and paper and have them sterilized with the filling apparatus.

**Preparation of Filling Room.**—Immediately before using the draft-free room for filling, wipe the table top and all flat surfaces and apparatus on which dust may collect, with a cloth dampened in 1-per-cent crude cresol. If the room has not been used for two or more days, give it a thorough preliminary cleaning on the day preceding the filling. At least twice a month clean the windows and wipe down the walls, door, and window frames with 1-per-cent crude cresol.

The following equipment and supplies should be readily available for use in the filling room: two chairs; one stool; a stand to hold the bottle of material to be filled; a Woulff bottle filled with 5-per-cent phenol solution; a cork press; a ring stand with two clamps to support the filling burette; one Bunsen and one

microburner; two enamel hand basins for 1-per-cent crude cresol; a metal supply box containing rubber gloves (surgeon's thin rubber); powder for gloves; forceps; scissors; absorbent cotton; rubber bands; tags, etc.; one can of sterile, plugged test tubes (180 by 25 mm.). Just inside the door is a mat in a metal tray, placed so that the workers step on it as they enter the room. It is kept moistened with 1-per-cent crude cresol, and is sterilized in the autoclave once in two weeks.

### *Filling Procedures*

**Preliminary Procedures.**—Bottles and stoppers, and gowns are taken to the filling room when they are removed from the sterilizers. The bottle of material to be filled is delivered to the filler by a member of the production group. Bottles of antitoxin and serum containing sediment are handled so as to disturb the sediment as little as possible. Before being filled the material is passed on by the head of the production group. The bottle and other supplies which have not been brought from the sterilizers are wiped off with 1-per-cent crude cresol just before being taken into the filling room.

Put on the sterile gowns and caps immediately before entering the filling room, adjusting the cap so that it covers the hair completely. If it is necessary to go out during a filling, remove the cap and gown just after leaving the room.

Compare the lot number and other data on the tag of the bottle of material with those on the record form, and note the appearance of the material. Report anything unusual to the head of the production group.

Assemble all necessary materials on the table in positions convenient for the workers—wooden trays for filled bottles, cork press, Bunsen and microburners, cotton strips dampened in 1-per-cent crude cresol, rubber gloves, basin of 1-per-cent crude cresol for sterilizing hands, forceps, sterility test broth and record forms for sterility tests, and miscellaneous supplies.

Wash the hands thoroughly using soap and running water before entering the draft-free room. Put on rubber gloves and rinse the gloved hands in 1-per-cent crude cresol after entering the room.

The head filler or a specially designated worker sets up the apparatus with the assistance of a second worker. Report at once any difficulty with the filling apparatus to the worker in charge of the group. Shake bottles containing bacterial vaccines to obtain a uniform suspension before the siphon is inserted. When sediment is present in products other than bacterial vaccines, keep the intake of the siphon well above the sediment.

Before using the filling apparatus make sure that a red "S" (sterilized)

has been marked on the tag. Open and spread out the paper case keeping the different parts on the sterile paper. Lift the siphon by the glass, air-inlet tube; pull out the pin holding the envelope in place and quickly slipping off the paper shield, insert the siphon into the bottle. Meanwhile a second worker, after flaming a pair of forceps and the top of the bottle, removes the stopper, and after flaming the opening, holds the flame in a horizontal position above it, while the siphon is being inserted. Make sure at once that the stopper fits snugly and wrap around it and the glass connections a strip of cotton wet in 1-per-cent crude cresol and thoroughly wrung out. Tie down the stopper and cotton guard. After adjusting the pinchcocks on the rubber tubing, clamp the burette in place. Remove the test tube from the air intake, and the rubber guard from the connection of the Woulff bottle, and after flaming the glass tubing of the latter, join the two. This joint must be tight.

Lift the bottle to be filled and the Woulff bottle carefully from the table to the stand. For convenience and to avoid errors, mark with pointers or narrow, red, gummed strips graduations on the burette at intervals corresponding to the volume dispensed. (Have the markings checked by a second worker.) Burn off the exposed part of the cotton plug in the burette and attach a short piece of sterilized rubber tubing. Open the pinchcock controlling the flow from the bottle, and start the siphon by gentle suction through the rubber tube. Draw off the muslin bag guarding the bell. Open the second pinchcock and allow enough material to run into the bottle for residues to displace the air from the filling tip.

Add to tubes containing sterility-test broth the required volumes for the burette test at the beginning of the filling. For procedure see "Sterility Tests of Biologic Products," p. 549.

Place a pail of bottles on a stool at the right of the filler and a pail of stoppers at the right of the second worker. Adjust the paper or muslin covering the contents of the pails to protect against air contamination.

**Technic of Filling.**—The filler: Fill the burette to the highest pointer. Take a bottle from the pail (in which it is packed mouth down), and hold it in an inverted position until it is under the bell, then turn it swiftly and place it under the filling tip of the burette. During this process inspect the bottle rapidly for cracks or obvious defects. Transfer it to the left hand, and with the right open the pinchcock. Take great care to deliver the exact volume. Then pass the bottle in a slanting position to the second worker.

The second worker: Pass the tip of the forceps through a micro flame, remove a stopper from the pail, take the bottle from the filler with the left hand and quickly insert the stopper. After pushing the stopper firmly in place with the press, place the bottle in a tray. Trays of two heights are used, to permit stacking when filled with small or large bottles.

Number the trays of bottles in the order of filling. As each tray is filled place in it an initialed slip stating the number of the tray and the number of bottles it contains. Place a marker after each hundredth bottle filled. Insert in the label holder on the tray a card on which is written the tray number, the date of filling, the kind and lot number of the product and in the case of antitoxins, the number of units dispensed ("1000," "3000" etc.). Send the trays to the boxing room, where the count is checked and the required data entered on the record form.

At frequent intervals during the filling flame the rim of the bell. When there is a pause in the filling, flame the neck of a sterile test tube and slip it over the filling tip. Place the forceps when not in use so that the tips do not touch surfaces which may be contaminated. At intervals dip the gloved hands in 1-percent crude cresol.

Replace badly fitting corks, if possible to do so quickly and without danger of contamination. Otherwise set the bottles aside as defective. Set aside also bottles which contain particles from the stopper ("floaters"), or which show other defects. Defective bottles may be used for the reserve stock, unless there is reason to suspect contamination. Defective bottles of diagnostic sera can generally be used by groups in the laboratory.

Test of volumes dispensed: In order to check the volumes dispensed, bottles are taken at frequent intervals from the supply in the cold room. The bottles are selected and measurements made by the worker in charge of sterility tests (see "Sterility Tests of Biologic Products," p. 558).

### *Procedures Immediately Following Filling*

**Sealing.**—Seal the bottles with glycerin-glue mixture as soon as they are brought from the filling room. Holding each bottle over the glue container, apply the glue with a small paint brush, so that it forms a thin uniform covering over the entire cork and the lip of the bottle. Allow the glue to dry before placing the trays of bottles in the cold room.

Formula for glue:

| | |
|---|---|
| Distilled water | 400 cc. |
| Gelatin | 160 grams |
| Glycerin | 80 cc. |
| Cresol | 10 cc. |

Mix the ingredients in a double boiler and heat until completely dissolved. Skim off the foam from the top. Allow to stand for one day before use. Keep in the double boiler, and warm over a flame before applying. If the glue becomes too thick, dilute with distilled water containing a few drops of cresol. The consistency should be that of a thick syrup.

**Residues.**—Combine in one bottle any antitoxin left in the bottle or burette, together with the material in discarded bottles and that which remains after taking sterility tests. Write "residue" on the original tag, and transfer it to this bottle. Record the approximate volume on the record form. Send the antitoxin and the empty bottle (for rinsing) to the concentration group. Pipette serum residue from the large bottle into one bottle since it usually contains precipitate; that from discarded bottles and bottles opened for sterility tests, into another. Label, record as above, and send to the production group. In the case of such sera as poliomyelitis serum, which is difficult to obtain, if directed, after removing material for sterility tests, close the bottle with aseptic precautions, label with the volume remaining, and prepare for distribution. In the case of diagnostic sera, send discarded bottles and bottles opened for sterility tests, without pooling the contents, to the production group to be used for tests. Discard bacterial vaccine and diphtheria toxin-antitoxin residues.

Antitoxin residues are held in the cold room for reconcentration. Serum residues are placed immediately in the cold room, and if in the judgment of the head of the production group a residue is in good condition and of suitable titer, it may be added to the next lot of the same kind of serum filtered.

**Care of Room and Equipment.**—Remove everything from the filling room except the permanent equipment, and clean the room thoroughly, including the floor, pipes, etc. Rinse the filling apparatus thoroughly with luke-warm tap water. After dismantling it, rinse again and store until needed. Wash the rubber gloves with soap and water and dust the inside with powder before putting them away. If a second filling is to follow immediately, everything connected with the first (filled bottles, sterility-test containers, filling apparatus, etc.) must be removed and flat surfaces such as table tops, etc. wiped with 1-per-cent crude cresol before preparation for the second filling is begun.

### *Tests for Sterility and Harmlessness*

Both cultural and animal tests are made on all biologic products distributed for prophylactic and therapeutic use; only cultural tests, on those used for diagnostic purposes. With the exception of tubes inoculated from the burette, sterility-test media and animals are in-

oculated by a worker from the sterility-test group assisted by a worker from the filling group. (See "Sterility Tests of Biologic Products," p. 543.)

Cultural tests: Material from the burette at the beginning of the filling is added directly to tubes of sterility-test broth by the filler.

Material from the final containers is added to both sterility-test broth and Hitchens' medium at the end of the filling, by a worker from the sterility-test group assisted by one of the fillers. When the test is to be made in the filling room the room is not disturbed until the test is completed. The sterility-test record forms are made out and the required media and pipettes assembled by the fillers. On being notified that a filling is completed, the worker from the sterility-test group selects the bottles to be tested. The inoculations may be made at once or on the following day.

Animal tests: Material from final containers is injected into a guinea pig; that containing phenoloid preservative, into a mouse also. The animals are inoculated in the operating room by a worker from the sterility-test group, assisted by a filler.

Retests: When there is any question as to sterility or harmlessness, retests are made by the worker in charge of sterility tests, assisted as before.

For detailed instructions see "Sterility Tests of Biologic Products," p. 543.

### LABELING AND BOXING

Bottled products are labeled and boxed as soon as the cultural and animal tests are satisfactorily completed. In emergencies, with the approval of the head of the production group, material may be labeled and boxed before the observation period is completed, if the sterility tests up to the fourth day indicate the material is sterile. Prophylactic and therapeutic products are in general distributed in individual packages.[2] Folding cartons are used for most of the products, special cardboard or wooden boxes for a few. A circular of directions is always enclosed. A form for reporting the results of the use of the material is usually included. Diagnostic sera and ox bile are not boxed in individual cartons and no printed matter other than the label is sent out with them.

Under certain conditions, products for human use may be sent to institutions, etc., in bottles not in separate cartons.

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[2] Diphtheria toxin-antitoxin mixture is also distributed in cartons containing twenty 10-cc. bottles for use when large numbers of persons are to be injected at one time. Arsphenamine is distributed in cartons containing 18 ampules.

*Preparation of Printed Matter*

Labels for each product are printed with general information and space for special data such as lot number, return date, unit content, etc. Circulars are printed, or for certain products of which few are distributed, mimeographed. Report forms with space for the lot number are usually printed. Folding cartons are printed with "Division of Laboratories and Research, N. Y. State Dept. of Health," all data concerning the contents being added at the time the cartons are used. A hand press is used for printing the special data on labels, report forms, and boxes. A sample of each, with the form of printing, approved by the bacteriologist in charge of the department is kept on hand. A high standard of work must be maintained in preparing the printed matter.

A small hand press with ink pad, and holders arranged to take several lines of rubber type is used. To avoid unnecessary changing of type several holders are kept set up with the data frequently required. Rubber type is blocked out in words commonly used.

Printing is always done a day in advance to allow time for the ink to dry. Labels for one lot of material only are on the work table at one time. Circulars and report forms are folded according to directions with edges true; the title always on the outside. A large supply of circulars and forms most used should be kept ready folded.

Circulars of directions are distributed with all products for human use; forms for the report on the use of the product, with all antitoxins and sera, with streptococcus toxin (scarlet fever) for the intracutaneous tests and for active immunization, and with arsphenamine. Printed circulars are 5 by 8 inches; report forms for diphtheria and tetanus antitoxins, 3 by 5 inches; for other products, 4 by 6 inches; mimeographed forms usually 8 by 11 inches. Reports on the use of the outfits for the Schick test and of diphtheria toxin-antitoxin mixture are sent directly to the Division of Communicable Diseases on forms provided by that Division. The printed matter contained in each package is given in the following list.

Antidysentery Serum:
(1) "Dysentery" circular, folded lengthwise.
(2) "Report on the Use of Antidysentery Serum," folded crosswise.
Antimeningococcus Serum:
(1) "Cerebrospinal Meningitis" circular, folded lengthwise.
(2) "Report on the Use of Antimeningococcus Serum," folded crosswise.
(3) Record of Potency (stating agglutinative titer), mimeographed (3 by 5 in.) from a sample prepared by the production group, folded lengthwise.
Antipneumococcus Serum: Type I (Therapeutic):
(1) "Pneumonia" circular, folded lengthwise.
(2) "Report on the Use of Antipneumococcus Serum," folded crosswise.
Arsphenamine:
(1) "Directions for the Use of Arsphenamine," mimeographed, folded crosswise.

(2) "Report on the Use of Arsphenamine," folded three-eighths lengthwise.

Ampules, wrapped separately in corrugated paper on which are printed the lot number and number of grams, are packed together in labeled boxes. A report form is wrapped with each ampule, two circulars of directions are included in each order.

Botulinus Antitoxic Serum:

"Botulism" circular, mimeographed, folded twice crosswise.

Diphtheria Antitoxin:

(1) "Diphtheria" circular, folded twice crosswise.

(2) "Report on the Use of Diphtheria Antitoxin," folded crosswise.

Diphtheria: Outfit for the Schick Test:

"Diphtheria—The Schick Test" circular, folded twice crosswise.

Diphtheria Toxin-Antitoxin Mixture:

"Diphtheria Toxin-Antitoxin Mixture" circular, folded twice crosswise.

Pertussis Vaccine:

"Pertussis" circular, folded twice crosswise.

Scarlet Fever: Antistreptococcus Serum (Therapeutic):

(1) "Scarlet Fever" circular, mimeographed, folded three times crosswise.

(2) "Report on the Use of Antistreptococcus Serum," folded crosswise.

Scarlet Fever: Antistreptococcus Serum (for Passive Immunization):

(1) "Scarlet Fever" circular, mimeographed, folded three times crosswise.

(2) "Report on the Use of Antistreptococcus Serum (for Passive Immunization)," folded crosswise.

Scarlet Fever: Antistreptococcus Serum (for Blanching Test):

(1) "Scarlet Fever" circular, mimeographed, folded twice crosswise.

(2) "Scarlet Fever—Report on the Use of Antistreptococcus Serum for the Intracutaneous Blanching Test," mimeographed (6 by 4 in.), folded crosswise.

Scarlet Fever: Streptococcus Toxin (for Active Immunization):

(1) "Scarlet Fever—Streptococcus Toxin for Active Immunization" circular, mimeographed, folded three times crosswise.

(2) "Scarlet Fever—Report on Active Immunization with Streptococcus Toxin," mimeographed, folded twice lengthwise and once crosswise. A circular is included in each individual package, one report form with each order.

Scarlet Fever: Outfit for the Intracutaneous Test:

(1) "Scarlet Fever—Intracutaneous Test for Susceptibility" circular, mimeographed, folded once crosswise and four times lengthwise.

(2) "Scarlet Fever—Report on Intracutaneous Test for Susceptibility," mimeographed (8 by 5½ in.), folded twice crosswise.

Tetanus Antitoxin:

(1) "Tetanus" circular, folded twice crosswise.

(2) "Report on the Use of Tetanus Antitoxin," folded crosswise.

Tuberculin:

"Tuberculin" circular, two mimeographed sheets.

Typhoid and Typhoid-Paratyphoid Vaccines:

"Typhoid and Paratyphoid Fever" circular, folded twice crosswise.

Convalescent Measles Serum:

(1) "Measles" circular, mimeographed, folded crosswise.

(2) "Report on the Use of Convalescent Measles Serum."

"Poliomyelitis" Serum (Human):

(1) "Poliomyelitis" circular, mimeographed, folded twice crosswise.

(2) "Report on the Use of Serum from Recovered Cases of Poliomyelitis," folded crosswise.

## *Labeling*

Preliminary inspection: Before labeling a bottle hold it to the light, shake slightly and examine carefully for imperfections such as particles from the stopper or other foreign matter, cracks, or leaky stopper. Label defective bottles of products other than vaccines, writing "defect" on the label with a red wax pencil. Return antitoxins to the concentration group, sera to the production group, holding one or two

bottles as required for the reserve sample, if suitable. Discard defective bottles of vaccines except those held as reserve samples, which are labeled and marked "defect" as above. Enter on the record form the number of bottles found defective.

Labeling by hand: Moisten the gummed label by pressing it on a wet sponge, taking care not to rub off the glue. Apply to the bottle, smoothing it with a piece of clean cheesecloth. Put the labels on straight and in a uniform position on the bottles. As the bottles are labeled, transfer them from one tray to another. When a tray becomes empty, transfer the information slip to the full tray.

Labeling by machine: Certain sizes of bottles as 5 and 10 cc. and larger are labeled by means of special attachments on the "World labeler" machine. Ungummed labels are used. The labeling is performed by a worker in the media department specially trained in the use of the machine, assisted by a worker from the filling group. A written request stating the number of bottles to be labeled and the approximate date on which they will be required is sent to the media department in advance.

*Boxing*

Three sizes of white cardboard collapsible cartons are used for products distributed in 2-, 3-, 5-, 10-, 20- and 30-cc. bottles ($\frac{15}{16}$ by $\frac{15}{16}$ by $2\frac{5}{16}$ in. for 2- and 3-cc. bottles; $1\frac{1}{4}$ by $1\frac{1}{4}$ by $2\frac{5}{8}$ in. for 5 and 10 cc.; $1\frac{1}{2}$ by $1\frac{1}{2}$ by $3\frac{5}{8}$ in. for 20 and 30 cc.). A heavy cardboard lining is inserted when the cartons are set up.

To box material in cartons, assemble the material and supplies and proceed as follows: Place folded circulars, etc. on the table at the right, cartons (previously set up) at the left, and a pile of tissue paper ($6\frac{3}{4}$ by $7\frac{1}{2}$ in. or 10 by 10 in.) in front. Again inspect the bottle for defects, and note the appearance of the material. Compare the data on the label with those on the carton, and on the report and record of potency forms if included. Place the folded circular on the tissue paper, the forms, if used, on top of the circular (with the folded edges together on the left), and lay the bottle on top with the neck towards the left. Roll all together, turning in the ends of the tissue paper before the roll is completed. Place right side up in the carton. Always keep the packages right side up. Stamp on the carton near the top on the side opposite the printing, the number assigned to the packer, as "Packer No. 4." Count the packages and enter the number on the record form and daily record card. Place the boxed products in white, tin supply boxes for storage, and attach to each box a tag giving

the kind and lot number of the product and volume or number of units, except small lots, which may be stored in trays with the necessary information on the label in the holder. Never store different lots in the same receptacle.

When products are boxed in other than the regular cartons, follow the same general procedure, making the necessary changes as given in the following paragraphs.

Antipneumococcus serum and normal horse serum: Place in shoulder boxes ($1\frac{7}{8}$ by $1\frac{7}{8}$ by $4\frac{1}{2}$ in.) with a printed label affixed. Pad the bottom and top of the box with cotton. Seal the intersection of the box and the cover with transparent adhesive tape.

Arsphenamine: Protect the tip of each ampule with cotton, and fold the report form about the ampule so that its folded edge is an additional protection. Roll in corrugated paper on which are printed the lot number and number of grams, fasten with a rubber band and stamp with the packer's number. Pack in boxes ($5\frac{1}{4}$ by 4 by $2\frac{3}{4}$ in.) holding 18 ampules, indicate the number and affix a label on the outside, and stamp with the packer's number.

Bacterial vaccine sets: Wrap the three bottles together without tissue paper in a folded circular and place in the special unlined carton (2 by $1\frac{5}{8}$ by $\frac{5}{8}$ in.) with the vials parallel to the open end.

Diphtheria toxin-antitoxin mixture (for special distribution): Wrap the labeled bottles in tissue paper without the circulars and pack twenty in a carton (3 by $4\frac{1}{2}$ by $5\frac{1}{2}$ in.). Enclose two circulars.

Outfits for the Schick test and for the intracutaneous tests for susceptibility to scarlet fever are assembled under the supervision of the toxin-production group, workers from the filling and boxing group assisting. For details see "Preparation of Outfits for the Schick Test," p. 483.

Tuberculin: Roll the vial in tissue paper and place between layers of cotton in a labeled cardboard box. Enclose the circular. Seal the box with transparent adhesive tape.

## STORAGE AND DISTRIBUTION OF PRODUCTS

### *Storage*

All biologic products are kept at a low even temperature. One cold room is used exclusively for the storage of these products. Additional storage space is assigned for finished material in large supply boxes. Cold-room doors must be closed completely by workers immediately after entering or leaving.

The main cold room is arranged especially for the storage of biologic products. On one side is a tier of shelves to hold the trays of bottles in the course of preparation; and the supply boxes of completed material awaiting release and transfer to the distributing drawers. On the other side are tiers of compartments, each holding two wooden drawers set end to end. These drawers contain only material which is released and ready for distribution. Separate compartments are re-

served for each kind of product and sized package distributed. As drawers are emptied, they are refilled from the released stock. Two lots of material are never put in one drawer. The kind of product, lot number, and volume, or, if antitoxin, unit content is designated on labels on all trays, metal boxes, or drawers.

### *Material Held in Reserve*

One or more samples of every lot of material released for distribution are retained for reference. Bottles sealed and labeled but not boxed are selected. Those containing floaters or with minor defects may be used for this purpose if there is no doubt of sterility. Each kind of product is kept together in separate, labeled supply boxes in the cold room.

Reserve from each lot enough final containers to give a total of at least 12 cc. of product, with the following exceptions: Reserve three bottles from a filling of antitoxin dispensed in volumes of less than 4 cc.; four vials from a filling of toxin for intracutaneous tests and of tuberculin; and one bottle from fillings of convalescent measles serum and of diagnostic sera. When material is filled in two or more different sized containers, reserve at least one of each size; in the case of vaccine sets, two complete sets.

Refer reserved bottles of globulins directly to the worker in charge of concentration. (These bottles are held for two years, and are inspected occasionally to detect cloudiness or beginning precipitation.) Hold other reserves until a date at least six months after the final return date. About once in six months refer to the production groups all material which has been held the required length of time.

Arsphenamine: Reserve 2 per cent of the total number of ampules of each sized dose dispensed (1.2, 0.6, or 0.4 grams) of every lot of arsphenamine. Hold for three months from the date of the first shipment, then release for distribution all but two of the ampules containing the largest dose dispensed in that particular lot. Hold these samples for one year, then refer to the preparation group.

### *Distribution of Products*

A product is released for distribution only after tests for sterility and harmlessness are satisfactorily completed, and the head of the production group has initialed and dated the record form. ("Antitoxin and serum record," fig. 49.)

One week after the filling refer the forms to the worker in charge of production for release. When the material is boxed, send the original to the shipping group; when distribution starts, the duplicate to the production group.

An emergency release, permitting material to be shipped in advance of the usual 7-day period required for the sterility and animal tests, must be approved and initialed by the bacteriologist in charge of the department. The words "Emergency release" must appear on the form, together with the signature and the date.

When shipments are to include biologic products, the "Memorandum of shipment" forms are referred by the shipping group to the filling and boxing group. The head of the latter group and two qualified assistants are authorized to remove from the cold room supplies for shipment. One of these workers is on duty at all times during laboratory hours. The supplies are checked by the shipper. The night watchman and certain designated workers are authorized to remove supplies at other times.[3]

Take the "Memorandum of shipment" and a tray into the cold room. Count out and place in the tray the required number and kinds of packages. As far as possible select material in order of date of preparation. After leaving the cold room, record the lot number of each product on the form. Initial, and leave the form and tray on the shipper's table.

**The Emergency Box.**—This box contains a few packages of each product, for distribution during the periods when no worker authorized to remove supplies from the main cold room is on duty. The worker on duty at such times, when removing material from the emergency box, enters on a mimeographed card the kind of material, number, size and lot numbers of packages, person to whom given or sent, and date; initials the card and leaves it in the emergency box. The worker in charge of the group and one assistant are responsible for keeping the box stocked. The contents are examined daily, and replenished if necessary. A list giving the products and the number and size of the packages is pasted on the inner side of the cover.

## RECORDS

The duplicate "Serum and antitoxin record" and "Bacterial vaccine record" forms contain information relating to the various steps performed by the filling and boxing group (see p. 526). When the material is ready for distribution the original record is transferred to the shipping group for entry of individual shipments and the duplicate is returned to the production group. The worker in charge of the group keeps a statistical record of the kinds and quantities of material filled

[3] Further detailed directions relating to the shipment of products are contained in methods on file in the office.

and boxed daily. The monthly report is readily compiled from these records.

*Permanent Records*

Daily records: A record is kept according to products, giving complete data in regard to each filling and the material boxed.

Filling and boxing report (monthly): A report (8 by 11 in., mimeographed) gives for each product: name of product, number of each sized bottle filled, total number filled, and total number boxed; and for all products combined, the total number of each sized bottle filled, and totals of all bottles filled and all bottles boxed.

The entries on the "Serum and antitoxin record" forms, p. 526, serve as further permanent records.

# CHAPTER 3

## STERILITY TESTS OF BIOLOGIC PRODUCTS[1]

The sterility of all biologic products is controlled by cultural tests made on material in the process of preparation and on the finished products as dispensed for distribution. When intended for human use, the harmlessness of the finished products is further controlled by the inoculation of animals with material from each filling. Filled material is not released for distribution unless the final cultural tests show the absence of growth during seven days, and the animal tests have been satisfactory for the same period.

A specially trained and responsible worker is in charge of all sterility tests assisted, when required, by another worker competent to assume charge of the work, if necessary. A qualified bacteriologist, designated by the bacteriologist in charge of the department, acts in an advisory capacity. The inoculation of culture media with products in bulk, is performed by the production and filtration groups; with finished products, by a worker from the sterility-test group, assisted by a member of the filling group, who also assists in injecting the test animals. The duties of the worker in charge of sterility tests include: general supervising of inoculation of media with material in process, selecting final containers for tests and inoculating animals and media with filled material; examining all media after inoculation and observing test animals; keeping records; reporting results of tests to production and filling groups; making necessary retests; and checking from time to time the volumes dispensed in the final containers.

### CULTURAL TESTS

#### *Culture Media*

In order to provide especially favorable conditions for the growth and detection of organisms which may be present, two kinds of media have been selected for routine tests of biologic products. The first ("Sterility-test broth") consists of a beef-infusion medium containing

[1] The standards for the tests differ somewhat from the official recommendations of the U. S. Hygienic Laboratory in Washington. As carried out in this laboratory, they have proved satisfactory.

0.1 per cent dextrose. The second (Hitchens' medium) (31), which is of a semifluid consistency, has as its basis the same broth medium, to which is added 0.1 per cent agar. Aerobic and anaerobic conditions may be obtained in both media. The medium in half the tubes of sterility-test broth is covered with a layer of paraffin-mineral-oil mixture. This mixture, which is semisolid at incubator temperature, has been found an efficient anaerobic seal when the tubes are stored for the usual period (two to three weeks).[2] In Hitchens' medium the physical conditions resulting from the small percentage of agar provide an exceptionally favorable environment for both aerobic and anaerobic growth, for studying colony formation, and for gauging, to some degree, the extent of bacterial contamination.[3]

**Routine Media.**—"Sterility-test broth": Beef-infusion broth, pH 7.2 to 7.8, containing 0.1-per-cent dextrose, is dispensed in 165-by-22-millimeter test tubes in 20-cc. volumes. The broth in the anaerobic tubes is covered with enough paraffin-mineral-oil mixture (about 1.25 cc.) to form a firm layer when cold.

Hitchens' medium: This medium is sterility-test broth to which 0.1 per cent agar has been added. It is dispensed in tubes (165 by 22 mm.) and in bottles; 20 cc. in tubes, 40 cc. in 50-cc. bottles, 80 cc. in 100-cc. bottles, and 160 cc. in 200-cc. bottles. The cotton plugs of the bottles are protected with paper caps. The medium is heated in streaming steam (98 to 100°C.) for one-half hour on the day of inoculation to expel oxygen and make it of a uniform consistency. Some flocculation usually occurs on standing. Lots with marked flocculation should not be used for sterility tests. After removal from the sterilizer, the medium is cooled at room temperature for at least two and not more than three hours before inoculation. If more than three hours is to elapse before use, the containers are held in a 55°C. incubator until about forty minutes before inoculation. The containers should not be packed so closely as to prevent uniform cooling.

**Solid Medium.**—For bacterial vaccines before dilution and for subculture, 0.2-per-cent dextrose beef-infusion agar is used. The agar is dispensed in 15-cc. amounts in 150-by-19-millimeter tubes for deep tube and pour-plate cultures. It may be advisable to supplement this medium with others, such as Hitchens' medium, various enriched media, or plain extract agar.

**Requisitions for Media.**—The media are requisitioned from the media department each day as required. Requisitions for Hitchens' medium, stating whether it is to be used in the morning or afternoon, are filed on the preceding day. This

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[2] While the standard Smith tube appears in certain regards as more satisfactory for sterility tests, its use in this laboratory has not yet proved as practical from all points of view as the present procedure. The matter is, however, still under consideration.

[3] Experimentation has shown that when inoculum sparsely seeded with such organisms as *B. tetani*, *B. botulinus*, streptococcus, and pneumococcus, was added to Hitchens' medium, growth readily developed, even when the concentration of phenoloid preservative was increased to 0.02 per cent.

medium is heated in the media department, and placed in the 55°C. incubator or delivered to the workers as directed.

**Tests of Media.**—All containers of media are incubated and inspected by the media department before being delivered. Media to be used for filled material are again inspected immediately before inoculation by the sterility-test group to detect possible contamination, and also to observe the nature and amount of precipitate, should any be present. Containers in which the medium shows more than a slight precipitate, and anaerobic tubes which have defective paraffin seals are rejected. When a new lot of sterility-test broth is first used, one aerobic

TABLE 37

*Preservative in biologic products*

| PRODUCT | PRESERVATIVE |
|---|---|
| | *per cent* |
| Globulin (antitoxins) | 0.3 cresol |
| Sera: | |
| Antidysentery | 0.3 cresol |
| Antimeningococcus | 0.3 cresol |
| Antipneumococcus | Chloroform (to saturation) |
| Antistreptococcus | 0.3 cresol |
| Botulinus antitoxic | 0.3 cresol |
| Normal horse | Chloroform (to saturation) |
| Convalescent measles | 0.5 phenol |
| Recovered poliomyelitis | 0.2 cresol |
| Bacterial vaccines | 0.3 cresol |
| Diphtheria toxin-antitoxin mixture | 0.4 phenol* |
| Streptococcus toxin (diluted for immunization) | 0.42 phenol (approximate) |
| Tuberculin | 0.3 cresol (50 glycerin) |
| Toxins for intracutaneous test | 0.5 phenol |
| Heated control for intracutaneous test | Negligible |
| Salt solution for intracutaneous test | None |

* The preservative content of diphtheria toxin-antitoxin mixture is so slightly in excess of 0.4 per cent (never more than 0.401 per cent) that the material is treated on this basis.

and one anaerobic tube, uninoculated, and two of each, inoculated with material of known sterility, are incubated as controls; when a new lot of Hitchens' medium, one 80-cc. bottle, uninoculated, and two 40 cc., inoculated with material of known sterility. Sterile serum controls may be repeated from time to time if advisable. Contaminations or variations observed in any lot of medium which might lessen its efficiency should be reported at once to the media department.

### *Quantity of Inoculum*

To avoid danger of inhibiting the growth of bacteria, the concentration of phenoloid preservative in the medium after inoculation is limited to not more than 0.01 per cent. In general, it is desirable to

test the maximum volume of material possible, within the limit. Since, however, an excessive quantity of serum or other material may give rise to a confusing precipitate or itself possibly inhibit the growth of certain organisms, the volume of inoculum is limited even when the material contains little or no preservative. Products containing chloroform are treated in the same way as those containing 0.3 per

TABLE 38

*Volumes of inoculum permissible in given volumes of Hitchens' medium*

| HITCHENS' MEDIUM | INOCULUM WITH A PHENOLOID PRESERVATIVE CONTENT OF | | |
|---|---|---|---|
| | 0.3 per cent | 0.4 per cent | 0.5 per cent |
| *cc.* | *cc.* | *cc.* | *cc.* |
| 20 | 0.7 or less | 0.5 or less | 0.4 or less |
| 40 | 0.8 to 1.3 | 0.6 to 1 | 0.5 to 0.8 |
| 80 | 1.4 to 2.7 | 1.1 to 2 | 0.9 to 1.6 |
| 160 | 2.8 to 5.5 | 2.1 to 4 | 1.7 to 3.2 |

The table is to be used to determine the volumes of Hitchens' medium required for a given volume of inoculum. In practice, when Hitchens' medium and broth are inoculated from the same bottle of filled material, the total volume of inoculum is withdrawn in the pipette, 2 cc. added to the broth, and the remainder discharged into the required volume of Hitchens' medium. The volume of medium selected is based on the actual volume of material dispensed when it is 5 cc. or less, when more, on the volume withdrawn, no allowance being made for the loss occurring when the material is transferred. The loss may, however, practically equal the volume to be tested in Hitchens' medium, in which case the Hitchens' medium test is omitted.

When more than one container of Hitchens' medium is required, the inoculum need not be distributed equally, but should be divided in such a way as to use as little medium as possible. Thus, when 8 cc. of material containing 0.3-per-cent phenoloid preservative is to be tested, 5.5 cc. should be added to a 160-cc. container and 2.5 cc. to an 80-cc. container. When the choice lies between one large and two small containers, however, the large container should be used even though there is some excess of medium. For example, 2 cc. of material containing 0.3 per cent is usually added to 80 cc. of medium rather than divided between a 40-cc. and a 20-cc. container.

cent phenoloid preservative. For preservative content of the various products, see table 37.

**In Sterility-Test Broth.**—Five-tenths cubic centimeter of inoculum is added to a broth tube (20 cc. broth) except when the phenoloid content of the material is greater than 0.4 per cent, in which case, 0.4 cc. or less is added. In practice, when 1 cc. of material containing more than 0.4 per cent preservative is to be tested, it is distributed

among two aerobic and two anaerobic tubes; when 2 cc. are tested, among three each.

**In Hitchens' Medium.**—The volumes of inoculum which may be added to various volumes of Hitchens' medium are given in table 38.

*The Tests*

**Material from Bulk Containers.**—Sterility tests are made of the contents of each bottle containing material in bulk. The sterility of un-

TABLE 39

*Sterility tests of products in bulk (from each container of 1 liter or over)*

| PRODUCT | WHEN TAKEN* | MEDIA INOCULATED BY | VOLUME INOCULUM† | |
|---|---|---|---|---|
| | | | In broth | In Hitchens' medium |
| | | | *cc.* | *cc.* |
| Serum | On receipt | Production group | 1 | 0 |
| Globulin (antitoxin) | After filtration | Filtration group | 2 | 8 |
| Serum | After filtration | Filtration group | 2 | 8 |
| Toxin | After filtration | Production group | 1‡ | 0 |
| Toxin or toxin dilution for human use | Before preliminary human intracutaneous tests | Production group | 2 | 6 |
| Diphtheria toxin-antitoxin mixture | After filtration (2 days) | Production group | 2 | 8 |
| Bacterial vaccine | After dilution | Production group | 2 | 8 |

* Should a bottle be reopened, repeat the broth tests at that time. See p. 547.

† When the material tested contains 0.4 per cent preservative or less, 1 cc. of inoculum is divided between one aerobic and one anaerobic tube, and 2 cc. among two aerobic and two anaerobic tubes; when it contains more than 0.4 per cent, 1 cc. is divided among two each, and 2 cc. among three each. For the volumes of Hitchens' medium required, see table 38.

‡ If a lot of toxin consists of less than four bottles, or if the toxin is filtered into 8-liter bottles, test 2 cc. of inoculum.

filtered sera, and vaccines before dilution is tested in broth; that of finished products, for human use, in bulk containers in both broth and in Hitchens' medium. If a bottle is opened, the tube test is repeated, except in the rare instances when part of the contents of the bottle

has been dispensed and sterility tests are to be made on the final containers. For further details and exceptions, see tables 39 and 40.

TABLE 40

*Sterility tests of products in bulk (from each container of less than 1 liter)*

| PRODUCT | WHEN TAKEN* | MEDIA INOCULATED BY | VOLUME INOCULUM† | |
|---|---|---|---|---|
| | | | In broth | In Hitchens' medium |
| | | | *cc.* | *cc.* |
| Serum‡ | On receipt | Production group | 1 | 0 |
| Serum for human use | After filtration | Filtration group | 1 | 2 |
| Toxin | After filtration | Production group | 1 | 0 |
| Toxin or toxin dilution for human use | Before preliminary human intracutaneous tests | Production group | 1 | 2 |
| Tuberculin | After filtration | Production group | 1 | 2 |
| Bacterial vaccine§ | After addition of preservative (1 day) | Production group | 1‖ | 0¶ |
| Polyvalent** and normal horse serum for diagnostic use. Ox bile | After filtration | Filtration group | 1†† | 0 |
| Monovalent serum for diagnostic use | After filtration | Production group | 1†† | 0 |

* Should a bottle be reopened, repeat the broth tests at that time. See p. 547.

† See footnote (†) to table 39.

‡ When a lot of serum consists of less than 200 cc. (special products such as human sera), test on receipt 0.5 cc. in Hitchens' medium, and omit the broth test.

§ Streak a blood-agar plate with the heated suspension just before adding preservative. For autogenous vaccine, streak a plate and inoculate an aerobic- and an anaerobic-broth tube, since the vaccine is diluted and dispensed immediately without further tests on the material in bulk.

‖ At the first examination, make subcultures from each broth tube by transferring 0.5 cc. to each of two similar tubes.

¶ Prepare one deep dextrose-agar tube and one dextrose-agar pour plate, adding five drops to each.

** Serum dispensed for therapeutic use, when outdated, may be pooled and refiltered.

†† When the volume is less than 500 cc., 1 cc. is tested; when from 500 to 1000 cc., 2 cc.

**Material from Burette.**—Two aerobic- and two anaerobic-broth tubes are inoculated from the burette at the beginning of a filling, with 0.5 cc. each when the phenoloid content of the material is 0.4 per cent or less; with 0.4 cc., when more.

**Material for Human Use from Final Containers.**—The sterility of all filled material is tested both in broth and in Hitchens' medium.

TABLE 41

*Sterility tests of products in final containers (for human use)*

| NUMBER OF BOTTLES FILLED* | BROTH AND HITCHENS' MEDIUM TESTS FROM SAME BOTTLES | | | | HITCHENS' MEDIUM TESTS FROM SEPARATE BOTTLES | |
|---|---|---|---|---|---|---|
| | Number of bottles | Volume inoculum from each bottle | | | Number of bottles | Volume inoculum from each bottle |
| | | Broth | Hitchens' medium† Balance contents to: | Total contents to: | | Contents to: |
| | | cc. | cc. | cc. | | cc. |
| 100 or less | 2 | 2 | 5 | 7 | 1 | 5 |
| 101 to 200 | 3 | 2 | 3 | 5 | 2 | 5 |
| 201 to 300 | 4 | 2 | 3 | 5 | 3 | 5 |
| 301 to 400 | 5 | 2 | 3 | 5 | 4 | 5 |
| 401 to 500 | 6 | 2 | 3 | 5 | 4 | 5 |
| 501 to 600 | 7 | 2 | 2 | 4 | 4 | 5 |
| 601 to 700 | 8 | 2 | 2 | 4 | 4 | 5 |
| 701 to 800 | 9 | 2 | 2 | 4 | 4 | 5 |
| 801 to 900 | 10 | 2 | 1 | 3 | 4 | 5 |
| 901 to 1,000 | 11 | 2 | 1 | 3 | 4 | 5 |
| More than 1,000 | One additional bottle from each hundred | 2 | 0 | 2 | 4 | 5 |

* For procedure in the case of very small fillings or when the volume is less than 2 cc., see text, p. 550.

† For exception made when products highly diluted with salt solution and to be administered in doses of 1 cc., or less, are dispensed in volumes of 5 and 10 cc., see text, p. 550.

The containers of filled material to be tested are selected by the sterility-test group. The number of containers selected and the volume of inoculum from each is based on a sliding scale and is determined by the size of the filling and the amount filled. Two bottles are selected at random from the first hundred bottles filled, and one from each succeeding hundred for the broth tests. Two cubic centimeters from each bottle are added to sterility-test broth. When the bottles contain

more than 2 cc., additional material from each is tested in Hitchens' medium. An exception is made when products highly diluted with salt solution, and to be administered in doses of 1 cc. or less, are dispensed in volumes of more than 2 cc., as in the case of diphtheria toxin-antitoxin mixture and bacterial vaccines. From such fillings, no Hitchens' medium tests are taken from the bottles from which the broth tests are made. One bottle from each hundred up to four hundred is selected for tests in Hitchens' medium alone; when more than four hundred bottles are filled, one bottle is selected for this test from the first hundred, one from the next to the last, and one from each of two other hundreds.

For the broth tests, the 2 cc. of inoculum from a bottle is divided among two aerobic- and two anaerobic-broth tubes, 0.5 cc. being added to each, except when the material to be tested contains more than 0.4 per cent phenoloid preservative when the inoculum is distributed among three aerobic and three anaerobic tubes. The amount of material tested in Hitchens' medium from these bottles depends on the total number of bottles filled. When one hundred bottles or less are filled, the remainder of the material in the bottle up to 5 cc. is added to sufficient Hitchens' medium to give a final concentration of not more than 0.01 per cent phenoloid preservative; when from 101 to 500 bottles, up to 3 cc.; when from 501 to 800, up to 2 cc.; and when from 801 to 1000, up to 1 cc. When over 1000 bottles are filled, the Hitchens' medium test is omitted. For the volumes of Hitchens' medium required, see table 38.

For the tests in Hitchens' medium only, the entire contents up to 5 cc. of each bottle selected is added to sufficient medium to give a final concentration of not more than 0.01 per cent phenoloid preservative. See tables 37 and 38.

When less than twenty-five bottles are filled or when from twenty-five to fifty bottles containing more than 12 cc. of material, two bottles only are selected for the test. Two cubic centimeters from each bottle are added to broth and the remaining material up to 10 cc. to Hitchens' medium.

When material is dispensed in less than 2-cc. volumes, 1 cc. only is inoculated from each bottle selected for broth tests. When material is dispensed in less than 1-cc. volumes, twice the usual number of bottles is selected for the broth tests: that is, two pairs of two adjacent bottles each from the first hundred, and one pair from each succeeding hundred.

When material is dispensed in more than one volume, at least one bottle of each size is selected for the tests.

**Material for Diagnostic Use from Final Containers.**—Material for diagnostic use is tested in broth and in Hitchens' medium. The tests

are taken by the sterility-test group except in the case of small lots dispensed by the production group. When between ten and fifty bottles are filled, two are selected for the tests; when from fifty to one hundred, three; and when more than one hundred, two for broth tests from the first hundred, and one from each succeeding hundred, and two for Hitchens' medium tests, one from the first hundred and one from the second or next to the last hundred.

For the broth tests, 1 cc. of inoculum is tested from each of the bottles selected when less than fifty bottles are filled; from each of two bottles of the three selected when from fifty to one hundred, and from each of the bottles selected for broth tests, when more than one hundred bottles are filled.

For the Hitchens' medium tests in the case of polyvalent and monovalent horse sera, normal horse serum, and ox bile, 2.5 cc. are tested from each of two bottles from a filling, except when less than ten bottles are filled, in which case 1 cc. is tested from the one bottle selected. In the case of monovalent rabbit sera, 1 cc. from one bottle of a filling of less than one hundred bottles is tested in Hitchens' medium. Should more than one hundred bottles be filled, 1 cc. from each of two bottles would be tested.

As in the case of material for human use, the inoculum is added to sufficient broth or Hitchens' medium to give a final phenoloid-preservative content of not more than 0.01 per cent. None of the products contains more than 0.3 per cent phenoloid preservative.

*Procedures Used in Inoculating Culture Media*

The procedures connected with the removal of material to be tested and the inoculation of the media must be carried on with rigid aseptic precautions in a draft-free room. The pipettes used must be freshly sterilized and from a container previously unopened. No container in which a satisfactory cobalt-ink control is not present is used. Two trained workers are required to carry on the procedure.

After the paraffin seals on anaerobic tubes have been loosened by warming an instant above the flame and tapping lightly, "A" carefully removes a pipette from the container, after first examining the cobalt-ink control and discarding it. Meanwhile "B," after flaming the neck of the bottle, removes the stopper with flamed forceps, and reflames the bottle neck. "A" draws into the pipette the required amount of material. "B" replaces the stopper after again flaming the neck. After removing the plug from the container of the medium, "B" flames the opening, and holds the tube or bottle in a slanting position, while "A" inoculates it. "B" then flames the opening and replaces the plug, pushing it firmly into the neck of the tube or bottle so that it will not be easily loosened or

pulled out. The paper caps are replaced on the bottles of Hitchens' medium and they are carefully rotated immediately after inoculation to insure distribution of the inoculum. Material left in the bottles is generally pooled with other residues from the filling. See "Preparation of Biologic Products for Distribution," p. 534.

STERILITY TEST RECORD

Date.....

| | |
|---|---|
| Ser. Dys. | Tox. Diph. |
| Men. | Tet. |
| Norm. | Strep. |
| Strep. | Anti. Diph. |
| Pnc. I | Tet. |
| II | Vac. Per. |
| III | Typh. |
| Diph. T.A.M. | T.A.B. |

.....

Lot. No.....

Unfilt. Filled.

Filt. 1.2. Retest 1.

.....

Bulk Container.

Burette Begin.

Bt. No.....

Sterility Test Broth.

Hitchens' Medium.

Report. Date.

Signed.....

FIG. 51. RECORD FORM

Take every precaution to avoid mixing sets of tubes or bottles. As soon as a set is inoculated, fasten the tubes together with a rubber band and insert a sterility-test record form which has been previously filled out. Attach a similar record form to each container of Hitchen's

medium. Place together all containers inoculated with the same product in a wire basket on one of the receiving shelves reserved for this purpose in the incubator.

### *Sterility-Test Record Form Accompanying Inoculated Media*

A form, figure 51, upon which are entered the data required for permanent records, p. 559, accompanies each set of tubes and each bottle containing inoculated medium. On completion of the test, the form with the results entered upon it, is sent to the production group.

**Directions for Filling out Sterility-Test Record Form.**—1. Use one form for each set of tubes and one for each container of Hitchens' medium.

2. Enter the date.

3. Underline the name of the product. If it does not appear on the form, write it on the blank line provided for that purpose. In the case of pneumococcus serum, type I (Pnc. I), indicate whether the serum is for diagnostic or therapeutic use.

4. Enter the lot number, indicating, if necessary, the bottle letter or any other data required for identification.

5. Underline the proper abbreviation to indicate the condition of the material (unfiltered, filtered, filled, retested). Indicate whether it is the first or second filtration or retest.

6. Underline to show whether the record form refers to a set of broth tubes or a bottle of Hitchens' medium.

7. In tests on material in bulk, underline the words "Bulk container."

8. In tests on material from the filling burette, underline "Burette Begin."

9. In tests on finished products indicate the bottle number which shows from which hundred bottles the sample was taken. In the case of Hitchens' medium, designate the containers inoculated from the bottles from which broth was also inoculated, by the numbers of the broth tests followed by an "H" ("1H" and "2H" from the first hundred, etc.); designate the other containers of Hitchens' medium by the numbers of the hundreds from which the bottles were withdrawn. (For example "1," "3," "4," and "6" designate bottles withdrawn from the first, third, fourth, and sixth hundreds.) In case of vaccine "sets," show in parentheses after the bottle number the volume dispensed.

### *Method of Recording Tests and Observations*

Records of all tests are kept in bound ledgers. The abbreviations used are listed in the front of each book. For convenience, records of tests of sera and antitoxins are kept in one book, those of toxins and diphtheria toxin-antitoxin mixture in another, while a third is used for vaccines.

The data from the sterility-test record forms are entered in the left-hand column of a double page, which is divided to allow space for each day's records with subdivisions for records of aerobic and anaerobic tubes inoculated from

each bottle, and space at the extreme right for the results of each test. (A section of the back of each book is ruled off in spaces convenient for entering the animal test records. See page 557.)

On the second day after inoculation of the media, arrange the sets of tubes in the order of inoculation. Then enter the data from the record forms in the space at the left; the name and lot number of the product, its condition (filtered, unfiltered, etc.), the date filled, and below, first the tests from the burette, then separately in order of inoculation each bottle tested in broth, or broth and Hitchens' medium, and finally each bottle tested in Hitchens' medium alone. Enter sterile serum controls, if made, at the end. Record in the second column, under the date of inoculation, the containers of medium inoculated from the burette and from each bottle.

Record the results of each observation as made. Indicate the absence of contamination by the symbol "OK." When contamination is suspected, enter the number of tubes questioned, and the subcultures made, "1?, 1 D.P." (deep tube, and pour plate) and later the presence or absence of growth in these. When inoculated broth is obviously contaminated, enter the number of tubes in the set in which growth is present, and record the type of organisms found, "2 C, sm. Gm.-bac."; when the Hitchens' medium, "C" and the type of organisms. Enter in the column at the extreme right "contam." and if a retest is made, "Retest" and the date. If no growth develops in a tube or bottle during the 7-day period, enter the final date, and initial. When the test is completed, record the results on the sterility-test record forms, date, and initial, and send to the production group; entering, if no growth has occurred, "OK"; if organisms have developed, "contam.," the kind of organisms and, in the case of tube sets, the number of aerobic or anaerobic tubes.

### *Incubation and Examination of Inoculated Media*

Incubate the inoculated media at from 35 to 36°C. for seven days. Examine broth tubes on the second, fourth, and seventh days. Examine Hitchens' medium inoculated from "separate bottles" daily, but record the results only on the second, fourth, and seventh days, except when contaminating organisms are found or suspected. Postpone for one day the examination of broth tubes falling on Sunday or a holiday; omit that of Hitchens' medium. If a Sunday and a holiday fall on successive days, examine all inoculated media on the day preceding and the day following. Requests for examinations on other than the regular days are received through the department secretary. Reports of such special examinations should be submitted promptly.

Each morning remove the media inoculated on the preceding day from the receiving shelf in the incubator to the compartment reserved for tests under observation. Examine the Hitchens' medium inoculated from the "separate bottles." On the second day (forty-eight hours) examine all the media, and enter the required data from the accompanying forms in the record book together with the results of the ob-

servation. In order to facilitate examination, incubate the tubes containing different types of products, and the bottles, in separate baskets, arranging the containers as far as possible in the order in which the tests are entered in the record books.

To avoid unnecessary cooling, remove only one or two baskets from the incubator at one time. Make examinations by daylight in front of a window. Examine one bottle of Hitchens' medium at a time and record the results of each observation before proceeding to the examination of another bottle. In the same way, examine only one set of tubes at a time. When examining a large set of tubes, separate them, as not more than three can be handled conveniently. After observing the appearance of the broth tubes, rotate them very gently to detect the presence of a precipitate or sediment suggesting contamination. Do not rotate containers of Hitchens' medium. Put a question mark with a red wax pencil on any suspected container which is to be observed with special care at the next examination. When the test is completed, enter in the record book the results of the final examination of each set of tubes and each bottle and initial, before removing the record form and entering the necessary data upon it.

Subject to further examination the medium in each container in which contamination is suspected. When it is necessary to remove a tube from a set, mark it plainly with the name and lot number of the product, together with any further data necessary for its identification. Do not remove the record slip from the remainder of the set. Since the examination of stained preparations of material showing slight indications of contamination is usually indeterminate, subject the suspected medium to cultural tests at once. Add 0.5 cc. to one deep 0.2 per cent dextrose agar tube and prepare a pour plate, using the same amount. For procedure see "General Bacteriological Technic," pp. 5, 8. Fasten together with a rubber band the original tube or bottle and the deep tube subculture, and incubate for five days, together with the plate culture. Make daily observations, entering the results in the record book only on the days of regular examination unless growth is observed.

When bacterial growth is obvious in a tube or bottle of inoculated medium, remove and mark the container as above. Make a slide preparation stained by Gram's method and record the type of organisms found. Supplement with a second slide preparation, using the spore stain, should the presence of spore-bearing organisms be suggested. Retain the culture in the cold room until the test is completed. Should the slide preparation fail to show bacteria, make subcultures as above.

If growth is present in several containers, make slide preparations from at least two, always from both kinds of media if both are contaminated.

*Retests*

Retests are made when the number of containers in which growth develops is so limited that there is doubt as to whether the organisms gained entrance at the time of the inoculation of the container or were present originally in the material tested. Retests are made by the worker in charge of sterility tests, assisted, in the case of bulk material, by a member of the production group, in that of filled material, by a member of the filling and boxing group.

In the case of bulk material, if the sterility tests indicate slight contamination in one bottle, the tests on that bottle are repeated; if in two or more bottles of a lot, the tests of the entire lot are repeated. If growth develops in one or more containers in the retest, the material is treated as contaminated.

Sera and toxins shown to be contaminated are filtered immediately; if in the judgment of the head of the production group, the extent or nature of the contamination is such as to raise any doubt of the suitability of the product for use, the bacteriologist in charge is consulted. Bacterial vaccines are always discarded, toxin-antitoxin mixture is discarded or filtered.

In the case of filled material, if growth has occurred in any container, the entire test is repeated. Should a retest also indicate the possible presence of contaminating organisms, the bacteriologist in charge of the department must be consulted as to the further treatment or disposition of the product. In certain instances, a second retest may be permitted. The presence of the same microörganism in the test and retest is considered evidence that the material is contaminated. In cases when there is doubt as to the advisability of following the routine procedure rigidly, the bacteriologist in charge may be consulted. Certain exceptions, such as a partial retest when "accidental contamination" (as growth in a cracked tube) has evidently occurred, may be allowed.

No material is released for distribution unless in the final test all containers have remained sterile.

## ANIMAL TESTS

In order further to insure the harmlessness of all biologic products for human use, material from the final containers prepared for distribution is injected into animals. Each lot is tested in a guinea pig and a

mouse except that the mouse test is omitted when the material contains no phenoloid preservative.[4] The animals are observed for seven days.

**Mice.**—Unused white mice weighing not less than 20 grams are selected. In case of shortage, healthy mice which have been injected previously with serum or other material containing no phenoloid preservative may be used. After injection, the mice are kept in separate metal boxes or glass jars.

**Guinea Pigs.**—Unused guinea pigs weighing not less than 300 grams, preferably over 350 grams, are selected. They are kept in cages assigned to the sterility-test group; not more than six animals in one compartment.

### *Injection and Dosage*

Make injections subcutaneously (for technic, see "The Use of Experimental and Test Animals," p. 35). For all products except bacterial vaccines and material containing 50 per cent of glycerin, inject 3 cc. into the guinea pig, 1 cc. into the mouse; for vaccines, 1.5 cc. and 0.5 cc. respectively; for tuberculin and any other product containing 50 per cent of glycerin, 0.5 cc. and 0.25 cc. respectively.

### *Method of Recording Tests and Observations*

Enter on a separate cage tag ($4\frac{3}{4}$ by $2\frac{3}{8}$ in.) for each animal, the date, name, and lot number of the product, quantity inoculated, name of worker; and for a guinea pig, the weight, ear-tag number, and a brief description in case the ear tag should be lost. Record on the tag any symptoms, and the date of discharge, or in the case of death, the date, and autopsy findings. At the time the animals are first examined, enter in the record book the kind and number of the product, dose, kind of animal, and for guinea pigs the number and weight. On the day of discharge or death, enter the further data from the tags.

### *Observation of Animals*

Keep the mice under observation for at least half an hour after injection. If an animal shows more than the slight or even moderately severe reaction which may be expected from phenoloid preservative, or does not recover promptly, notify the production group and repeat the test. Examine all animals daily at 9:00 a.m., except on Sundays and holidays, when the hour is optional. If any animals show evidence of illness, reëxamine at 5:00 p.m., except on Saturdays, Sundays, and holidays. Weigh any guinea pig showing marked loss in weight, daily;

[4] Certain exceptions are made: Autogenous vaccines and small lots (less than 200 cc.) of special products, such as human sera are not injected into guinea pigs; diphtheria toxin for the Schick test is not injected into guinea pigs nor mice.

all guinea pigs on the fourth day. Watch for general symptoms, including any indication of paralysis, and for local reactions especially at the site of injection. At the first evidence of illness or infection, isolate the animal in a separate box, and notify the production group. Unless the symptoms are slight and transient and appear to be of no significance, when a retest may be omitted, inoculate another animal with the same dose; two, should further control be advisable. If the condition of the animal has remained satisfactory, discharge it on the seventh day.

## REPORTS TO PRODUCTION GROUPS

Whenever growth occurs in cultural tests or an animal becomes ill or dies, notify the production group at once by submitting the following data on a "Preliminary Report" form; name and lot number of product, date of test, whether a first test or retest, the number of the bottle or bottles from which the medium was inoculated, the medium, and the type of organism found; and in animal tests, the nature and severity of the symptoms. A duplicate report is sent at the same time to the bacteriologist in charge of the department. In the case of filled material, if retests are to be made, notify the filling group also. The head of the production group in consultation with the worker in charge of sterility tests decides whether bulk material should be retested or treated as contaminated.

When the cultural tests (and animal tests when made) are completed, assemble all records, and after checking the entries on the record forms and animal tags with those in the record book, send the forms and tags to the production group, together with a "Final Report" form, on which are entered the name and lot number of the product and the date of release. Initial all reports. When the test is not satisfactory, do not include the "Final Report" form. Products are released for distribution by the heads of the production groups. This is done only after the reports from the sterility-test group show that all tests are satisfactorily completed. An emergency release, permitting material to be shipped in advance of the usual 7-day period must be approved by the bacteriologist in charge.

## TEST OF VOLUMES DISPENSED

As a control of the accuracy of the workers dispensing the material, bottles of filled material are selected at least once a month by the worker in charge of sterility tests, and the contents measured. Bottles containing less than 6 cc. are usually chosen.

Select at random two bottles from a recent filling, from the supply of unboxed material in the cold room. Measure the contents of each bottle in an accurate "to contain" pipette with a fine tip and a capillary stem graduated in hundredths, filling the pipette by means of the apparatus used in measuring toxins and antitoxins (see "General Instructions," p. 622). Draw the material into the pipette to the last drop, and take the reading quickly before air bubbles enter. Record on a record card (4 by 6 in.) the kind and lot number of the product tested, the volume required, and the volume found. Enter the same data on a report form and send at once to the bacteriologist in charge of the department.

*Permanent Records*

Cultural and animal tests: The record books (p. 553) are held as permanent records.

Tests of volumes dispensed: The original record cards (p. 558) are filed as permanent records.

## SECTION X

## CHAPTER 1

# CARE AND TREATMENT OF ANIMALS—SMALL ANIMALS

The procedures connected with the care and treatment of animals required in the work of the laboratory are given in the two following chapters. The first chapter deals with the small laboratory animals, which are used chiefly for diagnostic purposes, standardization tests of biologic products, and experimental work. The second chapter gives directions for the care of horses, which are used mainly in the production of antitoxins and other immune sera, and the different methods of injection and bleeding. At the end of the section brief reference is made to the care and treatment of cattle, sheep, and goats.

Quarters for the different animals are provided at the laboratory and at the laboratory farm eight miles from Albany. An experienced veterinarian, assisted by a second veterinarian and acting under the bacteriologist in charge of the antitoxin, serum, and vaccine laboratories, has supervision of the care and use of all animals. At the farm and at the laboratory a trained worker, with such assistance as may be required, is directly responsible for the care of the animals. These workers are also qualified to perform routine injections, bleedings, and autopsies, and to keep accurate records. Special care is exercised in the selection of workers to care for the animals; only those who show a natural interest in animals are chosen. In the care and handling of animals proper consideration and kindness on the part of all workers are expected and required. Any apparent infringement of this rule by a worker must be reported immediately to the bacteriologist in charge of the department. The worker is liable to instant dismissal.

### CARE OF SMALL ANIMALS

Procedures connected with the receipt and care of the stock supply of small animals and with the care of those under treatment are given under this heading. Some data in regard to breeding and the common diseases of laboratory animals are included. Operating-room procedures are given in a separate chapter, "The Use of Experimental and Test Animals," p. 30.

A large supply of guinea pigs, rabbits, white mice, and a small number of white rats are always kept in stock. As far as possible these animals are bred by the laboratory. A limited number of monkeys, Macacus rhesus (usually five or six), are held for special investigations. Other animals such as dogs, cats, and pigeons are obtained as needed.

### *Ordering and Receipt of New Stock*

Requisitions for the purchase of routine stock are placed with the purchasing group by the worker in charge of the animals after approval by the veterinarian; special orders are referred to the bacteriologist in charge of the department. On the requisitions are given the number, kind, and weight of animals required, and if desired, the dealer. To insure a definite supply of guinea pigs and mice, in addition to those bred by the laboratory, a contract for monthly shipments is usually made with one or two dealers of known reliability. Groups of workers needing unusually large numbers of animals are expected to give notice as far in advance as possible.

Upon receipt of a shipment, provide the animals immediately with food and water if required. Examine each animal carefully and notify the veterinarian at once if the condition of any is unsatisfactory. (Dead animals are autopsied to detect possible infections unless decomposition has occurred.) Enter the date of receipt, dealer, number of animals, (weight when required), and condition, in the "Stock Received" duplicating order book, and send the original to the purchasing clerk. Enter the shipment on the daily report sheet of laboratory animals. Report immediately in writing to the purchasing clerk, shipments found unsatisfactory upon receipt, and deaths occurring within seven days from causes for which, in the judgment of the veterinarian, the dealer is responsible.

Vagrant dogs or cats received by the laboratory are held at least as long as at the city pound. Attempts are made to locate the owners by following newspaper advertisements.

### *Quarantine*

Place the animals in clean, previously disinfected cages or boxes with a tag attached giving the date received, number of animals, and dealer's name. Hold each shipment in quarantine for at least seven days, preferably for fourteen, before transferring the animals to the stock quarters or distributing directly for laboratory use. Should one or two animals of a lot appear ill when received, or during the period of quarantine, isolate them at once. Make careful daily inspections.

Feed new stock in quarantine after the normal stock, but before the isolated and sick animals. Autopsy animals that die, and, as a rule, continue the quarantine for at least one week after a death from a suspected or known infection has occurred. Occasionally animals from recent shipments are carefully selected for breeding purposes.

*Animal Quarters*

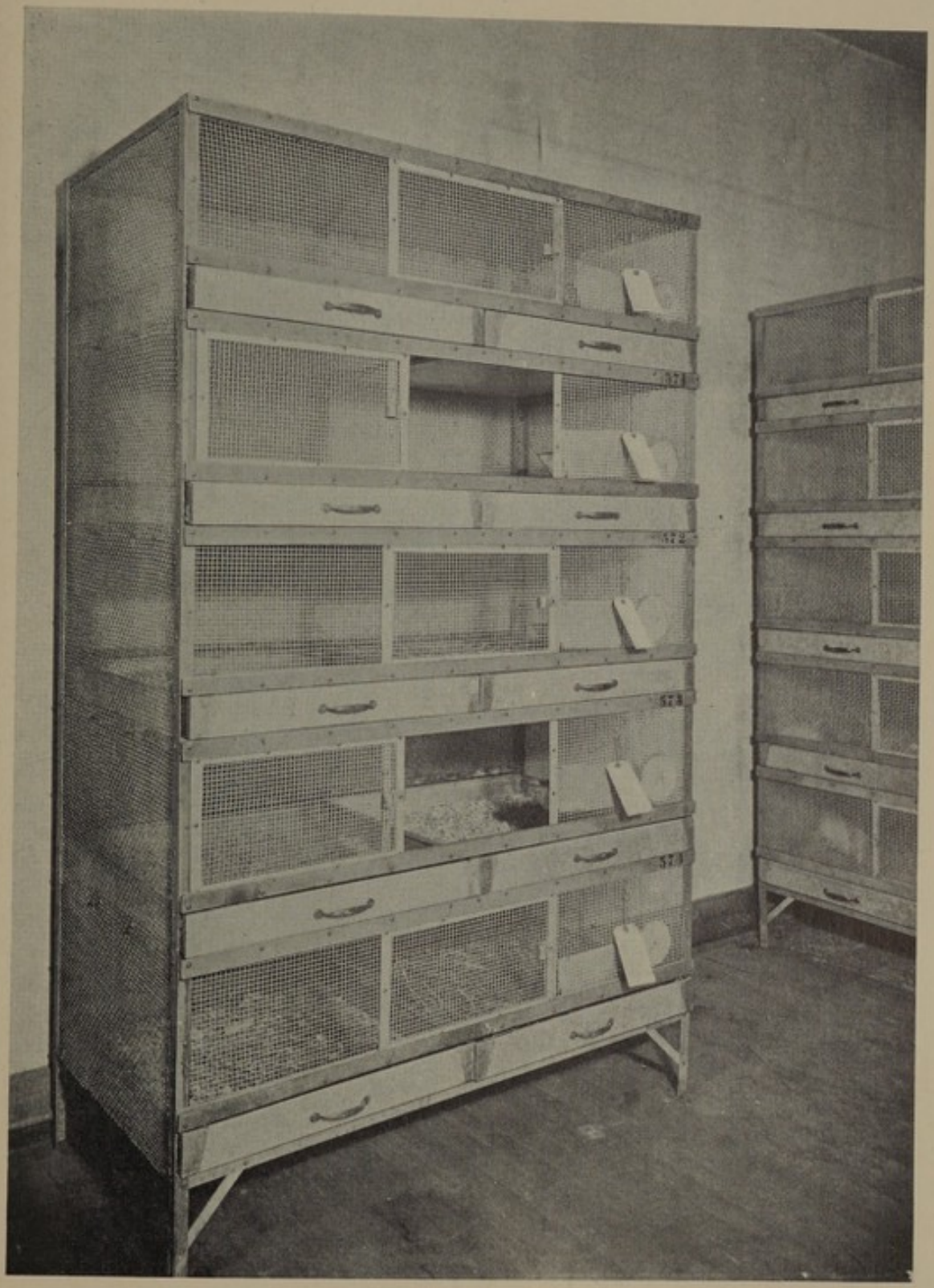
The animal quarters are divided into separate sections for (a) breeding animals; (b) normal stock; (c) test and experimental animals, with further subdivisions for those likely to spread infections; (d) detention quarters for new stock; and (e) observation and isolation quarters for sick animals. Quarters for special animals, such as dogs and cats, are provided as required.

The animal quarters must be kept clean, in order, and properly ventilated. All available light (and sunshine) should be utilized except during excessive heat. The rooms and cages must be cleaned frequently. Sudden changes of temperature and drafts must be avoided. In cold weather, if uniform temperature conditions can not be maintained in a room, ample bedding must be provided. As far as possible, a temperature of approximately from 60 to 65°F. is maintained for guinea pigs and rabbits; from 65 to 70°F. for mice, rats, and monkeys. The number of persons having access to the animal quarters is strictly limited.

Cleaning: Sweep the floors thoroughly each day, using moistened sawdust when necessary to prevent raising dust. Wipe flat surfaces, such as table tops and window sills, with a dampened cloth. Scrub or mop the floors of the test-animal quarters at least once a week, other floors as required. Thoroughly hose and scrub the floors where construction permits.

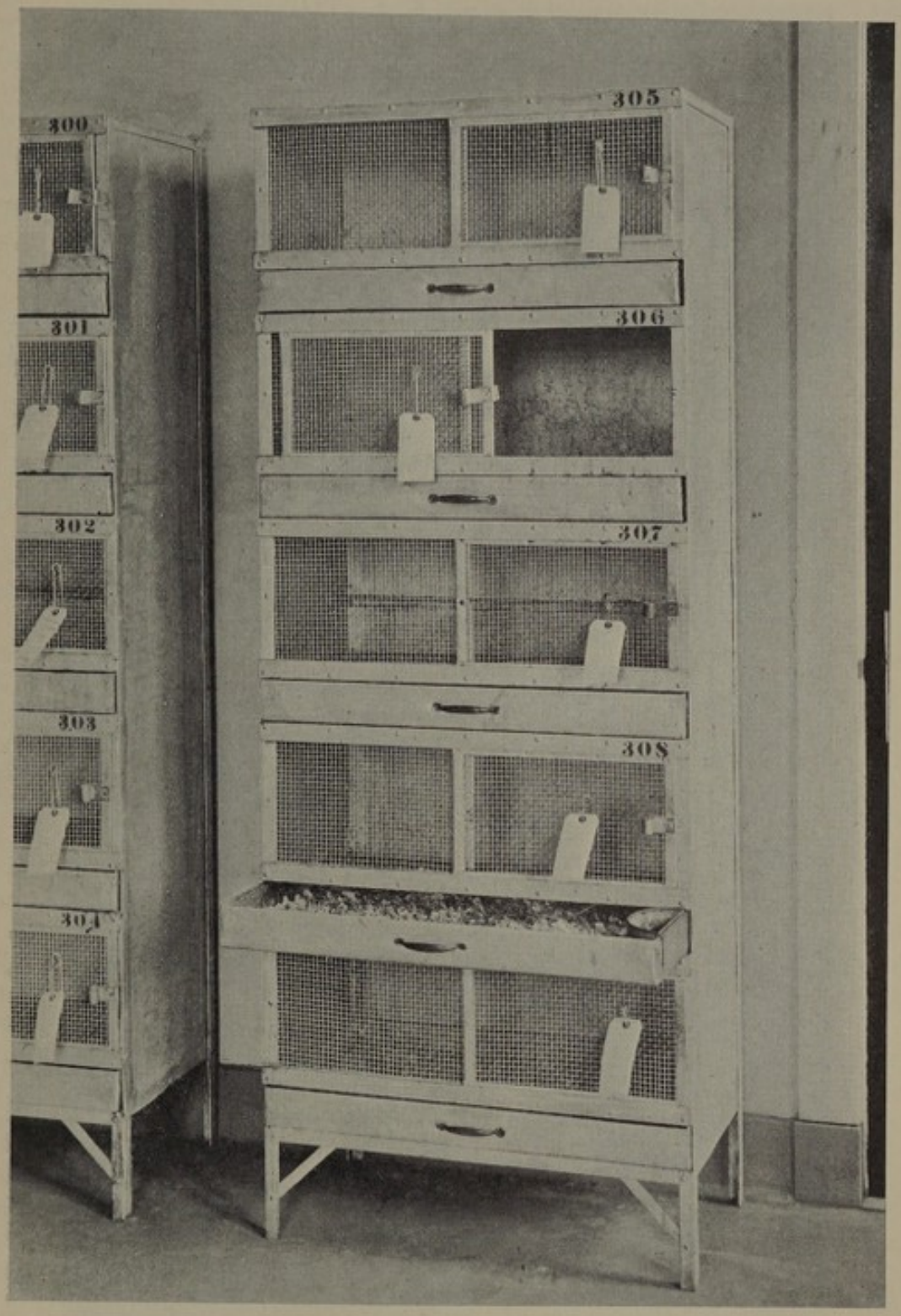
**Precautions Against Flies.**—Special precautions are taken to exclude flies, and to prevent the development of larvae brought in on food and other materials. When necessary, larvae may be destroyed by spraying the trays and inside of the cages with a borax solution (1 lb. to 1 gal. water). All windows and outside doors are screened, cages are cleaned at least once each week, and uneaten vegetables removed each day. Fly paper and traps may be of some assistance. Manure pits close to the laboratory are kept covered or screened and cleaned frequently. When necessary, uncovered manure pits may be sprayed with a solution of hellebore (1 lb. to 20 gal. water—10 gal. to 80 bu. of manure). When special precautions are required to prevent transmission of infection by flies, cages are screened.

**Precautions Against Vermin.**—Cages may become infested with cockroaches or bed bugs (*cimex lectularius*) brought in with shipments. When infestation once obtains a foothold, eradication of the vermin is difficult. Incoming ship-

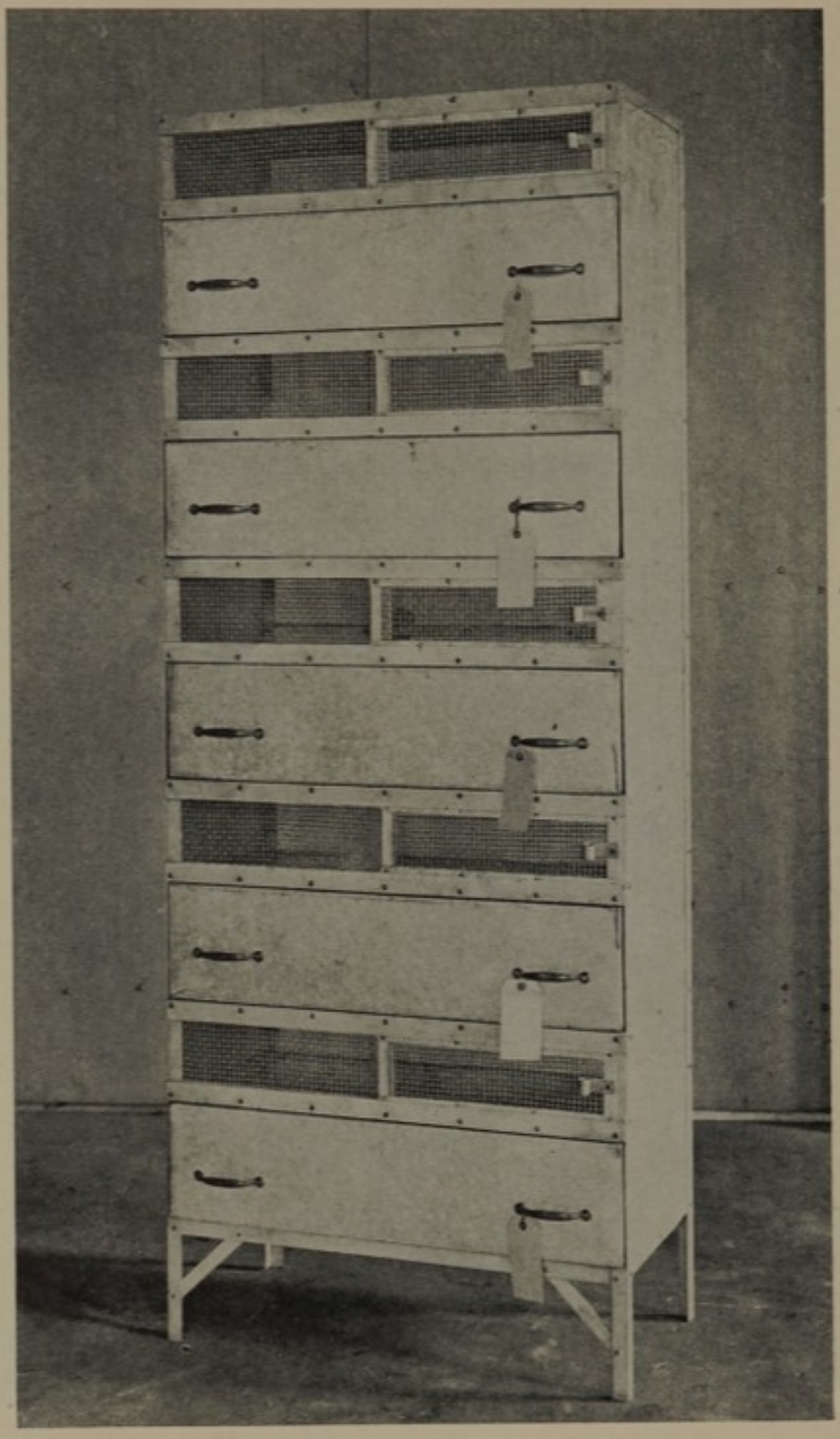


Stock Animal Cage

Wooden Runs for Small Animals




EXPERIMENTAL AND TEST ANIMAL CAGE



METAL CAGES WITH DEEP TRAYS

ments are carefully watched and boxes destroyed or sterilized. All cracks and crevices in which breeding may take place are, as far as possible, eliminated. At the first evidence of infestation in a cage, prompt action is taken. Immersion of metal cages and trays in a vat of boiling water or the application of live steam is the most effective method of extermination. Where such facilities are not available, thorough drenching with a heavy spray of 5-per-cent cresol compound, repeated at monthly intervals, has been used. Kerosene has been used similarly. Boric acid has been used with some success in eliminating cockroaches.[1]

### *Cages*

**Types of Cages.**—The standard metal cages and boxes used for stock and test animals are made according to special designs.

1. Metal cages of galvanized-iron wire (17 gauge No. 3 mesh) 78 in. total height, each a solid stack of five compartments (48 by 24 by 14 in.) built on a one-inch angle-iron frame with legs 8 in. high. Each compartment has a central sliding wire door, and for ease in cleaning, two removable trays (24 by 24 in. by 2 in. high) which rest on slides made from angle iron. A folded metal strip is slipped over the edges of the adjoining sides of the trays. Each compartment is designated by a number stenciled on the front of the cage. (See: plate VII.)

2. Metal cages of galvanized iron (19 gauge) of same size and type as No. 1 but with two sliding, wire doors, and a removable central partition, which may be used to divide each compartment into two divisions. Each division is designated by a number.

3. Wooden runs, each 30 by 60 by 10 inches high, with 12-inch legs (2 by 2 in.) which extend a further 8 in. inside the pen, forming supports for a second run. The runs are made of wood, painted with three coats of asphaltum paint. Three runs are stacked, forming a unit. Each run has two removable partitions so that they may be divided into three compartments, each 30 by 20 inches. (See: plate VIII.)

4. Metal cages of galvanized iron (19 gauge), 78 in. total height, consisting of a solid stack of five compartments (30 by 15 by 14 in.) having wire-mesh fronts with one sliding door in each, and a removable tray 2 in. high. A number is stenciled on each compartment. (See: plate IX.)

5. Metal cages with deep trays of same size and type as No. 4 except that the back is of galvanized-iron wire (17 gauge, No. 3 mesh), and the sides of the trays are 7½ in. high and each tray has two handles. The sliding door above the tray is used for feeding only. (See: plate X).

6. Metal cages (for monkeys) of galvanized-iron wire (11 gauge, 1-in. diamond mesh) 48 by 24 by 78 inches. Door (32 by 30 in.) divided horizontally into halves. Also cages 60 by 30 by 84 inches. Each cage is furnished with four round, wooden crossbars to be used as perches.

7. Metal boxes of galvanized iron (22 gauge) made from one piece with rolled edge top reinforced by heavy wire (6 gauge); wire mesh (No. 3) sliding covers with drop ring handle. Three sizes are used: 6¾ by 7½ inches by 5 inches

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[1] For further discussion, see Felt, E. P., N. Y. State Museum Bull., 1917, No. 194, p. 60 (160).

high; 15 by 13½ by 7½ inches high; and 13½ by 17½ by 12½ inches high. (For type of box see, plate XI and plate II.)

Racks for metal boxes 30 by 15 inches by 78 inches high; frame of ¾-in. angle iron, with eight adjustable shelves of galvanized round iron bars. (See: plate XI.)

8. Glass jars: For isolation of individual mice. Low pint preserve jars, covered with a circle of wire mesh held in place by the spring on the jar and a wooden block.

**Bedding.**—All cages and boxes are provided with an ample supply of clean bedding. Shavings are at present used in practically all instances. In winter, if a uniform temperature cannot be maintained, an extra supply of hay is placed in cages containing rabbits and guinea pigs.

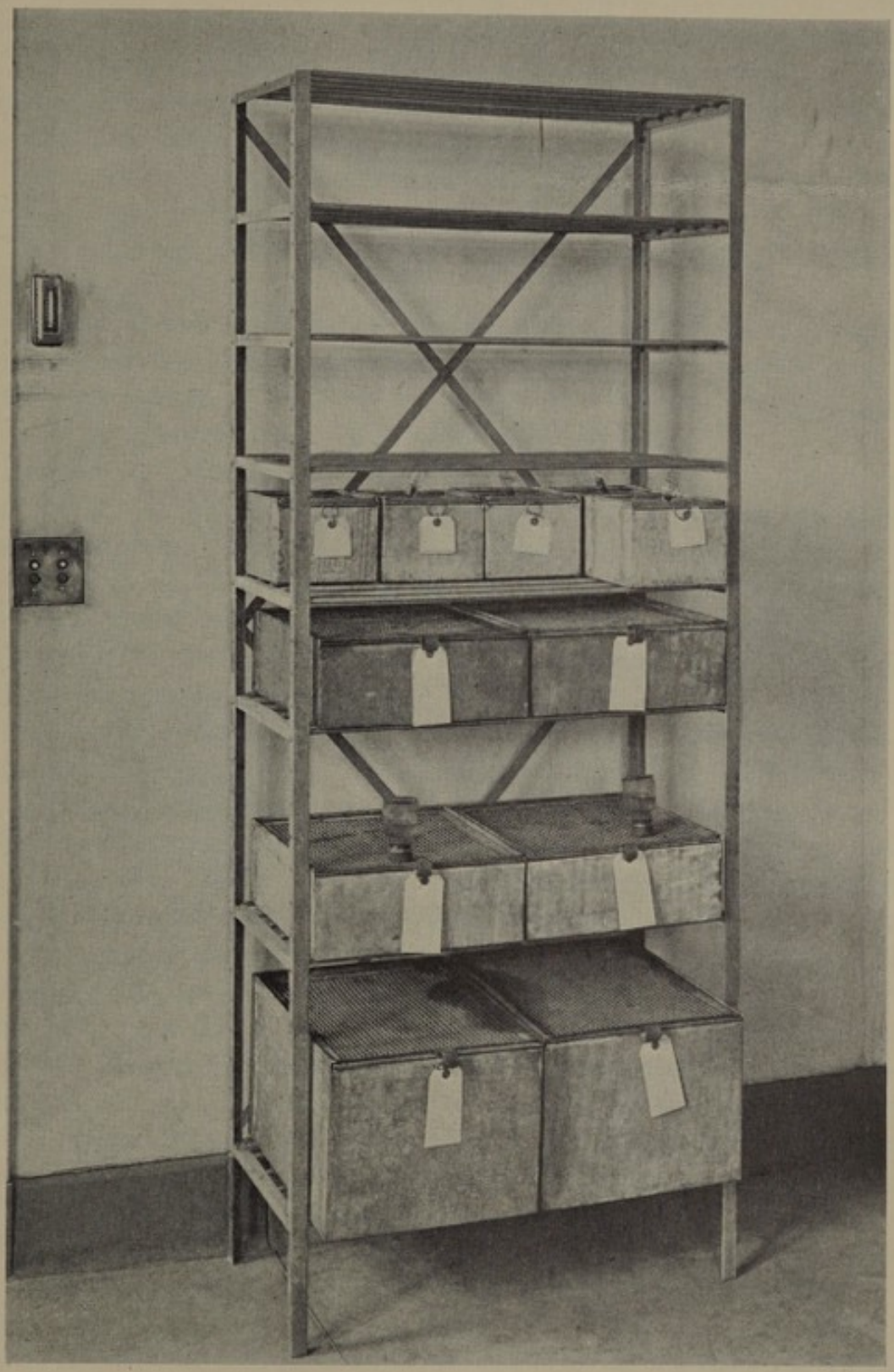
Use clean shavings which are not too coarse. Make the layer from 1 to 1½ in. deep. Change every seven days or oftener if necessary.

*Care of Cages*

All cages are given a thorough cleaning at least once a week. Compartments from which the animals have been removed are cleaned promptly. Special care is observed in cleaning cages which have contained test animals or animals suffering from spontaneous infections. From time to time as directed by the veterinarian, the cages are disinfected and hosed and, when required, repainted with aluminum bronze paint. The insides of trays (bottoms and portions of sides to include angles) are painted.

**Occupied Cages and Boxes.**—Normal guinea pigs and rabbits: To clean cages containing normal guinea pigs or rabbits, remove the animals carefully from the top compartment of a tier to a metal container, or if two trays are used, drive the animals into one. When the compartment is divided by a partition, remove the animals in each section to separate containers. Empty the bedding into a galvanized-iron receptacle placed on a hand truck. Scrape thoroughly the inside of the tray with a small hoe. Empty and clean the grain troughs. Boil, or otherwise sterilize, troughs or cups used for water. Brush all material from angles and walls of the compartment. Replace the tray, and after filling with fresh bedding, return the animals to the cage. Repeat the procedure with the next lower compartment. When all the compartments of a cage have been cleaned, commence at the top of the next cage.

Normal mice and rats: Transfer the animals to fresh boxes or, if kept in cages, transfer to metal containers and clean the cages as above.



ANIMAL BOXES AND RACK

Normal monkeys: Without removing the monkeys, clean the crossbars and floors and put in fresh shavings at least once each week.

Test guinea pigs and rabbits: Unless otherwise directed, clean cages as for normal animals, using special care to prevent scattering the shavings when removing the bedding.

Test mice and rats: Unless otherwise directed, do not clean boxes until the test is completed.

Test monkeys: Proceed as for normal monkeys unless special instructions are given.

**Vacated Cages and Boxes.**—Normal animals: Clean a compartment thoroughly as soon as possible after it has been vacated, and fill with fresh bedding. If infection among the animals was indicated, proceed as directed under "Infected Cages and Boxes."

Remove the bedding from boxes, then immerse the boxes and milk and water containers in the disinfecting tank containing 2-per-cent cresol compound[2] or sterilize them in the autoclave.

Test animals: Thoroughly clean the vacated cages and boxes as described under "Normal Animals." Sterilize mouse jars in the autoclave. Follow the procedure for infected cages and boxes, when directed by the worker responsible for the animals.

**Infected Cages and Boxes.**—Treat as infected all cages or boxes occupied or vacated by animals suspected of having, or known to have, an infection which might be transmitted to other animals.

Unless the condition of an occupied infected cage is such that cleaning is necessary, postpone all cleaning or removal of bedding until the animals have died or been disposed of. Should it be necessary to hold infected animals for some time, remove them to boxes. Dampen the bedding with 2-per-cent cresol compound before removing it. Wipe, spray, or brush the walls of the compartment with the same solution. Sterilize the tray by immersion in the disinfecting tank or by placing in the autoclave. If this cannot be done, spray the tray with 2-per-cent cresol compound solution and expose to the direct rays of the sun. Boil, or otherwise sterilize, the grain and water containers. Treat infected boxes in the same manner as infected cage trays.

**Infected Boxes Requiring Special Attention.**—Boxes or jars occupied or vacated by animals inoculated with material which contains, or is suspected of containing, *B. anthracis*, *B. mallei*, *B. melitensis*, *B. pestis*, *B. tularense*, or rabies virus, are cared for by the scientific workers

[2] When available, a tank with metal cover in which trays and boxes are submerged in boiling water heated by steam may be used. Soda is added to the water while hot, when necessary. A similar tank may be used for cages.

responsible for the tests. Such animals are kept in a separate room and each box is plainly labeled with a red tag "Do not handle or feed." A supply of food and bedding for these animals is left in cans easily accessible to the workers.

### *Feeding of Animals*

Since the condition of the animals depends largely upon the food which they receive, special attention must be given to providing a proper and well-balanced diet. Constant supervision is necessary. The worker in charge should inspect the cages constantly, and the veterinarian often enough to make sure that the proper kind and quantity of food is being supplied, and that there is no unnecessary waste.

The diet for the animals follows:

Guinea pigs and rabbits: A constant supply of oats or a mixture of oats and bran. In winter, hay (clover, timothy, or alfalfa) and chopped vegetables daily. Carrots and beets may be given on different days or as a mixture. Cabbage should be added at least three times a week. When available, cabbage may be given in place of the roots, but the change should be gradual. In summer, green alfalfa and clover or lawn grass are substituted for the roots and cabbage. When available, Swiss chard, lettuce, and spinach may be given. Seasonal changes in diet should be made very gradually. All cages containing breeding animals are provided with a piece of salt brick attached to the inside of the cage and with a container for water. Cages containing stock or test animals are provided with water only when green food is not supplied or does not contain sufficient moisture. These cages are usually supplied with salt when water is provided.

Mice: Bread every day, milk three days a week, more often for breeders. A mixture of oats, wheat, buckwheat and sun-flower seed is kept in the cages. In winter, a small amount of corn may be added. Occasionally cabbage, carrots, beets, or apples are provided. All boxes containing mice are provided with water bottles.

Rats: Oats, corn, and white bread every day, milk three times a week; raw beef and cabbage twice a week but not on successive days.[3]

Monkeys: Boiled white or sweet potatoes, carrots, cabbage, dry bread, apples, oats, whole wheat, and wafer. A mixed diet is advisable. As monkeys have individual preferences it may be desirable to try different foods, but sweetened food is to be avoided. Foods which may be used are: boiled rice, buckwheat, sunflower seed, cracked corn, bananas, oranges, cooked prunes, beets, and milk with lime water.

Dogs and cats: Water, dog biscuits, scrap meat, or meat from the media department after juice has been extracted for media, and milk. Cooked vegetables may be given.

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[3] The value of a synthetic diet such as that now recommended in the Miscellaneous Publication No. 22, U. S. Public Health Service (161) is being tested in a group of rats.

**Supplies.**—Practically all the roots and other green food and most of the grain are produced at the laboratory farm.[4] Farm produce for use at the laboratory is ordered by requisition signed by the head worker. Requisitions for supplies, such as bread and milk, from dealers are placed with the purchasing group.

Root cellars at the farm and the laboratory are provided with bins for carrots and beets, and racks with shelves for cabbage. During the summer months, a freshly cut mixture of green timothy, alfalfa, or clover is brought in daily from the farm. Grass from the laboratory lawns is also utilized.

Rat-feeding shelf: A removable wooden shelf (6 in. wide, 5 in. high) on solid wooden supports extending the length of the compartment is used in rat cages. Each shelf has two holes into which are set earthenware dishes for milk and water.

Water bottles: Glass bottles (10-cc. for test mice; 100-cc. wide-mouth specimen bottles for stock mice and rats and test rats in boxes) with rubber stoppers and straight glass tubes. The inverted bottle rests on the top of the box, the glass tube extending through the wire mesh. (See: plate II.)

Root cutters: Two types. One operated by a ½-H.P. electric motor attached to frame of cutter and entirely enclosed by sheet metal for protection against water; capacity 60 to 90 bu. per hour. The other, a hand-power cutter; capacity 20 to 30 bu. per hour. The root cutters should be hosed sufficiently often to prevent accumulation of dirt, and the machinery kept properly oiled.

Troughs for grain: Troughs of galvanized iron, 2 inches deep, 3 by 8 inches at the top, and tapering at the front and sides to a 2-by-6-inch bottom. The back extends 2 inches above the sides and front. The troughs are hooked or bolted to the sides of the cage.

Troughs for water: Troughs of galvanized iron, 2 in. deep, with a straight back 5½ in. across and a curved front 2½ in. at the maximum curve; attached as above.

**Procedure.**—Feed all animals, except monkeys, once a day and at regular hours. Feed monkeys twice a day. Never give the animals decayed roots or badly wilted green food. Scrub and wash roots, if covered with earth, before putting through the root cutter. Cut only enough roots for the day's rations. Gauge the amount of food to be placed in a cage by the number of animals in it. Always give an ample allowance but avoid unnecessary waste. If wilted vegetables are found in the morning before feeding, reduce the ration until none is found. Renew the supply of grain as exhausted. Wash and refill

[4] At the laboratory farm, which consists of 216 acres, approximately 90 acres are at present under cultivation; about 5 acres for roots and green produce used in feeding the smaller animals, the remainder for hay, grain, etc. A total area of about 50 acres of open pasturage and wooded grazing land is reserved for the large animals.

the water bottles as emptied; when troughs are used for water, clean and refill daily. Renew the salt when required. Always brush the floor of the rooms immediately after feeding.

At the time of feeding, always observe the condition of the animals. If any appear ill, or a dead animal is found, proceed as directed under "Detection and Isolation of Infected Animals," p. 568, or, for test animals, under "Removal of Dead Animals," p. 575.

Animals in isolation quarters or in quarantine should be fed and cared for after the healthy stock animals, or by a worker not in contact with the latter.

### *Detection and Isolation of Infected Animals*

Freedom from epidemics depends largely upon a self-maintaining stock, or, when it is necessary to introduce animals from outside, upon a rigid quarantine of the new stock for a sufficient time to prevent the introduction and spread among the laboratory stock of infection from outside sources. Prompt detection and isolation of such animals and of animals which are suspected of being normal carriers is also of the utmost importance. The following points should be carefully observed.

Proper inspection and complete physical separation of new stock for an adequate period (see "Receipt of Stock," p. 561 and "Quarantine," p. 561). Frequent inspection of all animals both at the laboratory and the farm. Immediate removal of dead animals. Prompt isolation, quarantine, or disposal of animals known or thought to be infected, and quarantine of contacts. (Under certain conditions it may be desirable to destroy the contact guinea pigs by assigning them for immediate bleeding for complement.) Immediate notification of the veterinarian when any unusual condition is observed among either the normal or test animals, particularly if there is any indication that an epidemic is commencing. Thorough disinfection of cages and quarters when required in the judgment of the veterinarian. Disinfection of animal carriers and food receptacles. Protection, as far as possible, of food and bedding in storage from wild rodents.

Caretakers in the normal-animal quarters observe the appearance of the animals at time of feeding. Deaths or illness are reported to the caretaker in charge who is responsible for arranging the proper quarantine or isolation and for notifying the veterinarian when necessary. In the test quarters the groups using the animals are responsible for promptly reporting spontaneous infections to the veterinarian.

### *Disposal of Dead Animals. Post-Mortem Examinations*

A record is kept of deaths among the normal stock. If only occasional deaths in scattered cages occur, the animals may be placed in the incinerator without autopsy. At the first indication of possible spread of infection in one or more cages, careful post-mortem examinations are immediately made by a specially trained and designated worker, to determine the cause of death and whether the deaths may be traced to the same factor, such as a common infective agent. Careful autopsies by an experienced worker are made upon all dead breeding animals, and upon dead animals from new stock held in quarantine. Dead animals to be autopsied are wrapped in paper, labeled with the date, cage number, and source of the animal, and placed in the cold room until the autopsy is made.

Records of all autopsy findings are made on cards. A statement in regard to the number and causes of deaths is included in the general report for each month.

### *Diseases of Small Animals*

A very brief description of certain of the diseases which are apt to occur among the smaller laboratory animals, together with their incitants has been included, since it is important that the workers responsible for the care of the animals or for their use in tests, should have some knowledge of the characteristic symptoms, and the usual lesions which may be met with at autopsy.[5]

**Diseases of Bacterial Origin.**—Acute and chronic respiratory infections are common among all small laboratory animals during the late autumn, winter, and early spring; other infections as those producing septicaemias may occur at any time but are probably also most prevalent during fall, winter, and spring. Some animals are undoubtedly carriers of the incitants of these infections throughout the year. The clinical manifestations of disease in small animals are variable and of uncertain diagnostic significance. Certain incitants are more or less characteristically associated with specific lesions.

*Streptococci:* Hemolytic streptococci have been recovered from most of the guinea pigs dying from pneumonia in this laboratory. At autopsy marked congestion of the whole lung with areas of consolidation may be found. The pleural

[5] For further information see Hutyra and Marek "Pathology and Therapeutics of the Diseases of Domestic Animals" (162); for discussion of certain specific diseases, recent articles such as the studies of *Bact. lepisepticum* and *B. bronchisepticus* by Webster, L. T., Jour. Exper. Med., 39, 837 (163) and later articles in the same journal; of *B. bronchisepticus* and pneumococcus by Smith, Theobald, Jour. Med. Res., 29, 291 (164); of epizootic lymphadenitis of streptococcus origin, by Boxmeyer, Jour. Inf. Dis., 4, 657 (165).

fluid is usually increased and blood stained. Pericarditis is often associated with pneumonia and pleurisy.

Local abscesses of streptococcus origin in the lymph glands (epizootic lymphadenitis) have been prevalent at different times among the guinea pigs. This condition occurs chiefly in the older animals; it has not been found in the very young guinea pigs. It is fatal only when the abscess by pressure or otherwise interferes with the functions of the vital organs. The abscesses, which may reach a considerable size, are encapsulated and contain a cheesy or creamy pus. Recovery may be rapid and apparently complete after external rupture and drainage of the subcutaneous abscesses. Incision with drainage hastens recovery.

*Bact. lepisepticum:* This organism is usually found in contagious nasal catarrh (snuffles) of rabbits. *B. bronchisepticus* is frequently associated with it. The first symptoms are sneezing and a slight nasal discharge, followed by general weakness and depression. There may be a rapid extension of the infection to the lungs with an acute fatal pneumonia; or the infection may follow a more chronic course. Fibrinous or purulent pericarditis and pleurisy may be associated with the pneumonia. Frequently, however, the infection does not extend beyond the upper respiratory tract and the animals recover.

*B. bronchisepticus:* Pneumonia induced by *B. bronchisepticus* is not uncommon in guinea pigs. The symptoms are a moist nose, roughened coat, and rapid breathing. At autopsy marked congestion or consolidation of the median, or lower borders of other lobes, is usually found.

*Microörganisms of the paratyphoid-enteritidis group:* Microörganisms of the paratyphoid-enteritidis group[6] frequently induce severe epizootics among guinea pigs, rats, and mice. Such infections among mice have usually been described under the name of mouse typhoid. The symptoms in guinea pigs are roughened coat, loss of appetite, and emaciation. In acute cases of short duration, symptoms may readily be overlooked. In mice the roughened coat is more noticeable and diarrhea is usually present. Autopsy findings vary with the duration of the disease. An enlarged spleen is the most constant finding; the increase may be slight or the spleen may be double the normal size. Multiple abscesses, usually of the spleen, and resembling the lesions of tuberculosis are occasionally found in cases of long standing. The microörganisms may be recovered from the spleen, peritoneal fluid, and the blood.

*Pneumococcus:* Sporadic cases of pneumonia among guinea pigs due to type-IV pneumococci have frequently been found; in two or three instances type-II pneumococci have been isolated. A fibrinous exudate over the spleen and liver is occasionally present.

*Tuberculosis:* Monkeys and rabbits may become infected and develop a pulmonary or generalized tuberculosis. Monkeys are extremely susceptible. A

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[6] Strains isolated in this laboratory have, with few exceptions, fallen into two distinct groups. One is agglutinated by serum produced against a human paratyphoid B strain in high dilutions, the other, only in very low dilutions. The latter is agglutinated by serum produced against *B. enteritidis*. Each is agglutinated by its homologous serum, but not by serum produced against the other, except in very low dilutions. Polyvalent vaccine prepared with strains isolated in this laboratory and used in the guinea-pig breeding quarters, while apparently effective, requires further trial before its value can be established.

new shipment should be kept under strict quarantine until there is reasonable certainty that none of the animals is infected.

**Parasitic Diseases.**—*Coccidiosis:* Coccidiosis is not uncommon among young rabbits; it may be nasal, hepatic, or intestinal. In the nasal type, the symptoms are sneezing and a mucous discharge from the nose and later, in some cases, an inflammation of the eyes. In coccidiosis of the liver and intestine, emaciation and diarrhea are the most pronounced symptoms. In the hepatic type at autopsy, whitish-yellow nodules are present in the liver; in the intestinal type, inflammation of the small intestine. Diagnosis may be made by finding coccidia in the nasal secretions, the liver nodules, or the feces. When animals are kept in groups, the use of runs with raised wire-mesh floors has been recommended, as effective in preventing the spread of coccidiosis.

*Worms:* Larval forms of the cat tapeworm are found encysted in the livers of mice and rats, and of the dog tapeworm (*taenia pysiformis*) in the liver or mesentery of rabbits. The condition of the animal does not seem materially affected by these forms (bladder worms) unless they are large or are present in large numbers.

*Ringworm:* Ringworm may occur among dogs, cats, rabbits, and mice. The animals are isolated and tincture of iodine or an effective ointment applied to the infested area. Workers caring for infested animals should protect themselves by wearing gloves.

*Mange:* Ear mange, which is not uncommon among rabbits, is caused by a mite (*psorptes cuniculi*). The infestation is confined to the inner surfaces of the external ear, where thick brown deposits filling the ear, are frequently found. The inflammatory processes may extend to the brain and cause death. The animals are isolated, and the ear treated with a solution consisting of mercuric bichloride, 1 part; glycerin, 100 parts; and 50-per-cent alcohol, 200 parts; or a kerosene spray may be used.

Tail mange has occurred among the rats and mice. The brown scabs, often edged with dry blood, appear first at the root of the tail. The scabs when removed leave a raw bleeding surface. Infested animals are destroyed and the cages sterilized.

*Lice:* Under existing conditions, infestation of the smaller animals with lice has never been a problem. When infestation has occurred in larger animals, such as goats and horses, cresol-compound solution has been used.

### *Breeding of Laboratory Animals*

Competent workers under the worker in charge are directly responsible for breeding of laboratory animals. All procedures connected with the breeding are closely supervised by the veterinarian. The present breeding stock has been developed from a limited number of carefully selected animals. With the special attention which has been given to mating, inbreeding has apparently not proved harmful.

At present, metal cages No. 2 are used for breeding rabbits; wire cages No. 1, with double trays, for guinea pigs; metal cages No. 4 for rats and mice. Water is provided for all breeding animals. A cage

record is kept on a ruled tag, attached to each compartment, of the number of births, deaths, and removals, and of the total number of animals in the compartment. In addition, a daily room record is kept of the total number of receipts, removals, births, and deaths. Cages are examined and the number of births estimated at from 5- to 7-day intervals. The births are entered as occurring on the day the examination is made.

**Rabbits.**—Period of gestation about thirty days. Average litter five or six young, though larger litters are not rare. Usually three to four litters are obtained in a year. Mate rabbits for the first time when six to ten months old. Keep each doe and each buck in a separate cage. Transfer a buck to a cage with a doe only long enough for mating. Provide plenty of hay for the nest. Do not disturb the young, as the litter may be destroyed by the mother, if handled. Remove the young about four weeks after birth and place the males and females in separate cages. Transfer breeding bucks and does to stock for laboratory use when from two to two and one-half years old.

**Guinea Pigs.**—Period of gestation sixty-eight days. Average litter two to three young; the first litter generally consists of one or two. Four to five litters are obtained in a year. Mate guinea pigs for the first time when from four to six months old. Keep one male with from five to seven females. Remove young when from about four to five weeks old and place males and females in separate cages. Select from these cages animals to be reserved for breeding. The other animals may be held in the breeding quarters until distributed for use or transferred to the stock quarters. Transfer old breeders (two and one-half to three years old) to stock for laboratory use.

**Mice.**—Period of gestation twenty-one days. Average litter from six to seven young. Six or seven litters may be expected each year. Mate the females for the first time when from two and one-half to three months old. Place two males in a cage with from twelve to sixteen females. Remove the young when about three to four weeks old and separate according to sex. Transfer old breeders (one to one and one-half years old) to stock.

**Rats.**—Period of gestation twenty-one days. Average litter from six to nine young. From five to seven litters may be expected from one breeder. (The reproductive function starts to decline when a rat is about twelve months old, and usually ceases at about the fifteenth month.) Breed for the first time at from three and one-half to four months. Place one male with from one to three females or two with from four to seven. Remove the young when from two and one-half to three weeks old and separate according to sex.

**Deaths among Breeding Stock.**—Special attention is given to evidence of illness or deaths occurring among the breeding animals (both breeders and young) and any increase beyond the occasional deaths which are to be expected, is reported immediately to the veterinarian. Two deaths occurring within a short time among animals in the same cage, or a number of deaths confined to animals in a few cages should be investigated for an infectious origin. In outbreaks of infection, the

pregnant animals are usually the first to die. Special note should be made of abortions and of the young dying soon after birth.

*Distribution of Animals for Laboratory Tests*

The animals to be used for experimental and test purposes are supplied by the worker in charge of animals to the various groups, on requisition. Signed requisitions giving the date, kind, number, and weight of animals, proposed test (when advisable), and day required, are collected from the baskets in the main office at 8:00 a.m. and 1:00 p.m. of each day. Orders for animals are usually placed one or more days in advance. Emergency orders must be filled with the least possible delay. Abuse of this service is reported to the veterinarian. Metal boxes, No. 7, are used for transporting the animals.

Keep stock animals from different sources separate, and, for ease in filling orders, make divisions according to weight as advisable. When requisitions specify definite weights, weigh each animal. Maintain as far as possible an adequate supply of animals of the weights usually required. Fill orders not requiring specified weights with animals of such weights as are in least demand, for example mice weighing over 22 grams.

Routine laboratory procedures requiring animals of definite weights are:

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| Standardization of diphtheria toxin, antitoxin, and toxin-antitoxin mixture, and botulinus toxin and antitoxin (subcutaneous tests) | Guinea pigs from 230 to 280 grams |
| Standardization of diphtheria toxin and antitoxin (intracutaneous tests) | White guinea pigs from 350 to 450 grams |
| Diphtheria virulence test (subcutaneous tests) | Guinea pigs from 250 to 350 grams |
| Diphtheria virulence test (intracutaneous tests) | White guinea pigs over 350 grams |
| Standardization of tetanus toxin and antitoxin | Guinea pigs from 330 to 380 grams |
| Complement-fixation tests | Guinea pigs over 600 grams,[7] used or normal |
| Tests for harmlessness of biologic products | Guinea pigs not less than 300 grams, preferably over 350<br>Mice preferably 22 grams or over |

[7] It is frequently desirable when a surplus supply of used guinea pigs of lighter weight is in stock to supply these animals for immediate use. When it is necessary to purchase animals, guinea pigs weighing between 600 and 800 grams are usually obtained.

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| Arsphenamine toxicity tests | White rats from 100 to 150 grams |
| Production of immune sera | Rabbits, usually from 1800 to 2400 grams |
| Pneumococcis-type-differentiation tests | White mice 16 grams or over |
| Standardization of antipneumococcus serum and pneumococcus and streptococcus virulence tests | White mice from 16 to 22 grams |

Three types of scales are used for weighing laboratory animals: (1) Chatillon spring balance with removable pan above dial; in three sizes, 1 to 60 grams; 1 to 210 grams; and 1 to 500 grams. (2) Troemer scale No. 80, 1 gram to 20 kilograms. (3) Toledo springless scale No. 4621, graduated on chart to 500 grams by 5, and on tare beam to 2 kilograms by 10 grams. The smaller scales should be tested for accuracy from time to time.

In general, fill an order with animals from one source only. If an order is filled with animals from different sources, place in separate boxes. Never put more than one order in a box. Enter the source and the number of the group requisitioning the animals on the box tag. Before animals are taken from the stock-animal quarters, enter on the daily-report sheet the kind and number of animals distributed, together with the group and requisition numbers. Record the date of filling on the requisition and send to the purchasing group to be filed.

## ANIMALS UNDER TREATMENT

Animals under treatment are kept during the tests in special quarters apart from the normal stock. A trained worker with the necessary assistance is responsible for the care of the animals, the animal quarters, and the adjacent operating rooms.

### *Receipt of Animals*

When the animals reach the test quarters, place the box containing them in the receiving section and notify the group ordering the animals.

The group is responsible for transferring rabbits and guinea pigs promptly to cages, and for making sure that all animals have food if they are not placed in the sections for test animals until after the last regular feeding of the day.

### *Assignment of Cages and Identification Tags*

Guinea pigs and rabbits are kept in cages (No. 4 or 5) and, for certain tests, in large metal boxes; rats, in boxes; and mice, in boxes or glass jars. (For description see p. 563.) Blocks of cages for test rabbits and guinea pigs are assigned to the different scientific groups

and to special research workers according to their requirements. One section is reserved, if possible, for use by workers whose assignment may be temporarily inadequate, or for special investigations. A list of the assignments is posted.

Attached to each cage containing guinea pigs or rabbits is a separate manila tag ($4\frac{3}{4}$ by $2\frac{1}{2}$ in.) for each animal, or one tag for all the animals, giving the date and purpose of the test (virulence test, tetanus antitoxin, etc.), ear-tag number, number of the group, and name of the worker responsible for the animals and, when desired for reference, the source. As a further means of identification, a brief description of the animal may also be given. (Cages containing guinea pigs to be bled for complement have only one tag giving the purpose for which the animals are used and the group and worker responsible.) Tags which have become soiled or erased must be replaced. Failure to comply with these rules should be reported.

An identifying number tag is placed in the ear of each test rabbit or guinea pig, except those bled for complement. One or more series of one hundred numbers (1 to 100; 101 to 200; etc.) are assigned to each group by the worker in charge of the animals.

Attached to each box or jar containing mice or rats is a tag giving the purpose of the test, date, and name of worker. (These animals are identified by marking different parts of their bodies with stains.)

### *Care of Animals*

The worker in charge of the test animals is responsible for the general care of the animals (feeding, cleaning of cages, etc.) unless special directions are given.

### *Removal of Dead Animals*

When feeding the animals on Sundays and holidays, remove the dead animals unless otherwise directed. Place dead rabbits and guinea pigs in a can in the cold room. Enter on the cage tag the date and hour found dead, and initial. Should the ear tag be missing, wrap the animal in paper and write the cage number and name of worker responsible on the wrapper. Wrap together dead test mice or rats from the same box; and write on the paper the date and hour found dead, number of the box, and name of worker responsible; initial and put in the cold room. A second can is reserved for mice.

Never remove dead animals from cages to which a red tag is attached, but when requested, record on the animal tag the hour at which the animal is found dead. Report animals found dead in cages not under

close observation to the group responsible for them. Inspect the cans for dead animals in the cold room frequently; clean and disinfect at least twice a week. Notify the veterinarian if dead animals are not removed promptly from the cans.

At night the night watchman, when so directed, inspects specified cages and removes dead animals. When inspection by the night watchman is desired, the compartment or box is marked with a green tag and a note giving the compartment or box number is left for him. In the case of sections where regular nightly inspections are required, the note is omitted. Unless otherwise directed, the night watchman makes two inspections. During laboratory hours, inspections are made and dead animals removed by the groups responsible for them.

### *Discharge of Animals*

As soon as a test is completed, the compartment or box containing the surviving animals is marked with a tag (later attached to the box in which the animals are transferred) on which are entered "Discharged" in red, and the date of discharge, number of animals (or ear-tag number of each animal), purpose for which they were used, and number of the group, and initials of the worker by whom discharged. The same information is entered on a requisition and sent directly to the worker in charge of the small animals. Remove discharged animals within twenty-four hours, except on Sundays and holidays, to designated cages in the used animal quarters. (A designated worker must see personally that the used animals are placed in the proper cages.) Report to the head of their department, workers who fail to discharge their animals after a test is completed.

### *Reassignment and Quartering of Used Animals*

Animals which have survived treatment may be used again for certain tests or experimental purposes. The use of such animals should be encouraged.

Keep the animals in clearly marked cages. Divide the guinea pigs when directed (on the basis of their previous treatment) into groups for reassignment. Used guinea pigs are supplied mainly to the complement-fixation group. They may, however, at times be used for other purposes. Before redistributing guinea pigs, remove the ear tags. Return the tags to the groups to which they belong, after sterilizing them in 1-per-cent crude cresol.

Place discharged rabbits together, leaving the identification tags in the animals' ears, and keeping the record of their previous use. When

an animal is reassigned or dies, return the ear tag (after sterilization) to the group to which it belongs.

Hold rats which have been used once for testing arsphenamine, since at the end of two weeks they may be redistributed for a second test when called for. Unless otherwise directed, chloroform rats which have been used twice for arsphenamine tests. Dispose of rats used for other purposes as directed.

### *Care and Use of the Operating Rooms*

The operating rooms adjoining the quarters for experimental and test animals are under the charge of a trained worker, supervised by the head worker. Cleanliness and order must be maintained in these rooms.

Keep the operating-room equipment in good condition, and the necessary supplies properly replenished. See that separate cans with closely fitting covers are provided for dead animals and for waste paper, etc., and kept covered. Empty these cans daily and keep them properly cleaned. Keep the instruments for special work in satisfactory condition for use. At the end of each day, brush or, if necessary, mop the floors and wipe table tops with a cloth moistened with 1-per-cent crude cresol. Clean the floors during the day if necessary. Mop or scrub the floors and thoroughly clean the rooms at least once each week.

The special instruments kept in a locked cabinet may be obtained for special procedures from the worker in charge of the operating room. They are not for general use, each scientific group being provided with its own instruments.

Workers using the operating rooms are expected to clean and disinfect operating trays, bleeding boxes, tables, etc., and to return bottles, trays, and other equipment to their proper places; to place dead animals (wrapped in paper and tied) and all waste, such as paper and cotton, in cans provided for the purpose, and to replace covers securely on the cans. Refer to the head of their department continued failure of workers to observe these directions. (See "Care of Operating Room and Equipment," under "The Use of Experimental and Test Animals," p. 44.)

The worker in charge of the operating rooms is qualified to assist in the procedures of routine inoculation, bleeding, and autopsy, so that he may be called upon when other assistance is not available.

### *Permanent Records*

Laboratory animals report (daily): A daily record (8 by 11 in., mimeographed) is kept of the numbers of guinea pigs, rabbits, mice, and rats, received from dealers and from farm, born, returned by staff, and total number of each kind added; numbers distributed to staff with group and requisition numbers for each

order, numbers sent to farm, died, destroyed, and total number of each kind removed.

Laboratory animals report (monthly): A monthly report (8 by 11 in., mimeographed) gives the numbers of normal and used guinea pigs, rabbits, mice, rats, and monkeys, and of sheep, goats, horses, and other animals; on hand at beginning of month, received from dealers, and from farm, born, returned by staff, and total number of each kind added; numbers distributed to each group and total to groups, numbers sent to farm, died, destroyed, and total number of each kind removed; balance; total number on hand by count and number unaccounted for. The use to which each horse and goat is assigned is given.

Farm report—small animals: On a monthly report are entered daily, the numbers of rabbits and guinea pigs and of other small animals, when kept; received, sent to the laboratory, and died. At present, the number of each kind born is determined by subtracting the number on hand at the beginning of the month from that at the end of the month plus the numbers distributed and died.

## CHAPTER 2

## CARE AND TREATMENT OF ANIMALS—LARGE ANIMALS

In this chapter are described procedures connected with the receipt and care of horses used for the production of antitoxins and other immune sera and the different methods of injection and bleeding. At the end of the chapter brief reference is made to the care and treatment of cattle, sheep and goats.

The horses are kept in stable units at the laboratory, and at the laboratory farm.[1] In general, animals receiving bacterial suspensions and whole cultures, and those used in connection with special studies, are quartered in the laboratory stables. Horses undergoing immunization against bacterial toxins such as diphtheria and tetanus, horses bled for normal serum and whole blood, and resting horses, are usually kept at the farm.

### *Horses Used. Sources from which Obtained*

It is essential that the horse be healthy and in good condition. The breed of horse is apparently of no special importance. Vigorous animals not more than from ten to twelve years old and weighing 1100 pounds or more are preferred. Horses belonging to the state police, which have become footsore or in some way disqualified for saddle

[1] The stable units at the laboratory, built of brick, accommodate from twelve to sixteen horses each. A number of box stalls, including several with Dutch doors opening on the outside of the building, are provided, as well as the straight stalls. The stalls are paved with wooden blocks, and each is furnished with a galvanized-iron combination hay and grain manger and a water bucket. A large operating room with smaller adjacent rooms is at the end of each unit. Concrete floors are used throughout. The stalls and operating rooms are drained by sewage connections. A loft for storage purposes, with concrete floor, extends the length of the unit. A basement under one of the units provides cold-storage facilities and a cellar for roots.

The present buildings at the farm consist of remodeled wooden structures which had already been built when the land was purchased. The facilities at present afforded necessitate some modification of a few of the procedures prescribed in the following methods. Plans for new construction at the farm are now being prepared.

Separate quarantine and isolation quarters are provided at the laboratory and farm. A paddock and two or three smaller enclosures are available at the laboratory; ample pasturage and several small paddocks at the farm.

purposes, but which are otherwise healthy, are obtained at a nominal charge. Animals may occasionally be secured from the state militia by transfer without cost on approval by the adjutant general. Purchase of horses from other sources is at times necessary. Mules also have been used for the production of antitoxins and diagnostic sera and have been found satisfactory.

*Method of Identification*

Horses are numbered consecutively in the order of their receipt, the number being branded on the right forward hoof. A description of the horse with its number is sent promptly to the veterinarian for entry on the horse record card.

*Quarantine*

New horses are given a thorough examination by the veterinarian and quarantined under observation for at least two weeks before being placed with other horses, or used for immunization. Should symptoms at any time develop suggesting infection requiring isolation, the animal is quarantined immediately and rigid precautions against spread of infection are observed.

*Mallein Test for Glanders*

Each new horse is tested by the ophthalmic test at once. All horses are tested every twelve months, or more often if indicated. A doubtful reaction is followed as soon as possible by the subcutaneous test, the horse meanwhile being isolated. If a horse is shown by the ophthalmic or subcutaneous test to have glanders, it is at once destroyed and a careful post-mortem examination is made.

Mallein is obtained from the Bureau of Animal Industry, State Department of Farms and Markets. Reports of all tests are made to the Bureau on special forms which it provides.

**Ophthalmic Mallein Test.**—The test is made by inserting mallein into the eye by means of a camel's hair brush. The left eye, if not inflamed, is used for the test, the right as a control. Reactions, indicating the presence of glanders, may appear in five or six hours and last from twenty-four to thirty-six hours. Such reactions consist of an inflammation of the conjunctiva with a discharge varying in character from a seromucous to a distinct purulent discharge gluing the eyelids. For detailed information see U. S. Department of Agriculture, Farm Bull. 166; N. Y. State Department of Agriculture, Regulations for Ophthalmic Test.

The brushes for the tests are placed in test tubes and sterilized in an autoclave. Immediately after use they are returned to the tubes, and later placed

in 2-per-cent cresol compound solution, then rinsed thoroughly in water and dried.

**Subcutaneous Mallein Test.**—Following the subcutaneous injection of a suitable comparatively small dose of mallein, horses infected with glanders show a rise of body temperature which begins in from four to eight hours and rapidly increases for from eight to fourteen hours, after which there is a gradual return to normal. Local and general reactions may frequently be observed. For a description of the subcutaneous test see "Pathology and Therapeutics of the Diseases of Domestic Animals," Hutyra and Marek. Reports of the subcutaneous test are made to the Bureau of Animal Industry, State Department of Farms and Markets, as in the case of the ophthalmic test.

A local and general reaction without increase in temperature indicates the presence of glanders. Animals showing atypical reactions and a temperature not above 103°F. are retested at the end of fifteen days or preferably at the end of one month. Two suspicious tests are regarded as a positive reaction. A subcutaneous test is not made on any animal showing symptoms of an acute disease or with a temperature above normal.

### *Prophylactic Injections of Tetanus Antitoxin*

All horses which are being injected or bled, except those actively immunized against tetanus toxin, receive intravenous injections of 2000 units of tetanus antitoxin every month. In order to avoid giving the dose between regular injections and bleedings, some leeway in the interval between the prophylactic doses is necessary, however. Tetanus antitoxic serum from horses whose plasma is considered to have an antitoxic content too low for concentration, or recently outdated antitoxin which has been returned from supply stations, is used for this purpose. See "Production and Standardization of Tetanus Antitoxin," p. 359.

### *General Care of Horses*

**Care of Stables.**—The stables must be kept clean and in good order. They should be kept properly ventilated but the horses should not be exposed to drafts. A temperature of about 60°F. should be maintained in winter.

Clean the stables thoroughly each morning. Clean the stalls once or twice during the day depending on the length of time they are occupied. At least once each week scrub and hose the floors, using hot water when advisable. Observe special precautions under the direction of the veterinarian when there is possibility of transfer of an infection.

**Feeding of Horses.**—In general, give as the daily forage ration for each horse: 12 pounds oats, 1 pound bran, 11 pounds hay and 3 pounds

straw. (Daily feedings of bran may be omitted and the total amount combined in one or two feedings each week. Occasionally oats may be replaced in part by corn.) Provide hay three times a day, a small feeding in the morning and at noon, and a large feeding at night. Give grain in the morning and at night only, except when the horse has been working or its general condition makes a noon feeding advisable. Omit the noon feeding when animals are pastured during the day. Horses kept in pasture may or may not require additional feeding, depending on the pasturage and the condition of the animal. Water the animals at least twice a day. If a constant supply of water is kept in the mangers, change it at regular and frequent intervals. Keep salt in brick form in the stalls and pasture.

**Air and Exercise.**—Let out the horses for at least four hours each day in a paddock or pasture where they may exercise. When the weather is unsuitable, this period may be reduced or omitted.

A moderate amount of work (saddle or harness) when intelligently given, is often of benefit, but the fact that the horse is primarily a producer of immune serum should never be lost sight of. No horse under immunization is permitted to do heavy work. When horses are given light work or otherwise exercised, the time in the paddock may be reduced or omitted.

**Daily Care and Observation of Horses.**—Take the temperature of each horse undergoing immunization every morning before watering. Enter immediately all temperatures as taken, upon a daily record form from which they are copied to the individual horse charts. When the horses are curried and brushed each morning look for bruises, foci of infection, or other conditions requiring treatment. Report immediately to the veterinarian, and to the group having the horse under immunization, abnormal temperatures, lameness, loss of appetite or weight, and other abnormal conditions or symptoms.

### *Assignments and Transfers*

Horses are assigned to groups requesting them by the veterinarian on approval by the bacteriologist in charge of the department. A signed duplicating order form is sent to the head of the group giving the number of the horse, the date available, and a record of previous treatment if any has been given. A detailed description of the horse for the horse protocol is obtained by the worker from the horse-record card kept by the veterinarian. When immunization of a horse is to be discontinued, a signed memorandum is sent to the veterinarian.

### *Immunization of Horses*

Toxins, cultures, or other antigens used in the production of immune sera are provided by the scientific groups responsible for the production of the sera. The schedule of immunization and bleeding is arranged by these groups. The technic of injecting and bleeding, etc. is given in this chapter, p. 585. Frequent examinations of all horses undergoing active immunization are made by the veterinarian. Any unfavorable symptoms or changes in the condition of a horse together with special examinations or treatments are reported promptly to the production group. Cultures of blood or exudates taken from horses undergoing active immunization are referred directly to the production groups for bacterial examination.

A separate schedule for each horse undergoing immunization against toxin is received shortly after the first of each month. (The schedules usually cover one week of the following month so that the results of trial bleedings taken during the month may be available before the new schedule is prepared.) The schedule on a duplicating order form gives the date, horse number, dates on which injections are to be given, amount of each injection, and dates of bleedings. It is filed with the temperature chart. (See "Permanent Records," p. 597.) The toxin is usually received in 4-liter bottles, each of which is accompanied by a separate duplicating order form giving the date of shipment; kind, lot number, titer, and volume of toxin, and a list of the horses for which it is to be used. The same data are given on the tag on the bottle.

For horses receiving whole culture or suspension of organisms, no general schedule is prepared. The antigens for single injections are received with duplicating order forms giving the date, horse numbers, kind or kinds of culture or toxin, number and size of doses. (In the case of killed organisms, the antigen and directions for several successive injections may be received at one time.) Orders for trial and whole bleedings are also given on these forms. The kind of organism and horse number are given on the label on the culture.

### *Temperature Reactions*

Rectal temperatures of all horses undergoing immunization are taken every morning before the animals are given water. In the case of a horse receiving bacterial toxins, the morning temperature is the only temperature taken, except when the horse requires special observation. In the case of a horse receiving intravenous injections of bacterial suspension or whole culture, the temperature is taken from three to five hours after every injection. The hour at which the temperature is to be taken is based, as far as possible, on that at which the highest temperature is obtained at the time of the first two or three series of injections, when temperatures are taken hourly from the

third hour until the decline commences. The temperatures following inoculation and the morning temperatures of horses receiving cultures are reported each morning to the production groups, so that the next doses may be changed if necessary. Any abnormal condition must be reported at once to the production group and to the veterinarian.

### *Rest Periods*

It is occasionally desirable to discontinue immunization for from one to three months. Before resting a horse, it should be decided whether the horse's general condition and the potency of the serum warrant the assumption that his later productive power will compensate sufficiently for the loss incurred during the periods of rest and reimmunization, or whether it is advisable to obtain all serum possible by bleeding out, or by taking two or three consecutive bleedings before holding the horse for reassignment after a period of rest. As a rule, while a horse is producing potent serum, injections and bleedings are continued, barring special disabilities, until two or more tests have shown a considerable decline in potency. Since, however, a horse producing potent serum can be rested and on reimmunization produce equally potent or only slightly lower material, it may at times be desirable to rest a horse producing potent serum. Thus it is usual to discontinue treatment of most of the horses at the laboratory units during part of the summer and to pasture them at the farm. Occasionally an interval of enforced rest may be necessary owing to an infection or accidental injury. It may be found advantageous to extend such an interval into a regular rest period.

### *Disposal of Unproductive Animals*

Horses which are not producing serum equivalent to the cost of their keep and which will be of no value on the farm or for special experimental work, are destroyed without unnecessary delay, usually by exsanguination while completely anaesthetized with chloroform.

### *Post-Mortem Examinations*

Autopsies are made by one of the veterinarians on all horses.[2] Gross specimens and tissues for microscopic sections are taken when of special interest; slide preparations and cultures are made whenever there is any evidence of an infectious process. Tissues for micro-

[2] Detailed procedures of post-mortem examinations are given in "Veterinary Post-Mortem Technic," by Crocker, W. J. (1918), Lippincott and Company (166).

scopic examination and gross specimens are treated according to methods given in the chapter "The Anatomic Examination of Tissue," p. 211. In the case of horses undergoing active immunization, the cultures and slide preparations are referred to the production groups. In the case of other horses these examinations are made by the veterinarian. The detailed findings are entered on a separate "Horse-autopsy card" (4 by 6 in.) and referred to the production group for entry on the horse protocol, after which the card is filed with the veterinarian.

The extent of an autopsy depends largely on the previous history of the horse, but an examination is always made of both body cavities. When the horse has received injections of living cultures of pneumococci, an especially careful examination is made of the organs of the pleural cavity, particularly the heart; when diphtheria toxin, of the peritoneal cavity, especially the liver and kidneys. When the horse has been injected with botulinus toxin the brain is removed and examined.

## METHODS OF INJECTION AND BLEEDING

### *Care of Operating Rooms*

Keep the operating rooms scrupulously clean and free from unnecessary apparatus and equipment. Make every effort to exclude flies. Clean the rooms thoroughly and hose and brush the floors at the end of each day's work. Use hot water when available, first removing blood, if present, with cold water. In the morning before work is commenced, again wipe off the table for supplies with a dampened cloth. When live cultures are injected, place contaminated apparatus immediately in the proper receptacles for sterilization, and use disinfectant on tables and floors as required. Always remove fecal and other matter deposited on the floor or in the stocks as soon as possible. Wear operating gowns, and clean the hands thoroughly. Prepare toxins and cultures, and the instruments and apparatus requiring sterilization, in the adjacent laboratory rooms.

The necessity for extreme care in handling material containing live pathogenic organisms must be impressed upon all workers in the operating rooms.

### *Preparation of Apparatus*

**Pipettes.**—Sterile pipettes are obtained as required from the media department.

**Syringes.**—Record or Luer syringes, 10 and 20 cc., are used for injecting small doses. Syringes and needles are sterilized by boiling. For cleaning and care, see "Use of Experimental and Test Animals," p. 44.

**Injecting Bottles.**—500-cc. bottles, graduated in 25 cc., are used in injecting, by positive pressure, toxin in doses of more than 40 cc. The bottle is closed by a

3-hole rubber stopper through which pass two glass tubes bent at right angles and a short straight tube. One tube, reaching just below the stopper, is for connection with (a) the stock-toxin bottles when the injecting bottle is being filled, or (b) with the pressure pump when the material is being injected. The second tube reaching almost to the bottom of the bottle is fitted with a 15-inch piece of pressure tubing into the end of which a needle (17 gauge, 2½ in.) is inserted and wired. To the third tube is attached a 2-inch piece of rubber tubing with clamp to allow the escape of air while the bottle is being filled. The bottles and attachments are sterilized in the autoclave or are boiled just before they are used.

**Metal Clamp.**—A screw clamp with two hinged arms is used to hold the stopper of the injecting bottle in place. On bottles with insufficient flange to hold the clamp, a metal collar is added.

**Pressure Siphon Apparatus for Toxin Bottles.**—The siphon apparatus consists of a 2-hole rubber stopper through which pass: a short bent glass tube to act as an air inlet, fitted with rubber tubing and attached clamp to connect with the Woulff bottle; and a long bent glass tube reaching nearly to the bottom of the bottle, through which the toxin is forced out, also fitted with rubber tubing and clamp to connect with the injecting bottle. The siphon apparatus is rinsed with water and boiled for fifteen minutes before being placed in a fresh bottle of toxin.

**Woulff Bottle.**—The air from the pressure pump is passed through a Woulff bottle half filled with 5-per-cent phenol solution before entering the toxin bottles. (For preparation see "Preparation of Biologic Products for Distribution," p. 529.) In addition to the inlet and outlet tube a short straight piece of glass tubing fitted with rubber tubing and clamp to allow the escape of air is inserted. When not in use, the ends of the inlet and outlet tubes are covered with rubber caps boiled and kept in 1-per-cent crude cresol.

**Apparatus for Intravenous Injection by Gravity.**—A special graduated cylinder (25, 50, or 250 cc.) or a glass funnel is used. It is connected with a needle (15 gauge, 1½ in.) by means of two 6-inch lengths of rubber tubing, joined by glass tubing which serves as a window. Sterilize in the autoclave the cylinder with rubber and glass tubing attached, and boil the needle and rubber connection for ten minutes just before using, or assemble and boil the whole apparatus just before using.

**Sterile Cotton Suction Filter.**—Used for filtering whole cultures: (For preparation, see "Production of Antipneumococcus Sera," p. 414).

**Bleeding Jars.**—Used for receiving blood for serum: Two sizes of Pyrex battery jars (diameters 6½ and 7 in., and heights 8½ and 10 in. respectively) to contain 3 and 5 liters of blood, are used. A tightly fitting felt band is adjusted around the top of the jar, over which fits a metal cover with a 1-inch overhang. The top of the cover has two holes, in one of which is fitted a rubber stopper with short glass inflow tube to which the tubing from the cannula is later connected. A flat octagonal weight of tinned copper, weighing about 550 or 750 grams, with ½-inch teeth on the lower surface, is suspended below the cover by a peg with eyelet extending through the second hole and held in place by a spring, brass, cotterpin. To release the weight, the pin is withdrawn by means of a string attached to it which extends below the edge of the heavy brown paper covering the top of the jar. The glass inlet tube projects through the paper and is protected by a cotton and a paper cap. (See: diagram of bleeding jar, fig. 12.) Orders for bleeding jars should be placed at least two days in advance.

**Bottles Containing Citrate Solution for Plasma.**—Used for receiving blood for plasma: Four- and eight-liter bottles with the required amounts of 17-per-cent sodium citrate ($(Na_3C_6H_5O_7)_2 \cdot 11H_2O$) solution, are prepared by the media department. The volume of citrate solution, usually 222 cc. for a 4-liter bottle, 777 cc. for an eight, is given on the tag.

**Bottles for Defibrinated Blood.**—500-cc. wide-mouth bottles containing ¼- to ½-inch pieces of glass rod; fitted with a 2-hole rubber stopper carrying an inlet tube with a needle (15 gauge, 2½ in.) and rubber tubing attached, and a cotton-plugged air-exhaust tube. The needle is placed in a cotton-plugged test tube which is tied to the side of the bottle. These bottles are prepared and sterilized by the media department.

**Siphon for Drawing off Blood.**—The siphon consists of a 12- to 18-inch piece of glass tubing with a sharply bent arm to which is attached about four feet of rubber tubing. The other end of the glass tube, which is inserted into the material to be drawn off, is bent slightly so that the opening can be directed to the side of the jar. The other end of the rubber tubing is attached to one of two glass tubes which pass through a rubber stopper of the proper size for the receiving bottle. The second glass tube in this stopper, bent and plugged, is used in applying suction to start the siphon. The siphons are assembled and sterilized by the media department, wrapped separately, and sent to the operating rooms in metal boxes; or, if necessary, are assembled and boiled immediately before use.

**Wooden Boxes for Shipping Bottles and Jars.**—Made by Fred C. White, Inc., New York City: Wooden boxes (8½ by 8½ by 16½ in. inside dimensions) with hinged covers which fasten, and reinforced corners to each of which is attached a spring compressor to hold the bottle in place. Boxes (19 by 19 by 14 in. and 20 by 20 by 15 in.) of the same general design, each arranged to carry four bleeding jars.

**Miscellaneous Equipment.**—Cannula (5 mm. bore and 75 mm. long) made by George Tieman & Co., New York City, according to special design. Needles (Nos. 4 and 8, half curved) and thread (Barker's 3-cord white linen thread, No. 25). Scissors, razor blades, scalpels, etc.

### *Preparation of Animal*

As a rule a horse is not placed in pasture nor exercised before an injection or bleeding. It is advisable not to give food for a period of four hours before bleeding. If a horse scheduled for a bleeding or an injection shows an abnormal temperature or other unusual condition, the fact is reported to the production group. Horses with a temperature over 101°F. are not bled for therapeutic serum.

Prepare the horse by brushing down and if necessary wipe or scrub with 2-per-cent cresol compound, before taking him into the operating room. Lead the animal into the stocks and tie the halter to a post in front and at the right of the stock in such a way as to give the head some freedom. If necessary, fasten the restraining strap over the back and a rope over the withers. Avoid the use of a nose twitch as far as possible. To avoid error, read aloud the horse's number from the direc-

tion slip, after the assistant has read aloud the number on the horse's hoof.

Stocks: The stocks are similar to those used at the New York State Veterinary College at Cornell University with the addition of a head post at each end and a reinforced concrete curb between the side posts. Constructed of 3-in. iron pipes (3½ in. outside diameter), they are 6 ft. (9 ft. including head posts) by 3 ft. by 8 ft. 5 in. high. A horizontal bar 3 feet 4½ inches from the floor connects the side posts on each side. Each of the upright posts extends 3 ft. into concrete below the floor level. Rings (⅜ in. iron, 2½ in. in diameter) are attached to the top horizontals; 6-in. cleats, to the uprights, middle horizontals and curbs. (See plate XII.)

### SUBCUTANEOUS INJECTIONS

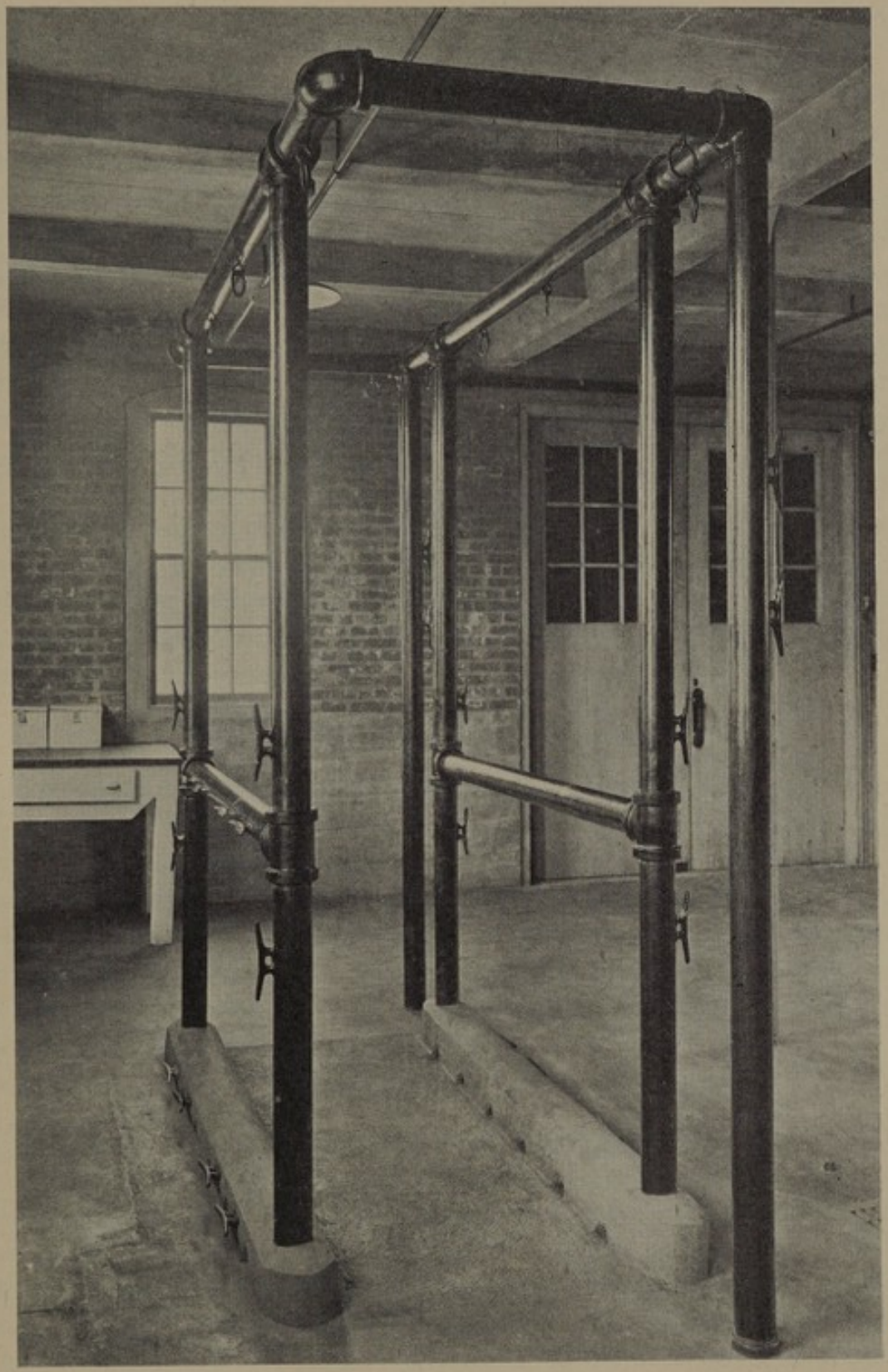
Routine injections of bacterial toxins such as diphtheria, tetanus, and botulinus are given subcutaneously for purposes of active immunization. Occasionally the same method is used for injections of cultures or desensitizing doses. To avoid mixtakes, the number on the toxin bottle is read aloud by one worker and checked by a second worker who reads aloud the number on the direction slip.

#### *Preliminary Treatment of Toxin*

**Small Volumes.**—For doses of less than 40 cc., pipette under aseptic precautions the required volume of toxin directly from the bottle into a sterile container. Then draw the toxin into a sterile, graduated syringe. When the dose is less than 20 cc., make up to that volume with sterile 0.85-per-cent salt solution.

When antitoxin is mixed with the toxin, as in the preliminary doses of tetanus toxin, pipette or siphon the specified volume of toxin into a sterile container, and add the required volume of antitoxin. Allow the mixture to stand at room temperature in the dark for one hour before injecting.

**Volumes Greater Than 40 cc.**—For volumes greater than 40 cc. use a sterile 500-cc. graduated bottle (injecting bottle, p. 585), and inject by means of positive pressure. With aseptic precautions, insert a siphon with stopper and connections in the stock bottle of toxin, covering the stopper with cotton dampened in 1-per-cent crude cresol, and tying it securely in place. Keep the same siphon in a bottle until the toxin is exhausted. Should a siphon be transferred to another bottle, rinse it with water until clean and boil for fifteen minutes. Bring the stock bottles of toxin from the cold room as needed, avoiding unnecessary exposure of toxin to light and heat. Before using, ex-



Stocks for Large Animals

amine carefully for evidence of contamination. (Small transparent crystals apparently of a phosphate nature may be disregarded.)

Assemble and boil the injecting bottle and siphon connections if not already sterilized in the autoclave. Insert the stopper firmly and clamp it in place. To transfer the toxin, connect the rubber tubing on the inlet tube of the toxin bottle with the outlet tube of the Woulff bottle, and that on the outlet tube of the toxin bottle with the inlet tube of the injecting bottle. Connect the Woulff bottle with the pump. After making sure that the air outlet of the Woulff bottle is closed and that of the injecting bottle open, force air into the toxin bottle thus driving the toxin into the injecting bottle. When the required volume has passed over, open the air outlet on the Woulff bottle, and close that on the injecting bottle. Keep the Woulff bottle connected until all the doses required during the day have been siphoned from the stock bottle. After disconnecting wipe and protect with sterile cotton moistened in 1-per-cent crude cresol the free ends of tubing attached to the toxin bottle and insert each in a sterile, plugged test tube (180 by 25 mm.). Cover the end of the outlet and inlet tubes of the Woulff bottle with rubber caps.

*Preparation of Site for Injection*

Avoid areas over the scapula, too near the vertebral column, too low, and further back than a line leading downward from the last dorsal vertebrae. After cleansing the sides of the neck and body with 2-per-cent cresol compound, prepare areas about $1\frac{1}{2}$ inches square on each side, the number depending on the amount of toxin to be given. Scrub and shave the "sites," then apply 70-per-cent alcohol, followed by tincture of iodine. (A safety-razor blade with dulled corners may be used for shaving.)

*Injection of Toxin*

Connect the pump to the inlet tube of the injecting bottle. Take the bottle in the left hand, and with the right, grasp the rubber tubing just back of the needle in such a manner that it will be easy to stop the flow of toxin by doubling the tubing. Hold the needle over a waste receptacle while the assistant forces air through the pump until a steady flow of toxin is obtained. Stop the flow by sharply doubling the tube in the right hand; give the bottle to the assistant and holding the needle at an angle of 45° with the point against the skin at one of the sites of injection, force the needle through the skin by a sharp, quick push. Move the needle about until it moves freely showing

that it is under the skin but not in the muscle. Release the grasp on the tubing and allow only enough pressure to force in the toxin slowly. (A gradual swelling will be noticed at the site of injection, indicating that the toxin is entering.) When the desired volume (usually from 25 to 50 cc.) has been injected, cut off the supply by doubling the tube sharply, at the same time withdrawing the needle. Repeat the procedure until the required dose has been given, having the assistant between each injection replace the needle with a sterile one, or wipe it off with cotton soaked in 2-per-cent cresol compound. Hold the bottle near enough to the horse to prevent the possibility of the connections breaking if the horse struggles. After each injection paint the site with iodine. When treatment is completed, place the horse in a stall or in pasture.

*Subcutaneous Injections of Agar and Living Organisms*[3]

Prepare an area from 5 to 10 centimeters in diameter, as previously described, on the back of the horse near the saddle. Draw up into a sterile syringe from 20 to 50 cc. of melted 1.5-per-cent infusion agar, which should be near the solidification temperature (about 40°C.). (The bottle containing agar to be injected and a control are usually received when the temperature is between 50 and 55°C. The material is injected when the correct temperature as shown by a thermometer in the control bottle of agar, has been reached.) Insert the needle with the point well under the skin and inject. The agar should harden quickly to form a large lump. After about five minutes inject the culture into the agar, inserting the needle through the skin and moving the point about to distribute the culture in the agar.

Keep the horse in special quarters assigned by the veterinarian. After an abscess has broken and is discharging, cleanse frequently the area around and below the site with 2-per-cent cresol compound. Take special precautions to prevent the entrance of flies into the isolation quarters. When toxin is to be injected, select areas on the other side of the horse or at some distance from the abscess.

INTRAVENOUS INJECTIONS

This method is used in the routine production of immune sera, such as antimeningococcus, antipneumococcus, and antidysentery, in which whole cultures or saline suspensions of bacteria are injected. It is used also for prophylactic injections of tetanus antitoxin and pre-

[3] Dochez Method, Dochez, A. R., Medicine, 1925, 4, 251 (167).

liminary doses of diphtheria antitoxin. Injections are usually made by the gravity method; for small volumes a syringe is sometimes used. Preparation of the different cultures and antitoxins is described in the standard methods for the production of the corresponding sera.

Keep live cultures at approximately incubator temperature until just before injecting. After injection place at once in 1-per-cent crude cresol, in a covered receptacle for autoclave sterilization, or in a sterilizer for immediate boiling, pipettes, funnels, filters, test tubes, and any other apparatus which has been used in connection with the preparation of live cultures.

### *Preparation of Antigens for Injection*[4]

**Killed Organisms in Suspension.**—Bacterial suspensions of dead organisms are received in proper dilution for injecting. Always shake the bottles containing the suspension immediately before giving the dose.

**Living Organisms Grown on Solid Medium.**—Suspensions of living organisms are usually prepared by the production group and received ready for injection. Occasionally, however, the cultures may be received on solid medium, in which case proceed as follows.

Meningococcus cultures: After comparing the set of cultures with the direction slip, place the tubes in a rack and add with a 10-cc. pipette, using aseptic precautions, 3 cc. of salt solution to each culture. Loosen the growth carefully with a wire loop, and stir until a uniform suspension is obtained, taking care not to break the agar. Pass the suspension through a sterile cotton filter (for preparation see "General Instructions," p. 624), previously moistened with a volume of salt solution equal to 1.5 cc. for every culture. Then pass through a volume of salt solution equal to that used for moistening the filter. Pipette the dose prescribed for each horse into a separate sterile 20-cc. bottle or test tube (150 by 19 mm.), add enough salt solution to bring the volume to 20 cc. and inject immediately. (If the dose for the day is given in two injections, allow an hour's interval between, keeping the second set of cultures at approximately incubator temperature.)

Dysentery cultures: Treat the cultures in the same way as the meningococcus, but add 2 cc. instead of 3 cc. salt solution to each slant and moisten the filter with a volume of salt solution equal to 1 cc. for

[4] Antitoxins are sent to the operating rooms in single doses ready for injecting, or in larger volumes labeled with the number of units per cubic centimeter.

each culture and wash with the same volume. Always give the entire dose in one injection.

Suspensions of other organisms are treated similarly. Specific directions are received from the production group in each instance.

**Living Cultures Grown in Fluid Medium.**—Whole culture (pneumococcus): The culture must be filtered immediately before injection. If not already filtered by the production group, draw the culture twice through a special cotton suction filter connected through a cotton air filter to a vacuum intake, or force the culture through the filter, using air pressure.

Salt-solution suspensions: Salt-solution suspensions of organisms from broth culture are usually received from the laboratory ready for injection. When filtration is required, pass the suspension through cotton in a funnel as in preparing meningococcus suspensions.

**Miscellaneous Antigens.**—Antigens which are used only occasionally, such as sheep cells, are generally prepared at the main laboratory and sent with instructions to the operating room.

### *Preparation of Site for Injection*

Cleanse the whole side of the neck with 2-per-cent cresol compound solution. Clip with curved scissors, or shave, a space about two inches square on the middle of the neck over the jugular vein. Swab with 70-per-cent alcohol, and paint with iodine.

### *Injections*

Boil the needle, and the cylinder and connections if not already sterilized, for at least ten minutes. Occlude the jugular vein by pressing the thumb of the left hand firmly into the jugular groove below the site of injection. Holding the needle and tubing with the right hand and supporting the cylinder on the right forearm, direct the point of the needle downward and inward, then force it through the skin with a sudden push. If the needle is in the vein, blood will pass through the tubing into the cylinder, quickly receding when the pressure on the vein is removed. If the needle is not in the vein, partly withdraw and push in again at varying angles until the vein is reached. When the needle is in the vein and while the tubing is filled with blood, have an assistant pour into the cylinder 10 to 15 cc. of 0.85-per-cent salt solution followed by the material to be injected. Control the rate of flow by raising and lowering the cylinder or by pressure on the rubber tubing. When the inoculum has nearly passed out of the cylinder,

pour in 10 or 15 cc. of salt solution. Take care not to allow air to be drawn into the vein. When the last of the salt solution has reached the glass "window," pinch the rubber tubing below it to stop the flow, and quickly remove the needle. Paint the site with iodine. After injection, place the horse in a stall without water until the temperature is taken.

### BLEEDINGS

Horses are bled from the jugular vein. Seven to nine liters of blood are taken at one bleeding for production purposes ("whole bleeding"), the amount depending to some extent on the size and condition of the horse. Plasma (blood from which the red and white corpuscles have been removed) is obtained by drawing the blood directly into sodium-citrate solution (nine parts of blood into one part of 17-per-cent citrate solution, giving 1.7-per-cent concentration of citrate); serum, by allowing the blood to stand until the clot is formed. The yield of serum is increased by exerting pressure on the clot. Smaller amounts of blood are taken for defibrinated blood. Samples ("trial bleedings") are taken during the course of immunization for test purposes.

#### *Technic of Bleeding*

**Whole Bleeding for Serum.**—Boil the cannula and rubber tubing, scalpel, and threaded needle. Select and prepare a site over the jugular vein (preferably on the left side) as for intravenous injections. With a sharp sterile scalpel make a $\frac{1}{4}$ to $\frac{3}{4}$ in. incision through the skin directly over and parallel with the jugular vein. Hold the thumb against the blade and a little below the point to prevent too deep an incision, and cut upward to prevent too long a cut should the horse move suddenly. Connect the free end of the tubing attached to the cannula to the inlet tube of the bleeding jar or bottle, which should be placed on a low stool in front and slightly to the horse's left. Press the vein below the incision with the thumb until it becomes prominent, then insert the cannula with an inward and upward movement until it is in the vein. Continue the pressure on the vein during the bleeding. When the first large jar is about three-quarters full ($4\frac{1}{2}$ liters), stop the flow by pressing the tubing between the thumb and forefinger while the assistant disconnects it from the inlet tube of the jar, and after covering the latter with a sterile cap, quickly connects the tubing to the inlet of the next jar. When the desired quantity of blood has been obtained, withdraw and pass the cannula to the assistant, holding the edges of the incision together with the thumb and index finger of

the left hand. With the right hand make a single suture, using a curved needle and white linen thread. Paint the site with iodine. Remove the tubing from the jar and replace the cap. Tag each container with the horse and bleeding numbers, and date, and record the date and volume bled on the horse chart.

Allow the jars to stand undisturbed until the blood has reached room temperature (three to six hours), and a firm clot has formed and shows separation from the wall. If the clot does not show complete separation, loosen it by giving the jar a quick turn or by cautiously tilting it; otherwise the weight when dropped may tilt and exert less pressure. (If the room temperature is low it may be advisable to cover the jars to prevent too rapid cooling.) Remove to the cold room and pull the string attached to the cotter pin, thus releasing the weight.

**Whole Bleeding for Plasma.**—Attach the free end of the rubber tubing connected with the cannula to a 12-inch piece of glass tubing and boil with the scalpel and threaded needle. Measure with a tape the height on the bottle to which the citrated blood should be brought and mark with a red pencil. Replace the stopper of the bottle containing citrate solution with a sterile cotton plug covered with gauze. Insert quickly the glass tube connected with the cannula, raising the cotton plug slightly, then reinserting and pressing it around the tube so that it protects against the entrance of contaminating organisms. Proceed to bleed as for serum; when the blood has reached the graduation mark on the bottle, stop the flow by pressing on the rubber tubing, raise the plug and transfer it quickly with the glass tube to a second bottle, keeping the tube perpendicular. At the same time transfer the stopper from the second bottle to the first. Repeat the operation as required, leaving the cotton plug in the last bottle. Flame the top of each bottle as filled, cover with a wad of cresolized cotton and replace the paper cap. Hold the bottles at room temperature for from four to six hours, then place in the cold room.

**Trial Bleeding.**—Proceed as above but omit the incision and use a needle (15 gauge, 1½ in.) instead of the cannula. Unless larger quantities are specified, draw from about 20 to 40 cc. of blood into a test tube (180 by 25 mm.). Attach a gummed label giving date and horse number. Unless otherwise directed, send trial bleedings without delay to the groups ordering them. When trial bleedings are held allow the blood to stand at room temperature, then place in the cold room. If the clot remains attached to the side, rim with a sterile loop. When a "trial bleeding" is taken at the time of a "whole bleeding," collect the required amount of blood in a sterile test tube before filling the larger containers.

**Bleeding for Defibrinated Blood.**—Prepare the site for bleeding as above. Use a needle (15 gauge 1½ in.) attached by tubing to a bottle for defibrinated blood (see p. 587). When the required volume of blood (usually 200 or 300 cc.) has been taken, stop the flow and withdraw the needle, replacing it in the test tube fastened to the bottle. Shake the bottle vigorously for ten to fifteen minutes. If the blood flows slowly, showing a tendency to clot, shake the bottle during bleeding. Attach a tag to the bottle stating the horse number and date bled, and send immediately to the laboratory.

**"Bleeding Out."**—Place the horse, preferably on an operating table, in such a position that the blood can drain from the hind quarters and abdominal region. Wrap a towel tightly about the nostrils and keep it saturated with chloroform. Use the usual aseptic precautions for bleeding. Make an incision over the ventral edge of the mastoid humeralis muscle in the lower third of the neck and by blunt dissection pull the jugular vein to one side, thus exposing the carotid. Clamp or ligate the artery at two points about four inches apart. (If the artery is ligated, use some form of knot which can be easily loosened.) Then insert the cannula directed against the blood flow into the carotid between the clamps. Remove the clamp from the proximal portion of the artery and allow the blood to flow through the cannula and tubing into the bottles or bleeding jars, depending on whether plasma or serum is desired. From 20- to 35-liters of blood can usually be obtained.

### *Drawing Off Serum and Plasma*

The same method is used for serum and plasma. The first drawing off of serum is usually at the end of forty-eight hours, the second after seventy-two hours. Plasma is drawn off but once, usually after forty-eight hours. The procedure should be carried out under strictly aseptic precautions in a room specified for the purpose.

Boil the siphon apparatus if not recently sterilized. Bring the separated blood from the cold room carefully to avoid disturbing the red cells or hemoglobin. Place a sterile receiving bottle at a lower level, remove the cotton stopper, flame the neck, and insert quickly the stopper and connections of the siphon outlet, wrapping around them a strip of cresolized cotton. Wipe the mouth of the bottle containing plasma, raise the cotton plug enough to insert the siphon and replace, pressing the plug around the siphon. To take serum from the bleeding jars, replace the inlet tube and stopper with the siphon and wrap cresolized cotton about the opening, or slit the paper and insert the siphon through the weight-peg hole and wrap with cotton.

Adjust the siphon so that the inlet is below the surface of the plasma or serum, then apply suction to the short glass tube of the receiving bottle until the product begins to come over. Take great care not to disturb the clot or to draw over the sedimented red cells, etc. This is especially important in the case of sera. Transfer the siphon from one bottle or jar to another with the same precautions (when siphoning from bottles take the cotton plug from bottle to bottle with the siphon). After use, wash the siphon immediately in cold water. Use a sterile siphon and connections for each bleeding.

When a receiving bottle becomes full or all the material has been drawn off, remove the siphon outlet, flame the neck of the bottle, and with forceps insert a rubber stopper previously boiled and dried in the flame. Flame and cover with cresolized cotton, tie down, and cap with brown paper. Attach a shipping tag to the bottle, giving horse and bleeding numbers, kind and volume of product, and date. Place immediately in the cold room. If the material is drawn off at the farm, forward it to the laboratory in special shipping boxes accompanied by a signed duplicating order form giving date, name of group to whom shipped, product, horse number, date of bleeding, and volume. Place the residues (clots or sedimented cells) in a container from which they are removed for fertilizer or otherwise disposed of.

When instructions are received to draw off the serum from a trial bleeding, pipette it carefully into a small sterile bottle, close and cap as above, and tag or label with the horse number, kind of product and date. The serum may usually be drawn off within from two to four hours after the trial bleeding is taken.

Enter the required data on the horse-temperature chart and the daily record.

## CARE AND TREATMENT OF CATTLE, SHEEP, AND GOATS

Several large rams are kept for bleeding to supply red blood cells for the complement-fixation test. A number of white goats[5] (short-haired, preferably Saanen, or Angora) are required for potency tests of streptococcus toxins and antistreptococcus sera. These animals are also occasionally used for the production of diagnostic immune sera. Calves and full-grown cattle are used for experimental or special test purposes. Many of the goats used are bred by the laboratory.

The sheep, and the goats which are in use, are kept in the laboratory

[5] To guard against the possible introduction of carriers into the laboratory herd, the blood from each new goat when received is tested for agglutinative reaction with *B. melitensis*.

stable units; goats which are not in use, usually at the farm; cattle, except those under treatment and requiring frequent observation by the scientific workers, at the farm.

Each kind of animal is numbered consecutively in the order of receipt. For sheep and goats the numbers are stamped on metal tags attached to a leather collar. A description of each animal and its assignment is kept by the veterinarian.

The animals are fed and given water twice a day. The rations for sheep and goats consist of clover or alfalfa hay, oats and occasionally corn, and in summer when not in pasture, cut grass several times a week. For cattle the rations are the same except that bran, ground oats, wheat middlings and occasionally corn meal are fed in place of the whole grains. Young calves are given pasteurized milk and commercially prepared calves' feed. Animals in pasture are fed as required by the condition of the animal and amount of pasturage.

Sheep, goats, and calves are injected and bled in a standing position in special stocks (see plate III) or while an assistant holds the animal's head. The procedures for subcutaneous and intravenous injections, and for bleeding, are those used for horses. The method for intracutaneous injection of goats is given in the chapter on the "Production and Standardization of Antistreptococcus Serum (Scarlet Fever)," p. 334. The following amounts of blood may be taken from the different animals: from sheep, from 300 to 600 cc. every seven to ten days; from calves, 100 to 600 cc. depending upon the size; from goats, 200 to 400 cc. When sheep are bled to supply red blood cells for the complement-fixation test, the blood is added to an equal volume of 2-per-cent sodium-citrate solution (2 per cent citrate in 0.85-per-cent salt solution). The blood is taken directly into bottles (500 cc. wide mouthed, with cotton plugs) containing 100 or 200 cc. of the solution. Occasional short intervals of rest may be advisable when frequent large bleedings are taken.

Grown cattle are usually placed in stocks for injection and bleeding. The procedures are those used for horses. From two to five liters of blood or occasionally more, may be taken at one time, depending on the size of the animal.

The post-mortem examination of these animals is in general the same as that for horses.

### *Permanent Records*

Temperature charts: The daily temperatures of each horse undergoing immunization are entered graphically on a weekly chart (8 by 3 in., printed in sets

of four on perforated sheets, fig. 52). Records of injections, bleedings and other treatment and unusual conditions are also entered on this chart.

Operating room reports: Records of daily injections and bleedings are entered on monthly "Operating-room report" forms (8 by 11 in., mimeographed).

At the end of each day's work the date, number of injections of each kind of toxin or other material given subcutaneously, and total volume of each, and number of injections of each kind of material given intravenously are entered in separate columns on the form for injections.

At the end of each day's work, the date, number of bleedings and volumes of blood taken, and the total volume of serum or plasma drawn off, are entered in a separate column for each kind of product, as diphtheria antitoxin, antimeningo-

Horse No..... Treatment..... .....19.....

| DATE | INJECTION | 99 | 100 | 101 | 102 | 103 | 104 | 105 | REMARKS | AMOUNT BLED | NUMBER |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | |
| ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... |
| ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... |
| ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... |

FIG. 52. TEMPERATURE CHART

coccus serum, beaded blood, normal serum, "media" serum (serum for media from horses previously under immunization), etc., on the form for bleedings. The number of trial bleedings is entered in one column.

Farm report: A monthly report (8 by 11 in., mimeographed) of the work at the farm gives the following data: totals of injections and bleedings; numbers of horses, mules, cattle, goats, and small animals (totals of the "Farm report—small animals") on hand, received from outside or from the laboratory, born, total number of each kind added, numbers sent to laboratory, died, destroyed, unaccounted for, total number of each kind removed, and balance; the purposes to which the large animals are assigned; and a summary of the farm produce harvested.

Laboratory animal report: A monthly report of the large animals at the laboratory units is included in the "Laboratory animals" report (see Permanent Records under "Small Animals," p. 577).

## SECTION XI

## CHAPTER 1

## BACTERIAL COLLECTION

The bacterial collection[1] consists of carefully selected representative strains of most of the pathogenic and of a few of the nonpathogenic species of bacteria. Certain species, such as the monilia, belonging to other classes of organisms, are also represented. The collection contains, in addition, supplementary strains of a number of the pathogenic bacteria obtained in connection with special studies or investigations and considered of sufficient interest to retain. The collection is maintained for the use of the staff of this laboratory and those of the local registered laboratories[2] in the state. The bacterial collection group, in addition to its work connected with the receipt, maintenance, and distribution of cultures and with related problems, coöperates in investigations and otherwise assists the workers in the various laboratories. Under the present plan of organization, the bacterial collection group is under the supervision of the bacteriologist in charge of the antitoxin, serum and vaccine laboratories.

In order to avoid duplication of work, the maintenance of strains used in the production of certain biologic products has been assigned to the production groups. From time to time other cultures are maintained by different groups in connection with special studies. These cultures may be received directly by the group, in which case they are given the accession number of the culture or of the specimen from which isolated, or they may be received through the bacterial

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[1] Much more study than has as yet been practicable will be required before the procedures used in the bacterial collection, especially those relating to the various species of bacteria, can be adequately formulated. The examination of a strain may, moreover, be limited to a large extent by the staff available. Thus the more precise differentiation of closely related microörganisms, such as those of the paratyphoid-enteritidis group, requiring absorption and other special tests, is at present impracticable. The classification of certain strains in the collection is, therefore, to some extent arbitrary. The present method, although tentative, serves a practical purpose and forms the ground work for future study.

[2] An act of the Legislature requires that all laboratories in the state where live pathogenic organisms are handled or cultivated shall be registered with the State Department of Health, Laws of New York, 1917, Chapter 411.

collection. A form, giving the kind and number and any additional data required in regard to cultures maintained by other groups, is received by the bacterial collection group semi-annually, so that information may be available. All requests for cultures, including those for cultures from outside sources, are referred to the bacterial collection group. If a culture is to be procured from outside, the letter requesting it is sent by the bacteriologist in charge of the department. The record of distribution of all cultures, including those distributed by the diagnostic laboratories for special purposes, is kept by the bacterial collection group. See "Distribution," p. 610.

### *Divisions of the Collection*

Cultures comprising the permanent collection are classified in one of two divisions, A or B. Cultures when received are placed in a temporary collection, Division C. Tests to determine the purity of the culture and to establish its identity are made promptly, so that the culture can be transferred or discarded without unnecessary delay. Occasionally, however, cultures received in connection with special studies are held in Division C until their value for further reference can be better determined.

Division A: A collection of carefully selected representative strains the identity of which has, within certain limits, been established. Each species is assigned a number.[3] Individual strains are designated by the species number followed by a letter.

Division B: An auxiliary collection made up of additional strains of "identified" organisms and of organisms of special interest the identity of which has not been determined. The method of numbering cultures in Division B is at present under revision. In general, cultures in Division B are assigned the same species numbers as those in Division A but the numbers are preceded by the letter "B." A subnumber preceded by a dash indicates the strain (B 144-1, B 144-2, etc.).

Division C: A temporary collection in which the strains are placed when received. Each strain is assigned a serial number (consecutive in order of receipt) preceded by the letter "C." This number is retained until the culture is transferred to the permanent collection, or discarded.

### RECEIPT OF CULTURES

When a culture is received, enter its temporary serial number on the label in red ink, and make the necessary entries on the "Accession record" card (p. 613). Write the temporary number in black ink (small script) just above "Collection no." on a history card (p. 615), and record on the back all data of significance available regarding the culture. Make the required examination of the culture with as little

[3] The present system of numbering is temporary, pending a revision of the method of classification.

delay as possible, and record the results on the history card. In making the examinations, follow the technic given in "General Bacteriological Technic," (p. 3) with the special modifications given under "Routine Transfers," p. 608.

If the results of the tests are satisfactory and the approval of the bacteriologist in charge of the department has been obtained, give the strain a permanent number which places it in the proper division of the collection. Record the number and the date in red ink on the accession card, and the number on the history card and on the labels of the cultures.

If, however, it appears that there are a sufficient number of such strains in the collection or if the results of the tests indicate that the culture is not satisfactory, notify the laboratory worker who referred it to the collection group or requested that it be obtained, and unless otherwise directed, discard it. Enter "Discarded," the reason, and the date on the history card and file it under "Cultures discarded, Division C"; enter "Discarded" and the date on the accession card.

If a contaminated culture is received, a fishing may be saved, and the fact noted on the back of the history card.

Store original cultures in the cold room or at suitable temperature, in a basket labeled "Original cultures," for three months before discarding them. Keep the original slide preparations for one year.

### IDENTIFICATION AND PURITY TESTS

The procedure varies with different species and depends to some degree upon the purpose for which the culture is obtained. The chart shown in figure 53, however, indicates the general procedure followed in the case of organisms growing readily in the usual culture media. Plates are incubated for five days,[4] and examined daily. In the case of anaerobic cultures a duplicate set of plates is incubated in an anaerobic jar, and usually examined only at the end of the 5-day period. The morphology of organisms grown in broth should in general be determined. For this purpose, methylene blue stain is usually adequate. When the reactions in carbohydrate serum waters are not characteristic, slide preparations from the cultures are made. The pathogenicity of cultures to be assigned to Division A is included when of special significance in determining the identity of the organ-

[4] Cultures which have appeared pure upon microscopic examination, have been found to be contaminated with slow-growing organisms when streak plates have been examined after several days' incubation.

ism. Further study as required of each new culture assigned to Division A is made, so that essential data relating to each strain may be available. The tests, thermal death point, degree of toxicity, cultural characteristics in other media, etc. depend on the microörganism. The use of a single cell method of isolation and study is at times advisable.[5] Data relating to periodic tests of purity are given under "Maintenance of Cultures," p. 609.

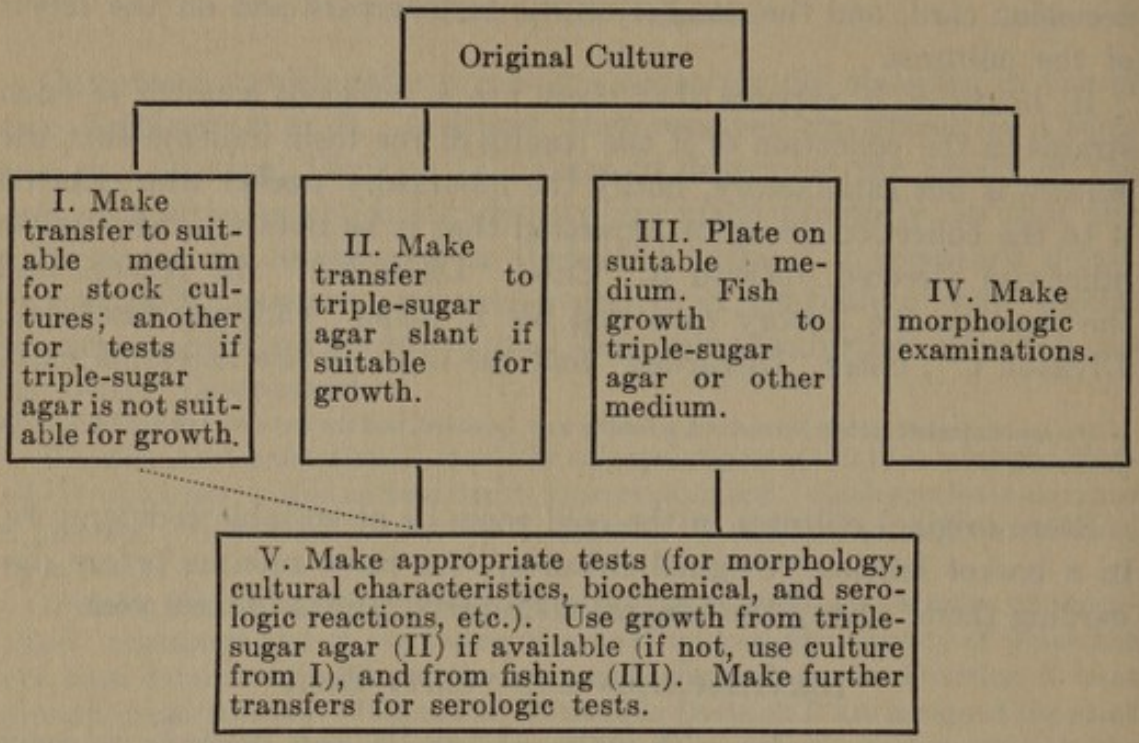


FIG. 53. GENERAL PROCEDURE FOR PURITY AND IDENTIFICATION TESTS OF NEW CULTURES

The procedures, in general, to be followed in studying the various species are indicated in the following list:

Gonococci: Gram stain. Plate on dextrose-beef-infusion agar[6] and testicular blood agar. Test in broth, milk, gelatin, and carbohydrate-serum waters, noting especially reactions in dextrose, maltose, mannite, levulose, and saccharose. Test agglutination reactions in specific sera when required, if available.

*M. catarrhalis:* Gram stain. Plate on dextrose beef-infusion agar. Test in broth, milk, gelatin, and dextrose-serum water, and on triple-sugar agar.

Meningococci: Gram stain. Plate on dextrose serum agar and blood agar. Test in broth, milk, gelatin, and carbohydrate-serum waters, noting especially

[5] The method described by Ørskov, J., Jour. Bact., 1922, 7, 537 (3), has been used in this laboratory.

[6] Unless otherwise specified "dextrose beef-infusion agar" refers to that containing 0.2 per cent dextrose.

reactions in dextrose, maltose, mannite, levulose, and saccharose. If no growth in broth, determine the morphology after cultivation in broth containing serum. If no growth is obtained in serum-water medium, test reactions on serum-agar medium containing the carbohydrates. Test agglutination reactions in monovalent group sera.

Pneumococci: Gram and capsule stains. Plate on blood agar. Test in broth, gelatin, milk, and carbohydrate-serum waters, noting especially the reaction in inulin. Test agglutination reactions in type sera and bile solubility.

Staphylococci: Gram stain. Plate on dextrose beef-infusion agar and blood agar. Test in broth, milk, gelatin, and carbohydrate-serum waters, and on triple-sugar agar. If pigment has not been produced, test on Loeffler's serum medium. Test solubility of pigment in alcohol, chloroform, and ether.

Streptococci: Gram stain. Plate on blood agar. Test in broth, milk, gelatin, and carbohydrate-serum waters, noting especially reactions in lactose, mannite, salicin, inulin, and saccharose. Test bile solubility. Test for toxin production and toxin-antitoxin neutralization, if required.

Sarcinae: Gram stain. Plate on dextrose-beef-infusion agar. Test in broth, milk, gelatin, and carbohydrate-serum waters, noting especially reactions in dextrose and saccharose, and on triple-sugar agar. Test motility. Note pigment formation.

*B. abortus and B. melitensis:* Gram stain. Plate on dextrose-beef-infusion agar. Test in broth, gelatin, milk, and dextrose-serum water, and on triple-sugar agar. Test motility, and agglutination reactions in specific sera. If difficulty in obtaining growth is experienced with new strains, the cultures may be grown under partial $CO_2$ tension.

Bacilli, acid fast: Except *B. tuberculosis.* Gram and acid-fast stains. Plate on dextrose-beef-infusion agar. Test in broth, milk, gelatin, and carbohydrate-serum waters. Test motility.

*B. acidophilus:* Gram stain. Plate on dextrose-beef-infusion agar. Test in broth, gelatin, milk, and carbohydrate-serum waters, noting especially reactions in dextrose, maltose, lactose, and saccharose, and on triple-sugar agar.

Bacilli, aerobic, Gram-positive, spore-bearing (*B. anthracis, B. asterosporous, B. subtilis*): Gram and spore stains. Plate on dextrose-beef-infusion agar. Test in broth, gelatin, milk, and carbohydrate-serum waters, and on triple-sugar agar. Test motility.

*B. anthracis:* See Bacilli, aerobic, Gram-positive, spore-bearing.

*B. asterosporous:* See Bacilli, aerobic, Gram-positive, spore-bearing.

Bacilli, anaerobic (*B. botulinus, B. histolyticus, B. oedematis maligni, B. putrificus, B. sporogenes, B. tetani,* and *B. welchii*): Gram and spore stains. Plate on blood agar, preferably agar containing 0.2 per cent dextrose, or on other media as required. Incubate for four or five days. one plate aerobically, the others in an anaerobic jar at from 34 to 37°C. Test in broth (tetanus broth, van Ermengem's broth, etc.) gelatin, milk, carbohydrate-serum waters that are considered differential for each species, and on Loeffler's coagulated-serum medium, incubating the cultures in the anaerobic jar where necessary. Test motility. Test *B. botulinus* and *B. tetani* for toxin production and toxin-antitoxin neutralization, when required, using for *B. botulinus,* type A and type B antitoxin.

*B. botulinus:* See Bacilli, anaerobic.

*B. bronchisepticus:* Gram stain. Plate on blood agar and dextrose-beef-

infusion agar. Test in broth, milk, gelatin, potato, dextrose-serum water and on triple-sugar agar. Test motility.

Bacilli, chromogenic: Gram stain. Plate on dextrose-beef-infusion agar. Incubate at from 35 to 36°C. and at room temperature. Test in broth, gelatin, milk, and carbohydrate-serum waters. Test motility. Make tests for specific pigment, as solubility in alcohol, water, chloroform, etc.

*B. coli* and miscellaneous Gram-negative bacilli: Gram stain. Plate on dextrose-beef-infusion agar, and Endo's medium when indicated. Test in broth, gelatin, milk, and the carbohydrate-serum waters that are considered differential. Test on triple-sugar agar. Test for indol production. Test motility.

*B. of Danysz:* See *B. typhosus.*

*B. diphtheriae* and diphtheroids: Gram and methylene blue stains. Plate on dextrose-beef-infusion agar, or dextrose-serum agar, if available. Test in broth, gelatin, milk, and carbohydrate-serum waters, noting especially reactions in dextrose, saccharose, and dextrin. Test motility. Test for toxin production and virulence, if required.

*B. dysenteriae:* See *B. typhosus.*

*B. enteritidis:* See *B. typhosus.*

Bacilli, encapsulated: (*B. lactis aerogenes, B. rhinoscleroma, B. mucosus capsulatus*): Gram and capsule stains. Plate on dextrose-beef-infusion agar. Test in broth, gelatin, milk, and carbohydrate-serum waters, and on triple-sugar agar. Test motility.

*B. histolyticus:* See Bacilli, anaerobic.

*B. influenzae:* Gram stain. Plate on coagulated-blood agar and transfer to a beef-infusion-agar slant. If growth develops on the latter, subculture, since blood pigment may have been transferred.

*B. lactis aerogenes:* See Bacilli, encapsulated.

*B. lepisepticum:* Gram stain. Plate on blood agar and dextrose-beef-infusion agar. Test in broth, milk, gelatin, carbohydrate-serum waters and on triple-sugar agar. Test motility.

*B. mallei:* Gram and methylene-blue stains. Plate on acid glycerin agar. Test in broth, and gelatin, and on potato and triple-sugar agar. Test motility.

*B. melitensis:* See *B. abortus.*

*B. mucosus capsulatus:* See Bacilli, encapsulated.

*B. oedamatis maligni:* See Bacilli, anaerobic.

*B. paratyphosus:* See *B. typhosus.*

*B. pertussis:* Gram stain. Plate on blood agar and on glycerin agar. Test in broth, milk, and gelatin, and on triple-sugar agar. Test motility.

*B. pestis:* Gram and methylene-blue stains. Plate on dextrose-beef-infusion agar or glycerin agar. Test in broth, and gelatin, and on triple-sugar agar. Test motility. Test agglutination reactions in specific sera when required, if available.

*B. pestis caviae:* See *B. typhosus.*

*B. proteus:* See *B. typhosus.*

*B. putrificus:* See Bacilli, anaerobic.

*B. rhinoscleroma:* See Bacilli, encapsulated.

*B. subtilis:* See Bacilli, aerobic, Gram-positive, spore-bearing.

*B. suipestifer:* See *B. typhosus.*

*B. sporogenes:* See Bacilli, anaerobic.

*B. tetani:* See Bacilli, anaerobic.

*B. tuberculosis:* Acid-fast and Gram stains. Test growth in glycerin broth. Inoculate a beef-infusion-agar slant and aerobic and anaerobic sterility-test broth. Test virulence if necessary.

*B. typhosus, B. paratyphosus, B. dysenteriae, B. of Danysz, B. pestis caviae, B. proteus, B. suipestifer,* etc.: Gram stain. Plate on dextrose-beef-infusion agar, and on Endo's medium. Test in broth, gelatin, milk, carbohydrate-serum waters and on triple-sugar agar. Test motility. Test agglutination reactions in immune sera as required. Make absorption tests when required.

*B. welchii:* See Bacilli, anaerobic.

*Sp. cholerae:* Gram stain. Plate on alkaline beef-infusion agar. Test in broth, milk, gelatin, and carbohydrate-serum waters, noting especially reactions in dextrose and saccharose and on Loeffler's coagulated serum. Test motility. Test for cholera red reaction. Test agglutination reactions in immune serum when available. Make absorption tests, if required.

Actinomyces: Gram stain. Plate on dextrose-beef-infusion agar. Incubate one plate aerobically, another in the anaerobic jar. Test in broth, gelatin, and carbohydrate-serum waters, and on triple-sugar agar. Test motility.

Leptospira and treponemata: Dark-field examination for morphology and motility. Inoculate aerobic and anaerobic sterility-test broth.

Treponemata: See Leptospira.

Monilia: Gram stain. Plate on dextrose-beef-infusion agar. Test in broth, milk, gelatin, and carbohydrate-serum waters and on triple-sugar agar. Test motility.

## MAINTENANCE OF CULTURES

Cultures which are not in frequent use are usually maintained in nutrient-gelatin medium, except when special media are required.[7] Table 42 indicates the medium at present used for each species. All cultures in nutrient-gelatin medium and many in other media are maintained in duplicate. In the case of cultures stored at incubator temperature the two sets are kept in different incubators.

Cultures in division A maintained by the bacterial collection group are generally arranged in special wooden racks of 30-tube capacity, constructed to hold a double row of tubes. At one end is a metal holder for a card on which are listed the numbers of the cultures. The racks are numbered, odd numbers being assigned to those containing cultures in nutrient-gelatin medium and even numbers to racks of cultures for which other media are used. So far as is practicable, only related organisms are placed in the same rack.

[7] The results of tests to determine the practicability of maintaining stock cultures not in active use, in a frozen state in sealed containers submerged in brine have been unsatisfactory. Work on the preservation of cultures by drying has not progressed sufficiently for the method to be included as a routine procedure.

TABLE 42

*Maintenance of cultures**

| CULTURE† | MEDIUM‡ | INTERVAL BETWEEN TRANSFERS | INCUBATION | | STORAGE TEMPERATURE | REMARKS |
|---|---|---|---|---|---|---|
| | | | Temperature | Period | | |
| Gonococci | Ascitic fluid semisolid agar | 1 month | 35–36°C. | 3–5 days | 35–36°C. | Transfer with pipette 0.1 to 0.2 cc. Stir every 2 weeks |
| Gonococci—duplicate strains | Hormone semisolid agar | 2 weeks | 35–36°C. | 3–4 days | 35–36°C. | |
| *M. catarrhalis* | Infusion agar | 1 month | 35–36°C. | 24 hours | 4–8°C. | |
| Meningococci | Ascitic fluid semisolid agar | 2 months | 35–36°C. | 24–48 hours | 35–36°C. | Transfer to dextrose serum agar, incubate 24 hours, transfer to ascitic fluid semisolid agar. Stir weekly |
| Meningococci—standard strains | Dextrose serum agar | 48 hours | 35–36°C. | 48 hours | 35–36°C. | |
| Pneumococci | Serum semisolid agar | 1 month | 35–36°C. | 24 hours | 4–8°C. | Transfer with pipette 0.1 to 0.2 cc. |
| Staphylococci | Nutrient gelatin | 3 or 6 months | 35–36°C. | 24 hours | Room temp. | |
| Streptococci | Blood agar | 1 month | 35–36°C. | 24 hours | 4–8°C. | |
| Sarcinae | Nutrient gelatin | 3 or 6 months | 35–36°C. | 24 hours | Room temp. | |
| *B. abortus* | Nutrient gelatin | 6 months | 35–36°C. | 2–3 days | Room temp. | |
| Bacilli, acid-fast—except *B. tuberculosis* | Nutrient gelatin | 3 months | 35–36°C. | 2–3 days | Room temp. | |
| *B. acidophilus* | Milk | 24 hours | 35–36°C. | 24–48 hours | 4–8°C. | Transfer with pipette 0.1 cc. |
| Bacilli, anaerobic—except *B. putrificus* and *B. welchii* | Dextrose semisolid agar | 3 months | 35–36°C. | 2–5 days | 4–8°C. | Transfer with pipette 0.5 to 1.0 cc. |
| *B. anthracis* | Nutrient gelatin | 6 months | 35–36°C. | 24 hours | Room temp. | |
| *B. asterosporous* | Nutrient gelatin | 6 months | 35–36°C. | 24 hours | Room temp. | |
| Bacilli, chromogenic | Nutrient gelatin | 3 or 6 months | 35–36°C. or room temp. | 24–72 hours | Room temp. | Two strains maintained on infusion agar—transferred weekly—incubated and stored at room temperature |
| *B. coli* and miscellaneous Gram-negative bacilli | Nutrient gelatin | 3 or 6 months | 35–36°C. | 24 hours | Room temp. | |
| *B. diphtheriae* and diphtheroids | Nutrient gelatin | 3 months | 35–36°C. | 24–48 hours | Room temp. | |
| *B. diphtheriae*—"toxin" strains | "Toxin" broth | 24 hours | 35°C. | 24 hours | Room temp. | |
| *B. dysenteriae* Flexner, Mt. Desert, Shiga—standard strains | Infusion agar | 1 month | 35–36°C. | 24 hours | 4–8°C. | |
| *B. dysenteriae* Flexner, Mt. Desert—other strains | Nutrient gelatin | 6 months | 35–36°C. | 24 hours | Room temp. | |

| | | | | | | |
|---|---|---|---|---|---|---|
| *B. dysenteriae* Shiga—other strains | Nutrient gelatin | 3 months | 35–36°C. | 24 hours | Room temp. | |
| Bacilli, encapsulated | Nutrient gelatin | 3 or 6 months | 35–36°C. | 24 hours | Room temp. | |
| *B. influenzae* | Coagulated blood agar, alkaline | 7 days | 35–36°C. | 24 hours | Room temp. | |
| *B. mallei* | Nutrient gelatin | 3 months | 35–36°C. | 24–48 hours | Room temp. | |
| *B. melitensis* | Nutrient gelatin | 3 months | 35–36°C. | 2–3 days | Room temp. | |
| *B. pertussis* | Coagulated blood agar, neutral | 3–4 days | 35–36°C. | 24–48 hours | 35–36°C. | |
| *B. pestis* | Infusion agar | 6 months | 35–36°C. | 24–48 hours | 4–8°C. | |
| *B. pneumosintes* | Smith tissue medium | 2 months | 35–36°C. | 2–4 weeks | Room temp. | |
| *B. putrificus* | Smith tissue medium | 3 months | 35–36°C. | 2–5 days | 4–8°C. | Transfer with pipette 0.5 to 1.0 cc. |
| *B. subtilis* | Nutrient gelatin | 6 months | 35–36°C. | 24 hours | Room temp. | |
| *B. tuberculosis* | Petroff's egg medium without dye | 2 months | 35–36°C. | 2–3 weeks | 4–8°C. | |
| *B. typhosus*, *B. paratyphosus* A and B—standard strains | Infusion agar | 1 month | 35–36°C. | 24 hours | 4–8°C. | |
| *B. typhosus*, *B. paratyphosus* A, B, and C—other strains | Nutrient gelatin | 6 months | 35–36°C. | 24 hours | Room temp. | |
| *B. welchii* | 2 per cent dextrose beef-infusion agar | 3 months | 35–36°C. | 24 hours | 4–8°C. | Transfer with pipette 0.1 cc. |
| *Sp. cholerae, metchnikovi*, etc | Infusion agar | 7 days | 35–36°C. | 24 hours | 4–8°C. | |
| *Sp. cholerae*—special strain | Alkaline infusion agar | 7 days | 35–36°C. | 24 hours | 4–8°C. | |
| *Sp. rubrum* | Nutrient gelatin | 3 months | Room temp. | 2–3 days | Room temp. | |
| Actinomyces | Nutrient gelatin | 3 months | 35–36°C. | 3–4 days | Room temp. | |
| *Leptospira* (*icterohaemorrhagiae* and *icteroides*) | Noguchi medium | 2 weeks to 1 month | Room temp. and 26°C.§ | 5–10 days | Room temp. and 26°C.§ | Transfer with capillary pipette |
| *Treponemata* (*pallidum* and *calligyrum*) | Smith tissue medium | 4–6 months | 35–36°C. | 2–4 weeks | 35–36°C. | Transfer with capillary pipette |
| Monilia | Nutrient gelatin | 3 months | Room temp. | 2–3 days | Room temp. | |

* A reclassification of the bacterial collection is under consideration. It has therefore been thought unwise at the present time to make any changes in the nomenclature used.

† Besides the cultures maintained *in vitro*, strains of *Treponema pallidum* and of vaccinia virus are maintained in rabbits by testicular inoculation.

‡ All media are filled in 150-by-16-millimeter tubes, with the following exceptions: serum and ascitic-fluid semisolid agar and diphtheria-toxin broth in 125-by-13-millimeter tubes; Petroff's egg medium without dye, dextrose-serum agar, coagulated-blood agar for *B. pertussis*, and 2-per-cent dextrose-beef-infusion agar for *B. welchii* in 150-by-19-millimeter tubes; Smith tissue medium in 200-by-14-millimeter tubes.

§ Since there is evidence that temperatures in excess of 26°C. may be injurious to *Leptospira icterohaemorrhagiae*, special care is observed in summer. If the incubator in which the cultures are stored is not artificially cooled, it is placed in a cool cellar. *Leptospira icteroides* is incubated and stored at room temperature and at 35–36°C.

Cultures in divisions B, and C, are arranged in wire baskets or racks, the grouping in each case depending on frequency of transfer and relationship of organisms.

All racks and baskets used for storing pathogenic anaerobes, and *B. abortus*, *B. anthracis*, *B. mallei*, *B. melitensis*, *B. pestis*, and *Sp. cholerae* are conspicuously marked with the name of the organism and the word "Caution." Such containers are kept in locked cupboards.

### *Routine Transfers*

Table 42 gives the general scheme followed in maintaining cultures; frequency of transfer, medium used, incubation period, storage temperature, etc. When certain strains of a species require more frequent transfer than others, the two intervals are given. The schedule for transfer of all strains is given on transfer cards. For technic of transferring, see "General Bacteriological Technic," p. 3.

When transfers are to be made, arrange the cultures in single rows in the racks. In the case of cultures kept in duplicate in the same rack, remove one set, usually retaining as seed tubes those which have not previously been opened. When the strains in the two rows of a rack are different, remove those from the second row to another rack. To prepare duplicate cultures, enter on the labels of two tubes of medium to be inoculated, the number of the culture at the extreme left of the rack and the date, and place them in a second rack behind the first. Repeat for each seed culture. Always compare the numbers on the tubes with those on the cultures, before making the transfers. Make two transfers from each seed tube, placing the freshly inoculated tubes in the front rack. When only one transfer is to be made from each culture, place the labeled tubes of medium in the back row of the rack and after making a transfer, return the tubes to the rack, in the reversed positions. When a transfer is made with a pipette, place the seed tube, after the necessary growth has been withdrawn, in a second rack behind the first. Then inoculate the tube of medium and place it in the front row of the first rack.

Seal with paraffin all cultures which are to be stored for two months or longer without transfer, unless the medium is covered with a layer of mineral oil. Reseal all tubes, including seed tubes which have paraffin plugs, by warming in a flame. Fasten together with a rubber band the seed tubes from each rack, mark the bundle with the number of the rack (or in the case of cultures not stored in racks, with an adequate designation) and store in wire baskets until the next transfer is made, when they are usually discarded.

After incubating the new cultures, observe carefully the macroscopic appearance of the growth, prepare and examine stained preparations if contamination is suspected, and make additional tests for purity

when necessary. If the cultures appear satisfactory, store them at the proper temperature. Attach a second label to cultures opened for purity tests, or which later are opened and retained, and enter on it the date opened, and initial.

Special procedures: Treponemata cultures: Examine a moist preparation of each culture over a darkfield before transfers are made. For procedure, see "General Bacteriological Technic," pp. 11, 14. If contamination is suspected, make preparations stained by Gram's method. Use a fresh capillary pipette each time a seed tube is opened. Pipette growth from the upper part of the growth and seed into the bottom of the tube of fresh medium, avoiding the introduction of air bubbles. Inoculate a tube of aerobic and another of anaerobic sterility-test broth with the material remaining in each pipette.

For these transfers two workers are desirable. "A" removes the cotton plug and flames the opening of the seed tube; "B" removes a capillary pipette from the container and then withdraws some of the growth from the tube. "A" flames the mouth and replaces the plug of the seed tube; then opens the tube of medium with the same aseptic precautions, and after "B" has discharged the growth, closes it again as above.

Leptospira cultures: Examine cultures over a darkfield before transfers are made. Make preparations stained by Gram's method, if contamination is suspected. If the culture is satisfactory, transfer some of the growth from the surface to the surface of a fresh tube of medium.

Pathogenic anaerobes: In transferring pathogenic anaerobes, take every precaution to avoid scattering material which may contain spores. When using a loop take particular care to avoid spattering when flaming. When a pipette is used, two persons should make the transfer. Hold a Petri plate containing cotton dampened with 5-per-cent phenol solution under the tip to catch any drops that may fall. Place contaminated pipettes in special jars; discarded cultures, and Petri plate, etc., in covered metal cans. Label with red pencil "Caution" and the name of the organism, and personally place in the autoclave.

*B. anthracis*, *B. mallei*, *B. melitensis* and *B. abortus*, *B. pestis*, *Sp. cholerae:* In transferring these highly pathogenic organisms take precautions similar to those observed for pathogenic anaerobes.

Control cultures for Gram-stained preparations: Transfer agar-slant cultures of *B. coli* and *Staphylococcus aureus*, daily except Sundays and holidays. Place the culture of each from which the transfer was made in a rack or basket accessible to the workers in the department.

### *Periodic Tests of Purity*

Besides the macroscopic examination of the cultures at the time of transfer, slide preparations, and streak plates are made as frequently as is considered necessary to insure, with reasonable certainty, the purity of the cultures. Slide preparations and special tests made when the strains are distributed, and in connection with the production of biologic products, act as a further control. Should, at any time, the presence of a contaminating organism in a culture be sus-

pected, a slide preparation and a streak plate, and further tests as indicated are made at once. Should contamination be found, transplants are made from a previous transfer when available, rather than from a fishing. Certain standard strains which are distributed frequently for use in diagnostic procedures and in connection with the standardization of diagnostic sera, are, in addition to the tests made at the time of distribution, subjected to careful periodic tests at intervals of six months or less.

The procedure for the periodic tests of certain standard strains which are among those most frequently distributed follows.

| | | |
|---|---|---|
| *B. typhosus* Bender | Collection No. | 270C |
| *B. paratyphosus* A | " | " 235A |
| *B. paratyphosus* B | " | " 236B |
| *B. dysenteriae* Flexner | " | " 114D |
| *B. dysenteriae* Mt. Desert | " | " 114E |
| *B. dysenteriae* Shiga | " | " 114F |

Plate these strains on dextrose-beef-infusion agar at least once in six months. Test the reaction of the whole culture and at least one fishing on triple-sugar agar slants; in milk, gelatin, and carbohydrate-serum waters. Test also for motility; and make macroscopic and microscopic agglutination tests with homologous and heterologous immune sera.

For the periodic tests of purity of standard strains maintained by the production groups (strains of meningococci, pneumococci, streptococci, *B. botulinus*, *B. diphtheriae*, *B. dysenteriae*, *B. paratyphosus*, *B. tetani*, and *B. typhosus*, used in the preparation of biologic products), see the methods for the production and standardization of the various products.

## DISTRIBUTION OF CULTURES

All cultures are distributed by or through the bacterial collection group. Cultures sent to laboratories in the state for the control of technic, or for use in the preparation of autogenous vaccines are prepared by the diagnostic department, which is responsible for their purity. A record of all cultures distributed is kept by the bacterial collection group on the "Distribution record" card.

The following highly pathogenic species, *B. anthracis*, *B. botulinus*, *B. mallei*, *B. pestis*, *B. tetani*, and *Sp. cholerae*, are distributed outside the laboratory only with the approval of the director; within the laboratory, after the request has been initialed by the bacteriologist in charge of the antitoxin, serum and vaccine laboratories. Strains of *Sp. cholerae* and *B. pestis*, or any cultures in fluid medium, may not be sent by mail. For further information regarding the shipment of cultures, see the United States Postal Regulations, 1924, section 461.

Cultures are distributed to the members of the laboratory staff when the request is initialed by a worker authorized to sign requisitions for general supplies, or, in the case of unusual requests, by the head of the department. In general, one transfer of a strain only is supplied. Cultures are also distributed to local laboratories in the state; and occasionally to laboratories outside the state when the request is approved by the bacteriologist in charge of the department. A culture requested by a physician in the state is sent to the registered laboratory designated by him.

Requests for cultures: Requests for cultures are received from members of the staff on "Memorandum of supplies" forms; from outside the laboratory, on telephone-message forms or by letter. Before referring a request for cultures from laboratories in the state to the collection group, the shipping group ascertains that the laboratory making the request is registered. Requests from outside the laboratory for highly pathogenic strains are referred to the director for approval. Unusual requests, requests for highly pathogenic strains from workers in the laboratory, or requests from unregistered laboratories or laboratories outside the state, are referred to the bacteriologist in charge of the department.

When a request is received, enter on the distribution record card the date, name and address of the person or laboratory making the request, culture numbers, and total number of cultures to be sent, and, in pencil, the probable date of shipment. When the request is received from outside, enter on it, the culture number and probable date of shipment; initial and return immediately to the shipping group; when from a member of the laboratory staff, hold the request until the cultures are distributed, then after dating and initialing it refer it to the shipping group.

### *Preparation of Cultures for Distribution*

Make a transfer from the latest stock culture of the strain requested to suitable medium. (Different media are kept in stock, dispensed in 125-by-13-mm. tubes for shipment.) At the same time, make transplants to other media when required to control satisfactorily the purity of the strain. After incubation of the culture for distribution, make a slide preparation and note results of the special tests. For more detailed procedures for certain cultures see the list which follows.

Cultures are distributed in the same media in which they are maintained (See: table 42) with the following exceptions: In general, the cultures maintained in nutrient gelatin medium are distributed on beef-infusion agar; gonococci, on testicular blood agar if available, or in ascitic-fluid semisolid agar; *B. pertussis*,

on alkaline coagulated-blood agar, since this medium is available in small tubes; anaerobic bacilli, in 2-per-cent dextrose-beef-infusion agar; and *B. diphtheriae* and diphtheroids, on Loeffler's coagulated-serum medium.

Seal the plug with paraffin. When a culture is to be sent out in semisolid medium, pipette on the surface of the medium enough of a melted, stiff agar to insure holding it in place. If cultures are to be distributed outside the laboratory in fluid medium, seal the tube carefully in a flame or insert a rubber stopper, and cap with fishskin. When advisable, as in the case of organisms which are grown with difficulty or those in frequent demand, prepare additional transplants, for distribution within two weeks.

Enter the name of the culture, collection number and date of transfer on a label with printed heading. After comparing the data with that on the tube, paste the new label over the original smaller label. For cultures to be distributed outside the laboratory, place on the tube another label giving the name and address of the person to whom the culture is sent. If the culture is to be used for autogenous vaccine, enter also the names of the patient and attending physician. Finally, record the necessary data on the "Distribution record" card and deliver the culture to the shipping clerk.

In the case of certain cultures, the following special procedures are carried out.

Meningococci: The production group prepares transplants on dextrose-serum-agar slants (125-by-13-mm. tubes), or if the cultures are to be more than two days in transit, in ascitic-fluid semisolid agar without oil. They also streak a blood-agar plate and inoculate a dextrose-serum-agar slant (150-by-19-mm. tube) for agglutination tests on the following day. If the agglutination reactions of the cultures have been tested within two weeks, this test may be omitted. The cultures for distribution and the plate are referred to the collection group before incubation. Upon satisfactory completion of the tests, the cultures are distributed.

Pneumococci (Standard, types I, II, III): Fresh cultures in serum semisolid agar are received from the production group at least once a month for use as seed cultures. When a request for a culture is received, a tube of broth is inoculated. On the following day, agglutination and bile-solubility tests are made and serum-semisolid-agar tubes inoculated, a blood plate being streaked with the growth remaining in the pipette. A Gram-stained preparation is made. If the tests are satisfactory, the culture is distributed without microscopic examination.

Streptococci (Standard strain, Dochez N. Y. 5): If available, a recent transfer is obtained from the production group from which cultures for distribution and a streak plate are prepared. Otherwise the cultures and streak plate are prepared by the production group and referred to the collection group before incubation. If tests are satisfactory the culture is distributed on the following day.

*B. acidophilus:* Transfers are made from the standard culture to milk and to

a beef-infusion-agar slant, and Gram-stained preparations are made from the cultures before the milk culture is distributed.

Bacilli, anaerobic: Two-tenths per cent dextrose-beef-infusion agar is pipetted into 120-by-16-millimeter tubes, boiled, and cooled to 43°C. The cooled medium is inoculated from the stock culture and incubated until good growth is obtained (from one to four days). A spore-stained preparation, if required, is made in addition to the Gram stain.

*B. diphtheriae:* ("Toxin" strain): The broth culture from which the latest transfer was made is obtained from the production group for use as a seed culture. Transfers are made to Loeffler's coagulated-serum medium. Slide preparations are stained with methylene blue.

*B. dysenteriae:* The tests described for *B. typhosus* are made, but the cultures are prepared only when a request is received.

Bacilli, encapsulated: Slide preparations stained for capsules by the Hiss method are made if required, in addition to the usual Gram stain.

*B. mallei:* A methylene-blue stained preparation for beaded forms is made when required, in addition to the usual Gram stain.

*B. pertussis:* Cultures are prepared and a blood-agar plate is streaked by the production group, and referred to the bacterial collection group before incubation.

*B. tetani* ("Toxin" strain): A comparatively recent, semisolid-agar culture is procured from the production group, and transfers are made to solid medium as described under "Bacilli, anaerobic." In certain cases, the original semisolid-agar culture, after slide preparations have been made, may be sealed and distributed.

*B. tuberculosis* and other acid-fast bacilli: An acid-fast stained preparation is made when required, in addition to the Gram stain.

*B. typhosus* and *B. paratyphosus* A and B: In order to have transplants of standard strains available for immediate distribution, several transfers to infusion-agar slants and a stab culture in triple-sugar-agar medium are made at least once in two weeks.

After incubation, a Gram-stained preparation of each culture is examined, the reactions in the triple-sugar agar are noted, and agglutination tests are made as follows (slide agglutination):

Mark off four sections on a glass slide. In the fourth space prepare a salt-solution suspension of organisms (several loopfuls of salt solution to one of growth from the triple-sugar-agar slant). Place a loopful in each of the preceding spaces and add a dilution previously found satisfactory, of typhoid, paratyphoid-A and paratyphoid-B immune serum respectively. Look for immediate macroscopic agglutination.

If results are satisfactory, the sealed and labeled tubes are kept in the cold room, ready for distribution.

Leptospira and treponemata: A dark-field examination of a recent transplant and a blood-agar streak plate are made. If satisfactory, the transplant is prepared for distribution.

### *Permanent Records*

Accession record card: The following data in regard to each culture received are entered on a card in order of receipt (5 by 8 in.): date received, species,

Collection No.....

Source:..... Date:..... Name:.....

| MORPHOLOGY | CULTURAL CHARACTERS | | BIOCHEMICAL REACTION | | | |
|---|---|---|---|---|---|---|
| | | | | Fermentation | Acid | Gas |
| Coccus, Bacillus, Spirillum... | Aerobe, Anaerobe..... | Broth..... | ..... | ..... | ..... | ..... |
| ..... | Colony: Agar, Gelatin | ..... | Indol..... | Milk..... | ..... | ..... |
| Division 1, 2, 3, planes..... | Surface..... | Blood Agar..... | ..... | Dextrose..... | ..... | ..... |
| Endospore..... ..... | ..... | ..... | Nitrates Reduced | Saccharose.... | ..... | ..... |
| Capsule..... | Deep..... | Blood Serum..... | ..... | Maltose..... | ..... | ..... |
| Motility..... | ..... | ..... | ..... | Lactose..... | ..... | ..... |
| Stain: Gram..... | Slant..... | Potato..... | ..... | Dextrin..... | ..... | ..... |
| ..... | ..... ..... | ..... | ..... | Mannite..... | ..... | ..... |
| ..... | Stab..... | ..... | ..... | Dulcite..... | ..... | ..... |
| Thermal Death Point..... | ..... | ..... | ..... | Inulin..... | ..... | ..... |
| | Liquefaction of Gelatin..... | ..... | ..... | Salicin..... | ..... | ..... |
| Pathogenicity (Toxicity):..... | | | ..... | ..... | ..... | ..... |
| ..... | | | ..... | ..... | ..... | ..... |
| ..... | | | ..... | ..... | ..... | ..... |
| ..... | | | ..... | ..... | ..... | ..... |
| Detailed history, special tests, etc. recorded on other side | | | | | | |

FIG. 54. HISTORY RECORD CARD

source, temporary collection number, and eventually (in red ink) the permanent collection number or "Discarded" with the date of final disposition.

History record card: A card (4 by 6 in., printed) is kept for each culture on which are entered: name of organism, date received, source, temporary and permanent (when assigned) collection numbers, results of morphological, cultural, and biochemical tests, and information as to pathogenicity; on the back, history of culture, references to correspondence, results of further tests or references to files where such results are recorded. (Fig. 54.)

Distribution record card: The following data in regard to each request for cultures are entered on a card (5 by 8 in.): date received, name and address of person or laboratory to whom cultures are to be sent, numbers of cultures, total number, and date request filled. (The probable date is entered in pencil pending final preparation for shipment.) Separate cards are kept for cultures distributed to members of the staff, elsewhere in New York State, and outside of the state.

## SECTION XII

## CHAPTER 1

## GENERAL INSTRUCTIONS

Instructions for certain procedures in general use in the antitoxin, serum, and vaccine laboratories have been brought together in this chapter, and also references to instructions for procedures in general use throughout the laboratory which are given in other parts of the book.

### *General Bacteriological Technic*

Follow the procedure given in "General Bacteriological Technic," p. 3, unless specific directions for other technic are given in the methods of the antitoxin, serum, and vaccine laboratories.

### *Use of Experimental and Test Animals*

Follow the procedure given in "Use of Experimental and Test Animals," p. 30, unless otherwise directed in the methods of the antitoxin, serum, and vaccine laboratories.

### *Stains and Methods of Staining*

Follow the procedure given under "Methods of Staining," p. 24.

### *Media*

For preparation of media used in connection with the production of the different biologic products, see "Preparation of Media," p. 64.

### *Aseptic Precautions*

In general, carry on all procedures connected with products for human use which require rigid aseptic precautions, in a draft-free room. Before entering the room, wash the hands thoroughly with soap and water, followed by glycerin if the skin is dry or rough. Wear sterile gowns and caps. Use the flame method throughout. Avoid unnecessary passing in and out of the room.

**Draft-Free Rooms.**—Always keep the doors of the draft-free rooms closed. In general keep the top ventilator closed and the bottom one open.

If the room has not been used for two or more days give it a thorough preliminary cleaning on the afternoon of the preceding day. In the morning before beginning work, wipe carefully the surface of the table with a cloth wrung out in 1-per-cent crude cresol.

After finishing work, remove all apparatus and supplies from the room. Wipe the floor and all flat surfaces with a cloth wrung out in 1-per-cent crude cresol. Wipe similarly the threshold of the door and the grooves in which it runs.

At least once a month, wash the windows and surfaces with a cloth wet with 1-per-cent crude cresol, or use a spray with the same solution. Then wipe the surfaces approximately dry with a cloth wrung out in the solution. Never permit dust to collect on any surface.

**Gowns and Caps.**—Place gowns and caps in muslin bags and paper, or paper alone, and have sterilized at 15-pounds pressure. Put them on immediately before entering the draft-free room and remove them just after leaving it. Adjust the cap to cover completely the hair.

**Pipettes.**—Use only freshly sterilized pipettes from containers which contain when first opened, a positive cobalt-ink sterility-control slip. Always remove the slip at once on opening the container. Use previously unopened containers when beginning work in the morning, and when beginning certain procedures requiring rigid aseptic precautions, as indicated in the various methods.

### *Orders*

Requisitions for supplies and orders for filling etc. are made out on special forms, and are initialed by the worker in charge of the group or other workers approved by the Laboratory Committee to sign requisitions for supplies. Unusual orders and all orders for special supplies or apparatus to be purchased, require the approval of the bacteriologist in charge of the department.

In making out requisitions give complete specifications covering each item. When time will be required to fill an order, make every effort to place it well in advance.

**For General Supplies.**—Order on requisition from the stock room, using the regular duplicating requisition books issued to each group. Give complete specifications on every item.

**For Media and Glassware.**—Use the "Media order" or "Glassware order" forms, and place with the media department.

**For Animals.**—Use the regular duplicating requisition book. For details see "The Use of Test and Experimental Animals," p. 30.

**For Cultures.**—Place the request with the bacterial collection group.

Use, for cultures to be distributed in the laboratory, a "Memorandum of supplies" form. If the cultures are needed in an emergency enter that fact on the request.

**For Filled Products from Stock.**—Place the request with the department secretary, using the forms as in requesting cultures. If the material is to be used for tests of sterility, or potency, etc., or research work within the department, indicate that it is to be recorded as "Withdrawn"; if to be distributed to other departments of the laboratory or used in tests on persons, "Distributed."

**For Filtrations and Fillings.**—Place instructions not later than Saturday for all filtrations and fillings to be made during the following week, consulting if necessary with the worker in charge of filtration or filling, respectively, in order that the work for all groups may be arranged to the best advantage. Use "Antitoxin and serum record" form (No. 15), see figures 49 and 50, or "Bacterial vaccine record" form (No. 25). When the material from one bottle is to be dispensed in different-sized doses (except vaccine sets) make out a separate order for each sized dose, adding a number preceded by a dash to the lot and bottle number to distinguish the doses (166A-1, 166A-2, etc.). Should only a portion of the material in a bottle be dispensed at one time, use "-a" to indicate the first filling, "-b" the second.

### *Records and Record Forms*

Keep careful records of all work, both routine and research, entering the required data in regard to each step as soon as they are available. Make the original entries directly on the permanent record unless there is definite reason why this is impracticable. In any case do not destroy the original record until the copied entries have been compared by a second person, and until a sufficient time has elapsed to insure that the permanent record contains all the data essential to a proper interpretation of the test. Make the entries in ink wherever practicable. In general keep the records of research work on 4-by-6-inch cards, following the procedure prescribed for the scientific files. For the forms to be used in keeping records of routine work, see the methods for the production of the various products, and the descriptions of certain forms in more general use which follow. In procedures involving calculations, such as vaccine dilutions, antitoxin tests, and concentration of antitoxin, make the original calculations on the backs of the record cards or sheets, and have them checked by a second worker.

**Duplicating Order Book.**—Order book (6 by 3½ in.) National No. 2810. Used for instructions accompanying cultures and toxins to the operating rooms, and shipments of serum, plasma, blood, etc., from the farm, and for other purposes as noted in the methods.

**"Antitoxin and serum record,"** figures 49 and 50. A working record in duplicate, accompanying each lot through the various steps of preparation and giving on the reverse side the names and addresses of the physicians or institutions to which distributed. This form is used for all products other than bacterial vaccines for which a similar form, "Bacterial vaccine record," is used. For further details see "Preparation of Biologic Products," p. 526.

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Date Rec'd.....Cost.....Age.....Sex.....Color.....
Weight.....Height.....Name.....
From.....Mallein test: ophthalmic.....subcutaneous.....
History.....Description.....

Horse No.....
Treatment.....
Method.....

| INJECTIONS | | | | | TETANUS ANTITOXIN TEMPERATURES, ETC. | SERUM TESTS AND BLEEDINGS | | | | | | | | REMARKS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Date | Inject. No. | | | | | Date taken | Date tested | Titre | Bleeding number | Amount | Serum | Plasma | Preparation number | |
| | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |

FIG. 55. HORSE RECORD

**Horse Protocol.**—Form 8½ by 11 inches (loose-leaf sheets, fig. 55). A description of each horse and a complete record of injections, bleedings, and other data relating to immunization are kept on these forms by the production groups. The autopsy findings are recorded on the reverse side of the last sheet.

## *Cultures*

The worker in charge of a group to which the care of standard strains has been assigned is responsible for their maintenance. Transfers should be made by him personally or by a trained worker who is definitely responsible. Rigid precautions must be taken against mixing the cultures. As far as possible the work with one strain should be completed before that with another is commenced. When a culture which has been opened is to be retained, the date of opening and initials of the worker are entered on the label.

Label every culture. Use a gummed label placed so as not to interfere with observation of growth, giving the kind and number of the culture and date of transfer.

When paraffin-sealed plugs are used, make sure the plugs are resealed after a transfer.

When cultures are maintained at incubator temperature only, keep two sets in different incubators.

Keep an open vessel containing water in each incubator used for cultures.

When cultures are to be placed in the incubator by the night watchman, place a signed note giving the hour and any other necessary data on his desk, and a duplicate with the cultures. The watchman enters on the duplicate the time at which he places the cultures in the incubator and his initials.

### *Material in Bulk*

Examine all bottles, flasks, and other apparatus for cracks or other imperfections before putting material into them.

Give a consecutive number to each lot of material in bulk and if a lot consists of more than one bottle, a letter to designate each bottle (166 designates a lot of one bottle only; 167A and 167B, a lot of two bottles). If a lot consists of two or more comparative batches, use Roman numerals to distinguish the batches (168I and 168II). If part of a lot, batch, or bottle is taken for further treatment distinguish the treated material by Arabic numerals in parenthesis. Example: A bottle divided and one portion only treated 166A, 166A(1); both portions treated, 166A(1), 166A(2).

Close all bottles containing "sterile" material in bulk with rubber stoppers previously boiled in 0.5-per-cent crude cresol and dried in the flame, cover with a wad of cotton dampened in 1-per-cent crude cresol, tie in place and cap with paper or with tinfoil and paper. When the bottle is to be reopened, remove the covering, and wipe the stopper and top of the bottle with this solution.

In general, when only an approximate estimate of the volume in a bottle is required, use a tape graduated for a bottle of that size.

In handling large bottles or flasks containing material, always support the container by one hand below; never lift it by the neck alone.

Ship all large bottles, as those with toxin for or plasma from the farm, in special wooden containers, see "Care and Treatment of Animals—Large Animals," p. 587. Accompany each shipment with a duplicating order form giving name of worker to whom shipped, date and

required data in regard to the shipment. On receipt of a shipment always compare the data on the form with the tags on the bottles.

### *Immunization of Horses*

As soon as a horse is assigned fill in the required information relating to it on a horse protocol sheet (see p. 619).

Before commencing the immunization of an animal take a small bleeding and hold the serum for comparison if required.

In general, when an injection or a bleeding would fall, according to the schedule of immunization, on a Sunday or a holiday, postpone it for one day, or even two, if the Sunday and holiday come together. When an animal is to receive a series of single injections at 3-day intervals, as a rule give the first injection on Wednesday, so that the scheduled injection will not fall on Sunday for three weeks.

If the morning temperature of a horse scheduled for injection exceeds 101°F., postpone the injection until the temperature is again normal. Do not have a bleeding for serum for therapeutic purposes taken from a horse with a temperature above 101°F.

Take care that cultures for immunization are not chilled during shipment. If necessary, use a special thermos jar, or wrap the cultures in a cotton and cheesecloth pad and pack in the copper container of a milk sample outfit. Fill the large can two-thirds full with warm water.

Accompany each shipment of cultures for immunization with a duplicating order form giving the date, name of person to whom shipped, kind or kinds of cultures, numbers of the horses to receive them, and the number and size of each dose.

Test for potency, sera from trial bleedings as soon as practicable after they have been received.

Store trial bleedings and samples from whole bleedings in separate baskets, and samples of finished antitoxin, according to kind and group (diphtheria antitoxin, pneumococcus serum type I, etc.). Keep trial bleedings for six months; the other samples, until the material has been released for distribution.

Enter promptly the autopsy findings of a horse dying while under immunization, which are received from the veterinarian on a "Horse autopsy" card (4 by 6 in.), on the back of the last sheet of the horse protocol, and return the card to the veterinarian.

### *Test Animals*

When the animals for a test vary in weight, in general, use the heavier animals for the doses representing the severer tests.

Place all dead animals which are not to be autopsied at once, immediately in the cans reserved for them in the cold room. Enter on the permanent record or the individual cage tag the time found dead, and initial. When the number of hours is of significance, if the animal is cold when found, place a minus sign before the hour as "–9 a.m.," and in calculating the time it lived, before the number of hours, as "−42 hrs." Wrap mice in paper, marking on it the number of the box or jar from which removed. Should the ear tag be lost from a larger animal follow the same procedure or attach a tag.

*Special Apparatus and Supplies*

**Apparatus for Filling "To Contain" Pipettes.**—The stand for filling and emptying "to contain" pipettes consists of (1) an iron ring stand with tripod base and rod 26 in. high; (2) a small clamp attached to the foot of the rod, holding a 5-cc. Luer syringe with air-tight plunger coated with a mixture of beeswax and vaseline; (3) a large clamp attached to the top of the rod, supporting an 8-in. horizontal glass tube with both ends bent downward 3 inches; (4) rubber tubing connecting one end with the syringe; (5) a short piece of hard rubber tubing of the required bore for holding "to contain" pipettes, attached to the other end. All parts can be adjusted to suit the convenience of the operator. With this apparatus the pipette is held in a position which enables the operator to see clearly the graduation and to draw the material exactly to it. See figure 56.

**Sterile Siphon and Burette with Stopcocks and Side Arm.**—Prepare the siphon for sterilization as given in "Preparation of Biologic Products," p. 529, except that the burette is not attached before sterilization. To sterilize the burette, place it in strong cleaning solution for at least half an hour, and rinse thoroughly with distilled water. Then fill it with 70-per-cent alcohol and allow to stand overnight, making sure that the alcohol penetrates between the stopcocks and sockets. Discharge the alcohol, smear the stopcocks with a sterile mixture of beeswax and vaseline, and connect the burette with the siphon, which has previously been placed in the bottle. When the apparatus is set up for continuous use, as when used for salt solution in the preparation of toxin and antitoxin dilutions, if drops adhere to the burette or the stopcocks stick, attach a pinchcock to the rubber tubing between the siphon and burette, disconnect the burette, protecting the end of the tubing with a sterile test tube, and clean as above. From time to time dismantle and clean the whole apparatus.

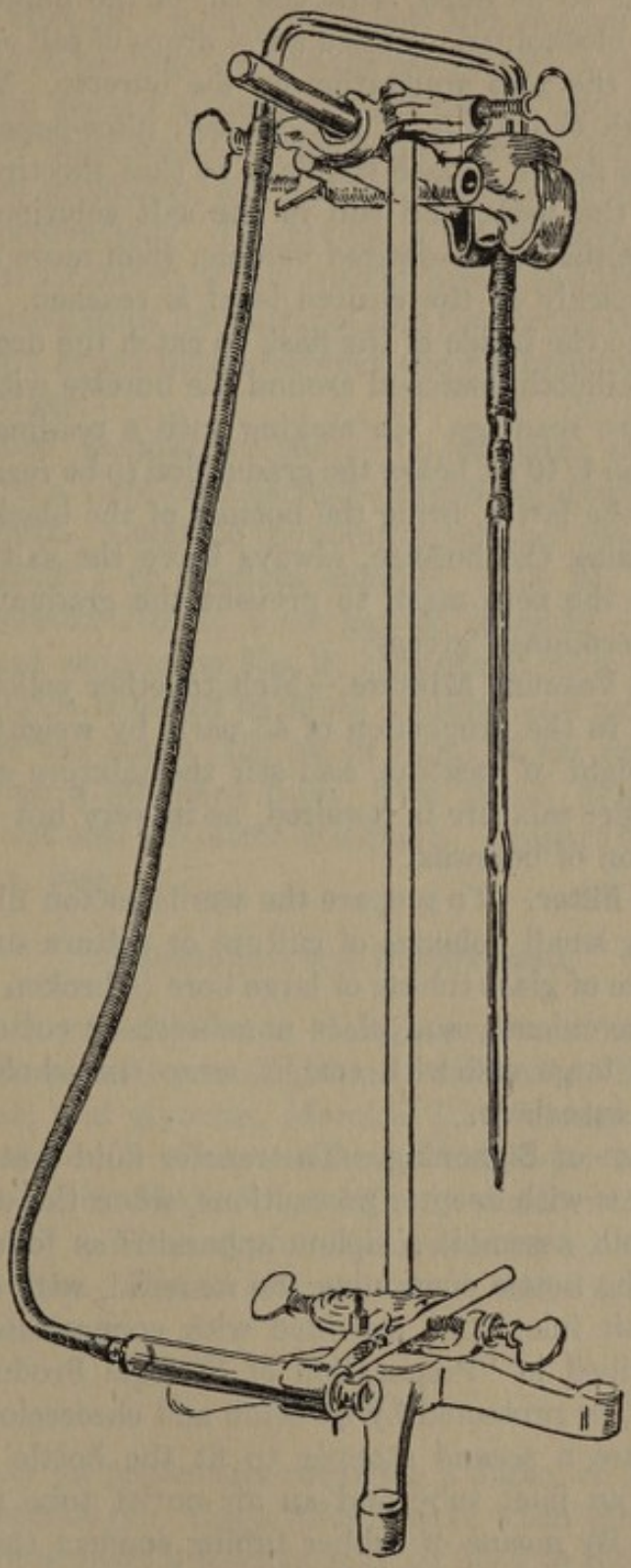

FIG. 56. "TO CONTAIN" PIPETTE

**Method of Filling Dilution Flasks from Burette.**—When a set of dilution flasks is to be filled, wipe the tip of the burette with cotton moistened with alcohol, and discard a few drops of salt solution. Bring the solution to the zero graduation on the burette. Wipe the tip of the burette with the inside of a sterilized, filter-paper cap removed from one of the flasks. Place the flask so that the tip of the burette extends below the neck, and run in the salt solution, rapidly until within a few tenths of the desired volume, then more slowly, turning the stopcock quickly as the desired level is reached. Touch the tip of the burette to the inside of the flask to catch the drop. Adjustable strips of black oilcloth, fastened around the burette with clips, may be used to facilitate readings. In making such a reading, place a strip at the graduation 1/10 cc. below the graduation to be read, and with the eye level with the latter, bring the bottom of the black meniscus just to it. After using the burette, always leave the salt solution some distance above the zero mark to prevent the graduated part of the burette from becoming "greasy."

**Beeswax and Vaseline Mixture.**—Melt together yellow beeswax and yellow vaseline in the proportion of 45 parts by weight of beeswax to 55 parts by weight of vaseline, and stir the mixture constantly as it cools. If a stiffer mixture is required, as in very hot weather, add a larger proportion of beeswax.

**Small Cotton Filter.**—To prepare the sterile cotton filters frequently used in filtering small volumes of culture or culture suspension, draw out a short piece of glass tubing of large bore (a broken 180-by-25-mm. test tube is convenient), and place nonabsorbent cotton in the small end. Plug the large end with cotton, wrap the whole in paper and sterilize in the autoclave.

**Closed System of Siphoning.**—To transfer fluid material from one bottle to another with aseptic precautions, when the use of a pipette is not practicable, assemble a siphon apparatus as follows: Prepare a stopper to fit the bottle containing the material, with a siphon outlet tube, and an air inlet tube provided with connections for a Woulff bottle as described in "Preparation of Biologic Products for Distribution," p. 529, or protected by a cotton and cheesecloth pad to filter the air. Prepare a second stopper to fit the bottle to receive the material, with an inlet tube and an air outlet tube protected by a cotton guard. By means of rubber tubing connect the siphon outlet tube in the first stopper with the inlet tube in the second. Wire all joints between rubber and glass. For the details of the connections of the second stopper, and also for the preparation for sterilization of

all rubber and glass connections, see "Filtration of Biologic Products; Filtration by Suction," p. 516. Protect the stopper and end of the siphon which is to be inserted into the bottle with a paper "envelope," and protect the second stopper with a cotton and cheesecloth pad. Wrap the entire apparatus in brown paper for sterilization.

To set up the apparatus, insert the stoppers in the respective bottles with aseptic precautions. If gravity is to be used, apply negative pressure to the air outlet of the second bottle to start the flow; or if gravity is not used, draw the material into the second bottle by negative pressure, using a guard bottle between the air outlet of the bottle and the vacuum intake.

**Barium-Sulfate Standards** (168).—To prepare, use a 1-per-cent sulfuric acid and a 1-per-cent barium-chloride solution prepared by the analytical chemist. Combine the solutions so that the following standards are obtained: 1 cc. barium chloride + 99 cc. sulfuric acid = standard suspension No. 1; 2 cc. barium chloride + 98 cc. sulfuric acid = standard suspension No. 2; continue in this manner until 10 cc. barium chloride is added to 90 cc. sulfuric acid making suspension No. 10. Select ten tubes (150 by 19 mm.) of the same diameter by fitting each over a standard tube. Put 10 cc. of each suspension into the tubes and seal the latter in a flame. Prepare fresh suspensions at least once a year.

### *Preservatives and Disinfectants*

Use high-grade preservative in biologic products; at present Phenol U.S.P. crystallized, in 1-pound bottles; Mulford's "Three cresols,"in 1-pound bottles; and glycerin, Merck's T.P. "Reagent" sterilized at 15-pounds pressure for forty minutes. Use 1-per-cent crude cresol (see "Preparation of Media," p. 106) as disinfectant except when otherwise directed. When alcohol is required as a disinfectant, use a 70-per-cent solution. Should iodine be used, make sure the surface is dry before applying it.

### *Miscellaneous*

When carrying on procedures requiring a flame, do not use a larger flame than is necessary. Use a microburner when practicable.

In special emergencies a product may be filled in the final containers before the sterility tests of bulk material are completed, provided the sterility tests have shown no contamination during the first two or three days. In general the heads of the production and of the filling groups

should plan so that the supply of boxed material on hand of each product is sufficient for at least a month.

Serum unsatisfactory for therapeutic use owing to the condition of the horse, or the titer or the age of the serum, may be used for diagnostic purposes provided it meets the requirements for such serum. No lot of serum should be discarded without the approval of the bacteriologist in charge.

Workers in the diagnostic department and in special research groups, using cultures and biologic products obtained from the antitoxin, serum, and vaccine laboratories, are expected to report immediately anything unusual which they observe in connection with the cultures or products, such as an apparent decrease in titer or lack of specificity of a diagnostic serum.

Syringes: All syringes, before being placed in stock, are tested for possible leakage around the piston and at the junction with the needle. Those showing more than very slight leakage are rejected. Syringes to be used in special work for accurate measurement of volumes, are tested for accuracy of delivery by measuring the volumes dispensed in an accurately graduated "to contain" cylinder; or when extreme accuracy is required, by weighing water delivered by the syringe. Syringes found to be accurate are reserved for purposes requiring them. Those showing slight inaccuracy which will not interfere with the usual laboratory work, are placed in stock separately. It has occasionally been necessary to reject syringes, showing marked inaccuracy.

Pipettes: Pipettes, except those for special purposes which are described, with their care, in the methods for the procedures in which they are used, are cleaned and sterilized in the media department. In general each group has its individual pipette containers, marked with the name of the worker in charge and the capacity of the pipettes. These are filled with pipettes from the general supply maintained in the media department. Additional pipettes and containers are obtained as required.

Place pipettes after use in a battery jar or wide-mouth 1-liter bottle in the bottom of which a piece of cotton, covered with 1-per-cent crude cresol, has been placed. Take to the media department all used pipettes, and all pipette containers holding pipettes.

Contaminated glassware: Place glassware, other than pipettes, which has come in contact with contaminated material, in pails provided by the media department for the purpose, or take the glassware personally to the media department for sterilization.

## PREPARATION OF DILUTIONS

The following methods and examples are given as a guide in preparing dilutions.

It is convenient to express in the form of a fraction or decimal the concentration of the reagent (serum, toxin, culture, etc.) employed in a test. For the purpose of calculation, decimals are most conveniently reëxpressed as fractions. The fraction 1/10 represents 1 part in 10, or 1 part of reagent plus 9 parts of diluent. The decimal 0.005, reduced to a fraction is $\frac{5}{1000}$ or, in its simplest terms $\frac{1}{200}$, which represents 1 part of reagent plus 199 parts of diluent.

It is very simple to prepare directly such dilutions as 1/10, 1/50, 1/100. If "M" represents the material to be diluted and "d" the diluent, these dilutions may be expressed as follows:

1/10 = 1 cc. M + 9 cc. d (0.5 + 4.5, or 0.1 + 0.9, etc.)
1/50 = 1 cc. M + 49 cc. d (0.5 + 24.5, or 0.1 + 4.9, etc.)
1/100 = 1 cc. M + 99 cc. d (0.5 + 49.5, or 0.1 + 9.9, etc.)

It is usually simpler to reduce a higher fraction to a simple fraction, the numerator of which is 1, but this is not essential. Thus:

3/100 = 3 cc. M + 97 cc. d.

When one reaches the larger fractions, such as 1/1000, it is necessary to proceed by intermediate dilutions. In fact, it is frequently desirable to do this in preparing lower dilutions, such as 1/100, particularly when but a small volume is needed, and a high degree of accuracy is not required. To make a dilution in two steps, the fraction representing the desired dilution is restated as the product of two factors one of which is the primary dilution used, the other the secondary. For example, to make a 1/1000 dilution: $\frac{1}{1000}$ may be stated as $\frac{1}{100} \times \frac{1}{10}$; $\frac{1}{50} \times \frac{1}{20}$; $\frac{1}{40} \times \frac{1}{25}$; etc. and a $\frac{1}{1000}$ dilution may be made by any of the following combinations:

| | |
|---|---|
| 1 cc. of a $\frac{1}{100}$ dilution + 9 cc. diluent | 1 cc. of a $\frac{1}{10}$ dilution + 99 cc. diluent |
| 1 cc. of a $\frac{1}{50}$ dilution + 19 cc. diluent | 1 cc. of a $\frac{1}{20}$ dilution + 49 cc. diluent |
| 1 cc. of a $\frac{1}{40}$ dilution + 24 cc. diluent | 1 cc. of a $\frac{1}{25}$ dilution + 39 cc. diluent |

A fact which should be considered in preparing dilutions where accuracy is essential, is that in general, the larger the volumes of material and diluent, the smaller is the percentage error. Where accuracy is necessary, it is undesirable to measure less than 0.5 cc. of the material to be diluted. In preparing a series of dilutions, it is usually advisable to use the highest dilution first. Thus, in preparing a 1/4000 dilution, the preferable method would be to make a 1/100 dilution, followed by a secondary 1/40 dilution. On the other hand, it is preferable to prepare only a moderately high initial dilution, to avoid the errors incident upon preparing a very low secondary dilution. For instance, in preparing a 1/400 dilution, where the alternatives are a primary dilution of 1/100 with a secondary 1/4; a 1/50 with a secondary 1/8; and a 1/20 with a secondary 1/20; the last would be chosen as involving the least error.

In repeating tests, or in performing tests whose results are to be compared, it is desirable to use the same or similar dilutions made in the same order.

The following is an example of a series of dilutions which might be used in an agglutination test, in which the quantities required are small (assume 3 cc.), and a high degree of accuracy is not essential.

| DILUTION DESIRED | PRIMARY DILUTION | FACTOR | VOLUME OF PRIMARY DILUTION | VOLUME DILUENT |
|---|---|---|---|---|
| 1/10 a | —— | —— | 1 cc. M | + 9 cc. |
| 1/50 b | 1/10 (a) | × 1/5 | 1 cc. a | + 4 cc. |
| 1/100 c | 1/10 (a) | × 1/10 | 1 cc. a | + 9 cc. |
| 1/200 d | 1/100 (c) | × 1/2 | 1 cc. c | + 1 cc. make (2 + 2) |
| 1/300 e | 1/100 (c) | × 1/3 | 1 cc. c | + 2 cc. make (2 + 4) |

The next series is an example of one in which greater accuracy is necessary.

| DILUTION DESIRED | PRIMARY DILUTION | FACTOR | VOLUME OF PRIMARY DILUTION | VOLUME DILUENT |
|---|---|---|---|---|
| —— | —— | —— | 1 cc. M | + 99 cc. a |
| 0.0005 or 1/2000 | 1/100 | × 1/20 | 1 cc. a | + 19 cc. |
| 0.0004 or 1/2500 | 1/100 | × 1/25 | 1 cc. a | + 24 cc. |
| 0.0003 or 3/10,000 | 1/100 | × 3/100 | 3 cc. a | + 97 cc. |

Equation: A simple equation may be used in more involved calculations to determine any one of three factors: (1) the quantity of reagent contained in a definite volume of a dilution of known concentration; (2) the volume of a dilution of a known concentration which contains a definite quantity of reagent; or (3) the dilution required to give a definite quantity of reagent in a definite volume.

If X represents the quantity of reagent, 1/Y the dilution, and Z the volume of the dilution, $X \div 1/Y = Z$, or simplified it may be stated as $X = Z/Y$; $Z = XY$; or $Y = Z/X$ (or $1/Y = X/Z$).

Examples:

(1) X is unknown; Y = 400; Z = 2.0 cc.

$X = Z/Y$, or $\frac{2.0}{400} = 0.005$ = amount of reagent contained in 2 cc. of a 1/400 dilution.

(2) X = 0.005; Y = 400; Z is unknown

$Z = XY$, or $400 \times 0.005 = 2.0$ cc. = volume of a 1/400 dilution containing 0.005 cc.

(3) X = 0.005; Y is unknown; Z = 2.0 cc.

$Y = Z/X$, or $\frac{2.0}{0.005} = 400$ $(1/Y = 1/400)$ = dilution required to contain 0.005 cc. of reagent in 2 cc.

(4) Procedure when a series of quantities to be tested must be contained in a definite volume, i.e. X and Z are known. The equation is $Y = \frac{Z}{X}$, or $\frac{1}{Y} = \frac{X}{Z}$.

If it is desired to have 0.002 cc., 0.003 cc. and 0.004 cc. of reagent contained in 2 cc. of the final dilutions

$$X = 0.002,\ Z = 2.0;\ \frac{1}{Y} = \frac{0.002}{2.0} \text{ or } \frac{1}{1000}$$

$$X = 0.003,\ Z = 2.0;\ \frac{1}{Y} = \frac{0.003}{2.0} \text{ or } \frac{3}{2000}$$

$$X = 0.004,\ Z = 2.0;\ \frac{1}{Y} = \frac{0.004}{2.0} \text{ or } \frac{2}{1000}$$

In the preparation of a series of dilutions such as the preceding, several alternatives are presented. In general, it is desirable, so far as possible, to start from the same initial dilution, and to prepare as few dilutions as possible. Thus of the following series 1 or 2 may be used, but 3 is not desirable.

Series 1, requiring four dilutions (one initial dilution)

$$\frac{1}{1000} = \frac{1}{50} \times \frac{1}{20}$$

$$\frac{3}{2000} = \frac{1}{50} \times \frac{3}{40}$$

$$\frac{2}{1000} = \frac{1}{50} \times \frac{2}{20}\ \frac{(1)}{(10)}$$

Series 2, requiring four dilutions (one initial dilution)

$$\frac{1}{1000} = \frac{1}{40} \times \frac{1}{25}$$

$$\frac{3}{2000} = \frac{1}{40} \times \frac{3}{50}$$

$$\frac{2}{1000} = \frac{1}{40} \times \frac{2}{25}$$

Series 3, given as an example of an undesirable series requiring five dilutions (two initial dilutions)

$$\frac{1}{1000} = \frac{1}{50} \times \frac{1}{20}$$

$$\frac{3}{2000} = \frac{1}{50} \times \frac{3}{40}$$

$$\frac{2}{1000} = \frac{2}{50} \times \frac{1}{20}$$

(5) Procedure when the volume of dilution need not be a definite quantity but should be less than a definite small volume, such as 2 cc. If it is desired to have 0.0003, 0.0005, and 0.0007 cc. of reagent contained in less than 2.0 cc. of the final dilution,

Let X be the maximum dose, 0.0007 cc., and Z the maximum volume, 2 cc.

$$Y = \frac{2}{0.0007} = 2857+ \quad \left(\frac{1}{Y} = \frac{1}{2857+}\right)$$

Any convenient dilution lower than 1/2857+ may be chosen. A 1/2000 dilution is satisfactory, since it gives simple final volumes.

$0.0003 \times 2000 = 0.6$ cc. of dilution to contain 0.0003 cc.
$0.0005 \times 2000 = 1.0$ cc. of dilution to contain 0.0005 cc.
$0.0007 \times 2000 = 1.4$ cc. of dilution to contain 0.0007 cc.

After the volumes of the dilution have been measured, they may be brought to a common volume, as is often required when animals are to be injected, by adding physiological salt solution.

(6) Procedure when a series of doses to be tested must be contained in volumes between definite limits; X is known and Z is limited.

If it is desired to have 0.003, 0.004, and 0.005 cc. contained in volumes of one dilution lying between 1 cc. and 2 cc.,

Let X be the maximum dose, 0.005 cc., and Z the maximum volume, 2 cc.

$Y = \frac{2}{0.005} = 400\ \left(\frac{1}{Y} = \frac{1}{400}\right)$, the highest dilution which may be used.

Let X be the minimum dose 0.003 cc., and Z the minimum volume, 1 cc.

$\frac{1}{0.003} = 333\frac{1}{3}$, $\frac{1}{Y} = \frac{1}{333\frac{1}{3}}$, the lowest dilution which may be used.

Any convenient dilution between these two may be used. If $\frac{1}{400}$ is chosen

$400 \times 0.003 = 1.2$ cc. of dilution to contain 0.003 cc.
$400 \times 0.004 = 1.6$ cc. of dilution to contain 0.004 cc.
$400 \times 0.005 = 2.0$ cc. of dilution to contain 0.005 cc.

# METHODS USED IN THE EXECUTIVE OFFICES

ILA M. DUTTON, *Executive Clerk*
ANNA M. SEXTON, *Secretary to the Director*

# INTRODUCTION

The administrative work of the laboratory is carried on by the Laboratory Committee, composed of the heads of the different departments, who choose from among their number a chairman and a secretary. The Committee meets once a week, or oftener, if necessary. In order to secure uniformity in the handling of detail, matters of general interest in the laboratory are discussed. Any change in policy or standards is referred to the director. The minutes of the meetings are kept in permanent form.

The executive offices are responsible for the executive, secretarial, and clerical work of the laboratory. All this work has been developed and systematized in accordance with approved modern practice. Consequently, procedures common to all business offices are but briefly outlined, and only those peculiar to this public-health laboratory are given in detail. The work of the executive offices is divided as follows:

I Recording and Reporting Results of Examinations and the Approval, and Registration of Laboratories
II Distribution of Laboratory Preparations and Outfits
III Purchase, Storage, and Distribution of Equipment and Supplies
IV Communication

Each of the four main divisions, with the necessary number of assistants, is under the immediate charge of a qualified secretary. Other secretaries are assigned to special work or to heads of departments. These secretaries compose the secretarial staff of the executive offices. They are required to have training in some branch of biologic science as well as in the technical phases of office work, and to have a reading knowledge of either French or German. They are capable of relieving the scientific staff of all clerical and secretarial work, and of assisting the library group in looking up data relative to research and to other problems, and thus they help to unify the whole laboratory organization.

## SECTION I

# RECORDING AND REPORTING RESULTS OF EXAMINATIONS IN THE DIAGNOSTIC LABORATORIES AND THE LABORATORIES FOR SANITARY AND ANALYTICAL CHEMISTRY; APPROVAL AND REGISTRATION OF LOCAL LABORATORIES

## RECORDING AND REPORTING RESULTS OF EXAMINATIONS

The results of the laboratory examination of specimens are reported to physicians and health officers in accordance with the Public Health Law and Sanitary Code. These examinations are classed as "routine" examinations and "miscellaneous" examinations. The routine examinations include those for diphtheria, gonorrhea, syphilis, tuberculosis, and typhoid fever. All others come under the head of miscellaneous examinations (See: specimens for miscellaneous examinations).

### RECEIVING SPECIMENS

Mail is collected from the post office five times each day, with the exception of Sundays and holidays, 5:00, 10:00, and 11:30 a.m., 4:15 and 10:00 p.m. The 10:00 and 11:30 a.m. collections are not made on Sundays and holidays.

**Procedure.**—Sort the specimens received in the 5:00 a.m., 4:15 p.m., and 10:00 p.m. collections according to their various kinds and enter the number of each and the time received on the sheet provided for that purpose. Place in the incubator immediately all cultures to be examined for diphtheria bacilli, and blood cultures in Keidel outfits, accompanied by a note of the hour and date received. Place all other specimens in the cold room. Deliver directly to the diagnostic laboratories all specimens received during the day, whether by mail, express, or messenger.

### ROUTINE EXAMINATIONS

An accession book is kept for each of the following routine types of examinations:

Diphtheria
  Morphological examination
Gonorrhea
Syphilis
  Complement-fixation test
Tuberculosis
  Microscopical examination
  Complement-fixation test
Typhoid fever
  Agglutination test
  Cultural examination

Data for the accession books are obtained from entries made by the technician and the examiner on the history blanks sent in by physicians with the specimens (see: fig. 57).

| CHECKED BY | LABORATORY NUMBER | NUMBER OF EXAMINATION | NUMBER OF SPECIMEN | RESULT | PATIENT'S NAME | PHYSICIAN'S NAME AND ADDRESS |
|---|---|---|---|---|---|---|
| | | | | | | |
| | | | | | | |
| | | | | | | |

FIG. 57. FORM FOR ENTERING DATA IN ACCESSION BOOK

History blanks are received at the accession books, from the workers in the diagnostic laboratories, two different times.

**Procedure.**—As soon as the blanks are received from the technician, enter the

Laboratory Number
Number of Specimen
Patient's Name
Physician's Name and Address.

When received from the examiner, enter the

Result of Examination
Number of Examinations
(For ready reference, enter the positive results in red ink.)

At the end of each month, total the items in the accession books for the monthly and yearly reports.

*Reporting*

Send reports on laboratory examinations in every case to the physicians who submit the specimens.

Send copies of reports of all routine examinations, except gonorrhea and syphilis, and of some miscellaneous, to the proper health officers. In the case of gonorrhea and syphilis, send only copies of the positive reports and of the negative reports on treated cases.

Occasionally, specimens are sent to the laboratory by persons who are not licensed to practice medicine in the state. In order to avoid delay, these specimens are examined. The results are reported only to the health officer and to the attending physician, if any, in charge of the case.

As the attorney general of the state has given an opinion that osteopaths are physicians within the meaning of the law, and are entitled, therefore, to receive reports, send them the results of the laboratory examinations of specimens which they have submitted to the state laboratory.

*Typing Reports*

The report forms used are uniform as to size and arrangement of data. The positive, negative, and "unsatisfactory" diphtheria report blanks (figs. 58, 59, 60, and 61) are shown to illustrate these standards.

The forms for positive reports in most instances are in colors[1] corresponding to the history blanks and labels on the outfits designed for the submission of specimens from persons suspected of having the communicable diseases mentioned in Chapter II, Regulation 2-a of the Sanitary Code.

| | |
|---|---|
| Diphtheria | blue |
| Gonorrhea | brown |
| Miscellaneous | pink |
| Pneumonia | grey |
| Syphilis | white |
| Tuberculosis | yellow |
| Typhoid fever | green |

All other forms are white with the exception of the unsatisfactory report blanks which are pink.

[1] The use of colors for positive reports connotes diagnosis which should never be made in the laboratory and some physicians so interpret them. For this reason the use of colors may sometime be abandoned despite the fact that they are otherwise very convenient in the office procedures.

**Procedure.**—Have all reports typewritten. Do not permit erasures in the results of examinations and of the patients' names. If corrections are necessary, retype the reports.

After the reports are typed, compare them with the history blanks to insure the correctness of the laboratory number, the patient's name

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DIVISION OF LABORATORIES AND RESEARCH
NEW YORK STATE DEPARTMENT OF HEALTH
*New Scotland Ave., Albany*

Laboratory number..... Date.....

In the examination of the culture from

Name.....

Address.....

Date taken.....

Culture from throat .....nose.....

Dr.....

Diagnosis..... Release..... Carrier.....

Diphtheria bacilli were found.
(*This report is based upon morphological examination alone*)

If the patient is a "carrier" with no history of illness or contact with a case of diphtheria, a culture may be submitted for virulence tests immediately.

AUGUSTUS B. WADSWORTH, M.D.
*Director*

Dr.....
*Health Officer*

---

FIG. 58. DIPHTHERIA REPORT FORM (POSITIVE)
(Blue paper)

and address, the date of taking the specimen, the name and address of both the physician and health officer, and the result of the examination. Check the carbon copies of the reports by the accession books to indicate that the reports have been sent.

Reports that are completed during the morning are sent by messenger to the post office at noon; those typed during the afternoon are collected by the postman and taken directly to the post office.

### *Telegraphic and Telephonic Reports*

Report by telegraph, charges collect, to both the attending physician and the health officer positive results on first cultures for the diagnosis

of diphtheria, if antitoxin has not been administered. If antitoxin has been given, notify only the health officer in this way. Send a confirmatory typed report in every instance. Otherwise, send telegrams only on request, or when a case seems urgent.

---

DIVISION OF LABORATORIES AND RESEARCH
NEW YORK STATE DEPARTMENT OF HEALTH
*New Scotland Avenue, Albany*

Laboratory number..... Date.....

In the examination of the culture from

Name.....

Address.....

Date taken.....

Culture from throat.....nose.....

Dr.....

Diagnosis..... Release..... Carrier.....

No diphtheria bacilli were found.

In an extremely small number of cases of true diphtheria, the diphtheria bacilli may not be found in the first culture, whereas in later cultures they are found. In laryngeal diphtheria, the pharynx may not have become involved but diphtheria bacilli may be found in a second culture.
In all doubtful cases, confirmatory cultures should be forwarded at once.

AUGUSTUS B. WADSWORTH, M.D.
Director

Dr.....
*Health Officer*

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FIG. 59. DIPHTHERIA REPORT FORM (NEGATIVE)
(White paper)

### *Reports on Sundays and Holidays*

The reporting of diphtheria, pneumonia, and positive typhoid-fever examinations on Sundays and holidays is an established routine. Other reports which are considered important by the examiners are also sent on these days.

## MISCELLANEOUS EXAMINATIONS

The procedure for receiving all specimens at the laboratory and typing reports of examinations is the same. A separate accession book is kept for recording miscellaneous examinations. They are entered under the following headings:

Animal autopsies
Animal tests—rabies

---

DIVISION OF LABORATORIES AND RESEARCH
NEW YORK STATE DEPARTMENT OF HEALTH
*New Scotland Avenue, Albany*

Laboratory number..... Date.....

The examination of the specimen of.....
from patient:

Name.....

Address.....

Date taken.....

Dr.....,

Was unsatisfactory for the following reason:
(See reverse side)

Another specimen should be forwarded at once.

AUGUSTUS B. WADSWORTH, M.D.
*Director*

Dr.....
*Health Officer*

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FIG. 60. REPORT FORM (UNSATISFACTORY)
(Pink paper)

(front)

Autogenous vaccines
Blood chemical
Blood cultures
Blood—Differential count, red and white cell, hemoglobin
Confirmatory examinations
Cultures for identification
Discharges, pus, sputum, etc.
Intestinal discharge

Laboratory investigations
Malaria
Pathological specimens
Pleural, ascitic, and other body fluids
Pneumonia
Rabies
Special investigations

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A—Received in poor condition.
I Serum medium dried, liquefied or contaminated.
II Tube broken. Not properly packed.
III Blood hemolyzed.
IV Specimen spoiled, or too old, delayed in transit.
V Insufficient material for test. Specimen leaked out in transit.
VI Specimen probably frozen in transit.
VII Culture contaminated (probably by organisms that were present in the mouth or nasopharynx of the patient).
VIII Specimen showed evidence of putrefactive changes.

B—Not properly taken.
I Insufficient material sent.
II Culture medium not thoroughly inoculated. Insufficient growth obtained.
III Film not properly made—too thick.
IV Not completely dried.
V Proper outfit for specimen not used; specimen spoiled.

C—Result of examination doubtful.
I Organisms atypical, or their presence of doubtful significance.
II Reaction partial.
III Nonspecific reaction obtained; specimen "anticomplementary."

D—Examination not made.
I See explanatory note.

E—Examination unsatisfactory.
I See explanatory note.

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FIG. 61. REPORT FORM (UNSATISFACTORY)
(Pink paper)

(back)

Spinal fluid
Stomach contents
Syphilis—Dark field
Syphilis—Dried preparations
Unclassified
Urine analysis
Vincent's angina

Virulence tests
Weil-Felix tests

When the number of specimens submitted for any one of the miscellaneous examinations becomes large enough to justify inclusion in the routine examinations, the change in classification is made, first in the diagnostic laboratories, and later, when a routine history blank is adopted for the type of specimen in question, in the office records.

### *Reporting*

Send the report to the physician who submitted the specimen and a carbon copy to the laboratory files.

If the report is on any of the communicable diseases mentioned in Chapter II, Regulation 1, of the Sanitary Code, have the reporter in the diagnostic office group indicate on the history blank by means of a rubber stamp that a copy of the report should be sent to the health officer of the district from which the specimen came. Should a health officer take a specimen, send a copy of the report to him as well as to the attending physician.

## LEGAL STATUS OF RECORDS

### *Tuberculosis*

Section 322, Article XVI of the Public Health Law and Regulation 8, Chapter VII of the Sanitary Code require the protection of records of examinations showing the presence of tubercle bacilli, in the custody of health authorities of cities, towns, and villages. It is assumed, by inference, that if certain records in the custody of local officials are required to be kept confidential, similar records in the custody of this department should also be regarded as confidential. Therefore, the following tentative procedure has been established.

**Procedure.**—Refer officials of organizations, other than of the Division of Tuberculosis of the State Department of Health, requesting access to, or copies of, tuberculosis records, to local health officers (who are specifically authorized by Regulation 8, Chapter VII of the Sanitary Code, to furnish information to representatives of certain organizations).

If subpoenaed to produce tuberculosis records in court, produce the records (Regulation 14, Chapter VII, Sanitary Code). Before disclosing any information, ask the court (presiding judge) for instructions as to whether or not questions calling for disclosure of such information should be answered.

If a physician states that he is the present attending physician in a case in which another physician submitted a specimen of sputum, and if he asks for the result of the previous examination, give him the information on the assumption that it will be in the interest of the patient.

*Venereal Diseases*

As Regulation 29-b, Chapter II of the Sanitary Code makes all records of this and of local health departments, relating to venereal disease, strictly confidential, the following procedure has been drawn up and is in force at this laboratory:

**Procedure.**—Do not permit any persons, other than those responsible for examinations or records in the laboratory, to have access to any records relating to venereal disease.

Upon receipt of a request from any person other than the physician who submitted the specimen or the patient from whom the specimen was taken, for information as to whether or not a specimen has been received for examination, tell the inquirer that we are prohibited by law from divulging such information.

If a physician attending a patient requests information regarding the results of examination of a specimen submitted by a physician previously attending the same patient, or for a copy of the report, advise him that state laws and regulations make such information confidential and that, in the opinion of the attorney general, we have no right to divulge it, except as specifically stipulated in such law or regulation, without evidence that the patient waives the privilege of having such information deemed confidential.

A letter from the patient, witnessed by the physician or another person, requesting that the information be furnished to the physician, is accepted as evidence that the privilege is waived.

If a legal order is received to produce venereal-disease records in court, have a laboratory representative familiar with these records take them to the place indicated at the appointed time. Before divulging any information, either as to the results of examinations or even as to whether any such specimen has been received or examined, call the attention of the court (presiding judge) to Regulation 29-b, Chapter II of the Sanitary Code, and to our understanding that all information with reference to venereal-disease specimens is to be deemed absolutely confidential, and ask for instructions as to whether or not the information should be given as required.

## WATER EXAMINATIONS

Water samples received during the day are taken immediately to the laboratories for sanitary and analytical chemistry. If samples are received during the night, they are placed immediately in the cold room assigned to the water laboratories and a memorandum to that effect is left on the table in the water laboratories, together with any other information received with the samples.

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NEW YORK STATE DEPARTMENT OF HEALTH

Matthias Nicoll, Jr., M.D.
Commissioner

Division of Laboratories and Research

Paul B. Brooks, M.D.
Deputy Commissioner

Augustus B. Wadsworth, M.D.
Director

WATER

*List of Examinations*

| LABORATORY NUMBER | C = CHEMICAL B = BACTERIAL | PUB. = PUBLIC PR. = PRIVATE INST. = INSTITUTION | MUNICIPALITY | SOURCE OF SAMPLE | DATE OF COLLECTION | BACTERIAL EXAMINATION | | | | | | REMARKS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Quantitative | | Qualitative | | | | |
| | | | | | | Number of Bacteria per c. c. | | Intestinal Bacteria | | | | |
| | | | | | | Gelatin | Agar | B. Coli Type | | | | |
| | | | | | | 20°c. | 37° c. | 10 c. c. | 1 c. c. | .1 c. c. | .01 c. c. | |
| ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | .... | .... | .... | .... | ..... |
| ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | .... | .... | .... | .... | ..... |
| ..... | ..... | ..... | ..... | ..... | ..... | ..... | ..... | .... | .... | .... | .... | ..... |

FIG. 62. ACCESSION BOOK FORM FOR WATER EXAMINATIONS

### *Recording*

Each sample is recorded in an accession book as soon as received in the laboratories for sanitary and analytical chemistry (fig. 62).

### *Typing Reports*

Type the reports on form 44 (fig. 63), with the necessary number of carbon copies, and mail them as instructed by the laboratories for sanitary and analytical chemistry. Always send a carbon copy of the report to the Department of Administration. (See: water.)

*Public Health is Purchasable. Within Natural Limitations Any Community Can Determine Its Own Death Rate*

NEW YORK
STATE DEPARTMENT OF HEALTH
ALBANY

Matthias Nicoll, Jr., M.D.
Commissioner
Paul B. Brooks, M.D.
Deputy Commissioner

Division of Laboratories and Research
New Scotland Ave., Albany
Augustus B. Wadsworth, M.D.
Director

Laboratory numbers..... Date rec'd..... Reported.....

Place.....
(City, town, village or institution)

Collected from.....

By..... Date.....

*Physical Examination* *Results in Parts per Million*

Color..... Turbidity..... Odor, cold..... Odor, hot.....

*Chemical Examination* *Results in Parts per Million*

Solids, total.....
Loss on ignition.....
Mineral residue.....
Iron.....
.....
.....

Nitrogen as:
Ammonia free.....
" albuminoid (total).....
" albuminoid (dissolved)....
Nitrites.....
Nitrates.....
Organic, total.....

Oxygen consumed.....
Oxygen dissolved.....
Chlorides.....
Hardness, total.....
Alkalinity.....
Chlorine, free.....

*Bacterial Examination* *Quantitative*

Bacteria per cubic centimeter, 48 hrs. at 20°C.....
Bacteria per cubic centimeter, 24 hrs. at 37°C.....

*Qualitative*

In.....cc. volume bacteria colon group were.....present.

Remarks:.....

.....

.....

AUGUSTUS B. WADSWORTH, M.D.
*Director*

FIG. 63. FORM FOR TYPING REPORTS

# APPROVAL AND REGISTRATION OF LOCAL LABORATORIES

## APPROVAL OF LOCAL LABORATORIES

Chapter II, Regulation 2-a of the Sanitary Code necessitates the prompt submission, by every physician, of specimens to the laboratory of the State Department of Health or to a laboratory approved by the by the state commissioner of health, from every person affected with or suspected of being affected with the communicable diseases mentioned therein.

Chapter III, Regulation 13 of the Sanitary Code, requires bacteriological counts of milk to be made in laboratories approved for the purpose by the state commissioner of health.

Chapter II, Regulation 44 of the Sanitary Code, provides for the inspection of laboratories by the state commissioner of health or his authorized representative.

Article 3, Section 25 of the Public Health Law regulates the reporting of infectious and communicable diseases by a laboratory making such diagnoses.

All laboratories wishing approval for the examinations mentioned in Chapter II, Regulation 2-a of the Sanitary Code should make application to the State Department of Health not later than December 1 of each year in order that the certificates of approval may be issued by January 1. Bacteriologists in charge of such laboratories should possess the educational requirements for a doctor's degree in medicine, science, or public health, from a school of satisfactory standing, and should have had approved special training or experience of at least two additional years in pathology and bacteriology or bacteriology alone. For the purpose of approval, the technic of methods used in the laboratory diagnosis of disease and in the analysis of milk and water, have been printed in agreement form and the bacteriologists in charge of the laboratories are required to sign the agreements covering the particular branches of work in their laboratories. When approval of a laboratory is requested for the first time, or when a change is made in the directorship, the director or bacteriologist in charge must sign agreements relative to the conduct of the work in the laboratory. Bacteriologists in charge of two laboratories should sign two sets of agreements.

APPLICATIONS AND AGREEMENTS

The following is a complete list of agreements.

Form No. 18 covers:

- Records
- Methods for laboratory examinations
- Anthrax
- Diphtheria
  - Morphological examination
  - Virulence test
- Gonorrhea
- Malaria
- Epidemic Cerebrospinal Meningitis
  - Examination of Spinal Fluid
  - Bacteriological examinations to determine carriers
- Pneumonia (type differentiation)
- Septic Sore Throat
- Syphilis
  - Complement-Fixation test
  - Questionnaire of the methods used in laboratories throughout the state for the complement-fixation test for syphilis
  - Direct examination for *Treponema pallidum*
- Tuberculosis
- Typhoid and Paratyphoid Fever (agglutination test)
- Typhoid, Paratyphoid Fever, and Dysentery (cultural examination of specimens of feces and urine)
- Vincent's Angina
- Agreement

Form No. 18-a covers:

- Agreement on conditions to be observed and methods to be followed in the determination of the bacteriological count of milk and cream.
- Questionnaire on the determination of the colony counts and the direct microscopic counts in milk.

Form No. 18-b covers:

- Agreement on conditions to be observed and methods to be followed in making the bacteriological examination of water.
- Questionnaire on the bacteriological examination of water.

Form No. 18-c covers:

- Agreement on conditions to be observed and methods to be followed in making the sanitary chemical examination of water.
- Questionnaire on the sanitary chemical examination of water.

**Procedure.**—Send two copies of each agreement form and of the application blank to every laboratory requesting approval, one of each to be filled out, signed before a notary public and returned to the state laboratory for filing, the duplicate set to be retained by the laboratory applying for approval.

When the signed agreements are received, refer them to the bacteriologist in charge of the diagnostic laboratories for consideration.

If the person in charge of the laboratory requesting approval meets the prescribed qualifications, specimens for comparative examination are then sent for the purpose of testing the technic employed. The laboratory is also inspected, if possible, by a representative of this (the state) laboratory.

If the methods used in the laboratory applying for approval are considered satisfactory, and if the report of the visit is favorable, and the reports on the specimens submitted for comparative examination demonstrate that accurate results are being obtained, type a certificate of approval in quadruplicate and send the original to the director of the state laboratory for signature. Mail the signed certificate of approval to the applicant and send a carbon copy of it to both the health officer of the place in which the laboratory is located and to the district state health officer of the locality.

Enter the necessary data on the "Approved Laboratory" cards (4 by 6 in.).

### *Reports from Approved Laboratories*

Every approved laboratory is required to submit a monthly report, on form No. 81, of the work performed by it and also an annual report during the first week of January. A supply of these forms sufficient for a year, is sent at the time the laboratory is approved.

**Procedure.**—Record the receipt of each monthly report on the cards (4 by 6 in.) provided for the purpose.

## STATE AID FOR COUNTY AND MUNICIPAL LABORATORIES

By the enactment of Chapter 638 of the Laws of 1923, state aid is provided for the establishment and maintenance of approved county and municipal laboratories. Before a grant of state aid can be recommended, it is necessary to have the following data on file: a formal application for state aid, the name and qualifications of the bacteriologist in charge of the laboratory, the detailed budget of the laboratory, a certified statement of the amount appropriated for the laboratory by the county or city, the names of the members of the board of managers, the area of the district to be served, and the fiscal year of the laboratory.

**Procedure.**—When an application for state aid is received, refer the information submitted to the bacteriologist in charge of the diagnostic laboratories and send the qualifications of the bacteriologist in charge of the laboratory to the Public Health Council for approval. If, upon inspection, and from the data given, the laboratory is found to meet the requirements specified in the law, type in quadruplicate a statement to the state comptroller certifying that the city or county making application is entitled to state aid. Send the original, after it is signed by the director of the state laboratory, and one carbon copy, to the purchasing clerk so that the original can be submitted to the state comptroller with a formal order reserving the necessary funds. Also send a carbon copy to the state commissioner of health.

Whenever a grant of state aid is made, record the necessary data on the cards (4 by 6 in.) provided for the purpose.

## REGISTRATION OF LABORATORIES IN NEW YORK STATE

Chapter 411, Laws of 1917, provides for the yearly registration and supervision of all laboratories and other places where living cultures of pathogenic microörganisms are handled, to prevent the use of bacteria for criminal purposes and to eliminate careless methods of transmitting cultures.

### REGISTRATION

Applications: Notify each laboratory of the expiration of its registration two weeks in advance and enclose an application blank in duplicate. When the application is returned in proper form accompanied by the required fee of one dollar, issue a notification of registration to the laboratory.

### PERMISSION TO DISPOSE OF PATHOGENIC MICROÖRGANISMS

Applications: Only registered laboratories are permitted by law to handle pathogenic microörganisms and permission to dispose of such cultures must be obtained from the Division of Laboratories and Research, New York State Department of Health. For this purpose, an application blank (form No. 19) has been drawn up.

**Procedure.**—Send a supply of these blanks with the notification of registration to each laboratory which is registered but not approved. Send a blanket permit to each laboratory which is approved for public-health service.

This permit—which is valid during the period of registration and approval of the said laboratory, unless sooner rescinded—sanctions the disposal of living cultures of pathogenic microörganisms to laboratories registered in accordance with Chapter 411, Laws of 1917, subject to the provisions of the statute and of the United States laws and regulations, and on the further condition that a record of all shipments or transfers shall be made to the State Department of Health with each monthly report of the laboratory.

Take the application for permission to dispose of living cultures of pathogenic microörganisms immediately upon its receipt to the bacteriologist in charge of the diagnostic laboratories. If the request is approved, send the applicant a notification of its approval.

## SECTION II

## DISTRIBUTION OF LABORATORY PREPARATIONS AND OUTFITS

### DISTRICT STATIONS

Chapter 637, Laws of 1923, provides for the establishment of District Laboratory Supply Stations for the distribution of antitoxins, sera, vaccines, miscellaneous preparations, and outfits for the submission of specimens, and for the appointment of the health officer of any municipality, the director or bacteriologist in charge of any public-health laboratory or other competent person located in the district, to serve as the custodian of the supply station. The law also permits the establishment by the custodian, with the approval of the State Depart ment of Health, of as many substations as may be necessary for the proper distribution of laboratory supplies to the physicians practicing in the district.

In accordance with this law, the entire state is divided into districts served by district supply stations. It is the duty of each custodian to maintain in his own station, and in each substation, a sufficient supply of laboratory outfits and preparations in good condition, to meet ordinary demands. Such supplies are required to be accessible to physicians at all times and under no circumstances are they to be sold. Perishable supplies are required to be kept in a refrigerator, except that in stations maintaining small quantities, when facilities for refrigeration are not available, such supplies may be kept in a cool, dark place, not subject to extremes of heat or cold.

Custodians are required to submit monthly a list of physicians to whom antitoxins and sera have been distributed (see fig. 64), that the laboratory may check its record with reports from physicians on the use of such products. Custodians are also required to render a semiannual report of supplies on hand in their stations and substations.

Each station is inspected annually, and if it has been maintained and operated satisfactorily, a certificate to that effect is forwarded to the custodian, whereupon he is entitled to receive from the municipal or county treasurer, as the case may be, certain fees and expenses provided by law.

## FILLING ORDERS

Since New York City is more accessible than Albany to the supply stations located on Long Island, in Westchester, Rockland, Orange, and Putnam Counties, emergency requests coming from these counties for supplies, are sent to the branch laboratory in New York City. All other orders are received at the Albany laboratory. So far as possible all orders are filled the day they are received. There are two express collections daily, one at 10:30 a.m. and the other at 3:30 p.m. In addition, a special messenger from the laboratory takes the

DISTRIBUTION OF ANTITOXINS AND SERUMS FOR MONTH OF.....19.....

Supply Station.....

Custodian.....

| DATE | PHYSICIAN | ADDRESS | AMOUNT | LOT NO. | MATERIAL |
|---|---|---|---|---|---|
| | | | | | |
| | | | | | |
| | | | | | |

To be filled in and forwarded on the first of every month to the Division of Laboratories and Research, New Scotland Avenue, Albany, N. Y.

FIG. 64. REPORT FORM FOR CUSTODIANS

late afternoon shipments to the express office at 4:15. Special attention is given to telegraphic and telephonic orders, shipment being made, if possible, by the first train leaving Albany after the receipt of the order.

## RECORDS

Keep a perpetual inventory record of all laboratory preparations. Check monthly the record of the biologic products with the actual stock on hand.

Keep a record of the production and distribution of each lot of antitoxin, serum, vaccine, and miscellaneous preparations.

Record for special study and for statistical purposes the reports on the use of antitoxins and sera.

Summarize all records for the monthly and annual reports.

## SECTION III

# PURCHASE, STORAGE, AND DISTRIBUTION OF EQUIPMENT AND SUPPLIES

## PURCHASES

### STANDARDS

On most items of equipment and supplies used in the laboratory, standards have been established and complete specifications drawn up. These standards and specifications are strictly observed when purchasing stock supplies.

### REQUISITIONS

Requisitions signed by the director or the heads of departments furnish the necessary authorization for purchases. Microscopes are purchased only upon request of the director, and requisitions for equipment of unusual or expensive nature are submitted to the director for approval.

### BIDS

Bids are solicited on all purchases.

## STORAGE AND DISTRIBUTION

### OPENING PACKAGES AND TESTING QUALITY

As packages are opened, compare the material carefully with the specifications and, if satisfactory, count and place it in the proper compartment in the stock room; refer special apparatus to the member of the staff who signed the requisition, for approval. Report the quantities and condition of supplies received, to the office daily for inventory record and auditing purposes.

### STOCK-ROOM HOURS

Requisitions are received at the stock room daily between the hours of 9:00 and 9:30 a.m. and 2:30 and 3:00 p.m. and deliveries are made as soon thereafter as possible.

## FILLING ORDERS

Each department of the laboratory is furnished as many requisition books as needed for the purpose of obtaining supplies from the stock room. Complete specifications must be given on all requisitions for supplies that there may be no error in filling them.

**Procedure.**—Never disburse stock without an official requisition covering such disbursement, and do not give out microscopes or their parts except on orders approved by the director, the bacteriologist in charge of the diagnostic laboratories, or of the antitoxin, serum, and vaccine laboratories.

| NUMBER OR SIZE | ARTICLE | | | SECT. | UNIT | MAX. / MIN. |
|---|---|---|---|---|---|---|
| Date | Firm | Price | Received | Balance | Disbursed | Department |
| | | | | | | |
| | | | | | | |
| | | | | | | |

FIG. 65. PERPETUAL INVENTORY AND PRICE CARD

## INVENTORY

The form given in fig. 65 shows the perpetual inventory and price card used in the laboratory.

Maximum and minimum quantities have been established on all articles regularly carried in stock. For instance, if it is desired to carry a six months' stock of any article, the maximum quantity is determined by taking one-half of the number or quantity used during the previous twelve months.

That sufficient time may be given the purchasing group to secure bids and place orders, the minimum quantity covers at least one month's supply. When the stock on hand reaches the minimum quantity, the maximum quantity is purchased.

As already mentioned, definite specifications have been drawn up on most of the supplies and equipment, so that articles in general use may be interchangeable between departments, and that the number of different articles carried in stock may be reduced to a minimum. These specifications are entered on the inventory cards.

**Procedure.**—Each morning enter on the inventory cards the materials received during the previous day, the prices paid, and draw the proper balances. Enter also the quantities disbursed on laboratory requisitions and deduct them from the balances.

## AUDITING

The rules and regulations of the state comptroller determine the methods used in auditing and presenting bills for payment, in keeping appropriation balances, and in compiling the annual budget for the expenses of the laboratory.

## SECTION IV

## COMMUNICATION

To facilitate the prompt handling of specimens and the distribution of laboratory preparations and outfits, five special collections of mail from the post office are made during a twenty-four-hour period: 5:00, 10:00, and 11:30 a.m., 4:15 and 10:00 p.m. The 10:00 and 11:30 a.m. collections are not made on Sundays and holidays.

### DISPOSITION OF MAIL

When mail is received at the laboratory, it is taken to the desk of the mail clerk.

**Procedure.**—Open all letters except those addressed to individuals and marked "personal." After the date has been stamped on the back of each piece of mail, take it to the director's secretary who will read it and then pass it on to the executive clerk, whose duty it is to read it and mark on the upper-left corner of each letter the name of the individual to whom it is referred for attention. Then take it to the worker on the accession book for recording. (See: fig. 66.)

Stamp the same serial registration number on the letter as in the book.

As soon as this record is completed, distribute the mail to the persons interested. When the completed correspondence is returned for filing, check the corresponding registration numbers on the letters and in the book.

### STENOGRAPHY

In general, in correspondence from the laboratory the use of the third person is preferred. Stenographers and typists are required to follow definite forms for the letters, memoranda, scientific papers, and reports. As particular emphasis is placed on the arrangement of letters, a semi-straight-edge style has been adopted and stenographers should observe the following directions carefully:

**Procedure.**—Place the date directly under "Albany" on the letterhead.

Place the introductory address six spaces below the date and two spaces above the salutation.

Single space and block the address.

Use the colon after the salutation.

Leave a double space between the salutation and the first paragraph.

Leave a one-and-one-fourth-inch margin on the left of the page and a one-inch margin on the right of the page.

Indent all paragraphs ten spaces, irrespective of the length of the salutation.

Use double spacing for one-page letters, single spacing for letters of greater length.

Use two spaces only between sentences.

Use plain paper for all pages of a letter except the first, which must be a letterhead. Type the name of the addressee in the left corner of all sheets except the first, the date in the right corner and the number of the page in the center.

Leave a double space between the last paragraph and the complimentary close.

| FROM WHOM RECEIVED | POST OFFICE | DATE OF LETTER | DATE RECEIVED | REMARKS | REFERRED TO | REGISTRATION NUMBER |
|---|---|---|---|---|---|---|
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |

FIG. 66. FORM FOR ENTERING DATA IN ACCESSION BOOK

Center the complimentary close.

Block the complimentary close and title (when used).

Type the initials of the person dictating the letter and of the stenographer typing the letter in the lower-left corner of the last page of each letter.

Outgoing mail is signed only by the director, the assistant director, or the head of the department concerned.

## FILING

The geographic filing system is used for the majority of the laboratory records, as information regarding the work is more accessible when arranged in this way. This applies particularly to the filing of history blanks which accompany specimens. For these a separate file is kept for each type of routine examination and for the miscellaneous examinations. A three-guide system is used; the first guide bears the name of the city or village in which the attending physician or institution is

located, the second bears the name of the attending physician, and the third that of the institution.

All files are open for reference work between the hours of 9:00 and 10:30 a.m. and 2:00 and 3:00 p.m.

**Procedure.**—File the history blanks in the following manner:

Sort them according to the various types of examination.

Arrange each lot alphabetically according to the city or village in which the attending physician or institution is located.

File the history blanks behind the proper geographic and physicians' or institutions' guides, subfiling them under the names of the patients.

In case the name of the attending physician accompanies that of the institution, file the history blank behind the institution's guide, and cross-index the names of the physician and institution on each guide.

## SERVICE-RECORD LAW

The Civil Service Commission has prescribed certain rules and regulations, in compliance with the provisions of Chapter 653 of the Laws of 1917, regarding the service record of workers in the competitive service of the state.

In accordance with these requirements, a detailed system has been formulated. A time record and a capacity record are kept of each worker in the competitive class and are filed with the Civil Service Commission twice a year, on May 15 and November 15. A personnel board, consisting of the heads of the different departments, has been appointed to review the ratings in accordance with the Service Record Law and to consider appeals which may be made by the staff.

A similar record is kept of noncompetitive and exempt workers.

Promotions in rank or salary are based on the service record.

## VOLUNTEER WORKERS

Volunteer workers with the necessary educational qualifications, who wish to fit themselves for positions in public-health laboratories, are accepted for training. Besides a prescribed two-year course for such workers, shorter courses may be arranged to meet the needs of experienced students.

## VISITORS

Visitors are received at the entrance and shown to one of the reception rooms. The person in charge (usually the telephone operator) notifies the members of the staff or their secretaries if the visits are for them. Visitors for the director are referred to his secretary.

### VISITORS TO SEE THE LABORATORY

If the visitors wish to see the laboratory, a member of the office staff is assigned to conduct them through the building and to show them in regular sequence the laboratory rooms and the equipment and apparatus of general interest.

Before being conducted through the building, visitors are asked to register in the visitors' book.

Visitors are not permitted to go about the laboratory unescorted.

### VISITORS COMING FOR TRAINING AND OBSERVATION

Visitors from laboratories and, in special instances, others who have had proper training in scientific subjects, are given opportunity to observe the different branches of the work. These visitors are referred to the director's secretary. If they are from local laboratories, they are referred to the secretary of the laboratory department which they wish to visit.

These visitors are asked to fill in a blank prepared for the purpose, giving a record of their training and experience, including an outline of the work they wish to accomplish during their stay. Copies of this information are made and sent to the heads of the departments concerned.

After the blank has been filled in, the visitors are conducted through the building by the assigned member of the office staff, and are then referred to the library where they are shown the facilities for looking up literature and are instructed in the use of the library's resources.

It is understood that the heads of departments will advise the director's secretary concerning the transfer of a visitor as far in advance as possible so that the next department head can be notified.

When a visitor leaves a department, the head of the department should prepare a report for the director.

Visitors are expected to make a report when leaving a department, such report to be read by the head of the department and forwarded to the director.

The secretaries should keep in touch with these workers and should see that the office has a record of the work accomplished by them.

# METHODS USED IN THE RESEARCH, PUBLICATIONS, AND LIBRARY DEPARTMENT

MURIEL ALMON, *Scientific Research Secretary*

MARY WILLIAMS, *Librarian*

# INTRODUCTION

The research, publications, and library department consists of two divisions: the publications section, and the library, closely allied and jointly serving one purpose, but each with certain definite aims and specific functions.

The publications section is devoted to the formulation of the results of research. It aims to establish and maintain a high standard in the presentation and publication of scientific papers. The manuscripts of the reports, circulars, and bulletins issued by the laboratory, and all scientific papers written by members of the staff are filed in the publications room, together with original record cards, outlines, and reports of investigations. This material constitutes what is known in the laboratory as the "scientific file." A research secretary has charge of this section and is ready to help members of the staff in every possible way to prepare outlines, reports of progress and of completed investigations.

The library section offers members of the staff the opportunity to study the scientific literature bearing on their work. Its function is to provide the workers with the material most helpful to them. With this end in view, the librarian indexes current literature, compiles lists of publications, puts information into compact and easily accessible form, and calls the attention of members of the staff to all matters that may be of interest to them.

Besides books, periodicals, and pamphlets, the resources of the library include photographs, maps, and lantern slides.

The staff of the publications and library department consists of a librarian, a research secretary, and several assistants, all of whom have had either library training or experience in editorial or secretarial work in addition to academic education, and thus are fitted to help the technical workers in formulating the results of research and in their study of the scientific literature.

All procedures involved in the work of the publications and library group are in accordance with the accepted principles of office practice and library economy as adapted and applied to a highly specialized institution. Each member of the group keeps a set of detailed directions for doing the work, and these directions are revised from time to time whenever a method is changed or a new one adopted.

A condensed copy of the directions which are kept in the library for the use of members of the scientific staff, follows. Rules for the guidance of secretaries and stenographers will be found in the standard methods of the executive offices.

## SECTION I

## PREPARATION OF REPORTS AND PUBLICATIONS

### RESEARCH

#### *Preliminary Work*

When study of a research problem is suggested by a worker or proposed to a worker, the approval of the head of the department or of the director must be obtained. The worker then applies to the publications section for information as to what, if any, work has already been done in the laboratory on the subject in question. The request for this information should be in writing and should state the problem clearly and concisely.

On receipt of such a request, the research secretary makes an appointment for a conference with the worker, before which time she examines the material in the scientific file and makes a note of any papers there that bear on the proposed problem. These notes she brings to the worker's attention and discusses with him the advisability of studying the literature on the subject in the library.

This initial conference should result in a clear understanding between the research secretary and the scientific worker as to the exact nature of the proposed investigation; whether a bibliography should be prepared and, if so, how extensive it should be; whether he needs any help in his study of the literature, such as abstracts or translations.

The research secretary informs the assistant librarian of the proposed investigation and the latter procures whatever material is asked for on the subject. She notifies the worker as soon as it is available and he consults it in the library.

#### *Preparation of Outlines*

After reading the literature on the subject in question, the scientific worker discusses the problem with the director or with the head of his department and prepares an outline.

The outline should state:

(1) the problem;
(2) the purpose of the investigation;
(3) the conclusions to be drawn from a study of the literature;
(4) the technic it is proposed to use.

### *Approval and Filing of Outlines*

The outline must be approved by the head of the laboratories in which the work is to be done and by the director. Any comments made by the director upon the outline should be taken up with him in person by the worker.

If the outline is approved, it is typed by a stenographer connected with the department in which the work is to be done. One copy is sent to the publications room to be filed, one is given to the scientific worker, and one to the head of his department. If there are no facilities in that department for filing this copy, it is sent to the publications room to be filed.

Complete original records of the work as it progresses are made on 4-by-6-inch cards which are filed in the scientific file when the study is finished.

References to literature are kept in regular form on the standard library cards.

### *Monthly Reports*

A monthly report of progress is made by the worker and given to the head of his department, who sends it to the publications room on the first of each month. The research secretary reads each report in connection with the outline and, if the proposed technic is not followed, she confers with the worker and asks that the reason for the deviation be embodied in the report.

When the report conforms to the outline or a satisfactory reason for any change has been given, it is sent to the director for approval. After being initialed by him, it is returned to the publications room and filed.

### *Final Reports*

When the work on a research investigation is finished, the worker summarizes it in a final report. This is treated in the same way as a monthly report of progress.

The passing back and forth of outlines and reports is done entirely by the secretaries of the laboratory departments.

## PUBLICATION OF PAPERS

### *Preparation of Papers*

When the final report on a research problem has been approved, the worker consults the head of his department and the director as to the advisability of publishing a paper. If it is decided to do so, he con-

sults the research secretary as to the form and character of the paper and the most suitable place for publication.

After the paper has been written and approved by the head of the author's department, it is sent to the publications room.

All manuscripts undergo final revision by the research secretary before they are sent to any printer, editor, or publisher. The purpose of this revision is to make the papers conform in diction and general form to the standard adopted by the laboratory, and the research secretary has the authority to make any changes necessary to achieve this end. No statement of fact may be changed nor any alteration made in the substance of the papers without the approval of the head of the department from which the paper in question came. When a paper has been initialed by the research secretary, it is sent to the director for approval and then returned to the publications room. The research secretary sends it to an appropriate journal.

### *Scientific File*

After a paper has been made ready for publication, a copy is given to the secretary in charge of the scientific file, together with the original record cards, additional references if there are any, also any microscopic preparations, specimens, maps, charts, etc.

The material and all manuscripts are kept in the scientific file which thus constitutes a complete record of the research done by the laboratory staff.

### *Reading of Proof*

Galley proof is read first in the department from which the manuscript came and reasonable changes in the subject matter may be made there. It is then read in the publications room and the research secretary is responsible for form and typographical accuracy.

Page proof is read only in the publications room.

### *Reprints*

Before galley proof is returned to the printer, the author is asked whether he wishes any sewed reprints with covers. If so, they will be ordered and given to him when received in the publications room. Four hundred unbound reprints of every paper are ordered, 350 of which are kept to be bound later as collected studies. Whether reprints with covers are ordered or not, ten uncut, unsewed reprints without covers are given to each author as soon as received.

## REPORTS

### *Annual Reports*

The head of each laboratory department makes a written report of the year's activities to the director early in January. If there are special phases of the laboratory's activities which the director wishes to have emphasized, a memorandum enumerating them is sent to heads of departments not later than the previous October.

Annual reports are sent to the publications room as soon as completed. Any changes made there or in the director's office are submitted to the heads of departments for approval before the reports are sent to the printer.

Proof of the annual reports is read in the same way as proof of scientific papers.

### *Routine Reports*

Monthly reports are sent to the publications room by the sixth of the month. They record the progress of the routine work, special items, and changes in standard methods.

Monthly reports are read and initialed by the research secretary as soon as they are received and are forwarded to the director without delay. They are finally returned to the office to be filed.

### *Special Reports*

Special reports are required from visiting workers who spend a certain prescribed time in one or more departments of the laboratory. They are approved by the head of the laboratory department in which the visitor has worked and, after they have been sent to the director, are returned to the publications room to be filed.

### *Reports of Visits*

Reports of visits made by members of the laboratory staff to other institutions are sent to the publications room, initialed by the research secretary and forwarded to the director. They are sent to the office to be filed.

## GENERAL INFORMATION

If a worker wishes information on material in the scientific file, the secretary in charge will give him, on request, a list of the papers in the file bearing on the subject in question. If necessary, he may consult

the papers themselves in the library. Unpublished reports are confidential and may not be withdrawn from the scientific file without the director's approval.

### *Changes in Methods*

A proposed change in standard methods is based on facts established by experience, or that are recorded in the literature. The results of special research are frequently applied to the improvement of a method. A suggested change is embodied in a routine report or is written as a separate memorandum. It is sent to the publications room and must be approved by the director before it is adopted. When approved, it is returned to the department from which it came and finally to the publications room to be filed.

### *Clerical Work for Other Departments*

Research workers who have no facilities for typewriting in their departments send their papers in manuscript form to the publications room where they are typewritten.

Requests for clerical service should be made of the research secretary.

## SECTION II

## METHODS USED IN THE LIBRARY

### CURRENT LITERATURE REVIEW

Periodicals are examined by the librarian or an assistant as soon as they are received and papers of interest to members of the staff are noted. Typewritten cards bearing the titles of the papers and the names and dates of the journals containing them are distributed to the laboratory departments once a week.

Heads of departments give the cards to the workers whom they consider best fitted to abstract the papers. They read and abstract the articles in the library and return the cards with the abstracts on the back to the librarian within a week.

### REFERENCE QUESTIONS

#### *Simple Questions*

Simple reference questions, such as the mere verification of some name, date, or quotation, which do not require much search and to which answers are desired at once, need not be written. On receipt of a verbal request for such information, the assistant librarian will give it her immediate attention. As soon as the information is found, she will notify the worker who asked for it.

#### *Questions Requiring Search*

All requests for bibliographies, abstracts, translations or for information about which the worker can give only inadequate data should be in writing. In the case of an obscure reference question, the worker should state any facts that might be helpful in a search for information and, if he himself has consulted any sources, he should name them and give the dates covered and the subjects under which he has looked. The assistant librarian notifies him as soon as she has found the information and inquires a few days later whether it was satisfactory. When he has finished with the material collected for reference, he notifies the assistant librarian.

In the case of bibliographies, abstracts, and translations, the research secretary will confer with the worker in question and together

they will decide on the scope of the work and the best method to pursue. This matter is left entirely to the discretion of the research secretary and the worker. When requests are received from other divisions of the health department or from approved laboratories, the assistant librarian will prepare a list of references and, when necessary, will abstract or translate an article containing the needed information.

## LIBRARY INSTRUCTION FOR NEW WORKERS

All new workers come to the library on entering the laboratory. Those who enter for less than two weeks are not required to follow any course of reading but are introduced to the librarian before they begin work in any department, and are briefly instructed in the use of the library.

Workers who enter for longer periods are required to spend at least three half days there. If they cannot complete the prescribed course in that time, they may be required to give additional hours to reading and study.

## CATALOGUES AND INDEXES

The first card in every catalogue drawer contains an explanation of the arrangement of the cards in the drawer and directions for using them.

The drawer marked "General index" contains cards on which are entered the place where information on many subjects can be found. In searching for material, a worker is advised always to consult the general index first.

## LOANS FROM OTHER LIBRARIES

A request for material from the State Medical Library is given to the assistant librarian on a slip of paper. If the reference is for a book, author, and title, if for a journal, author, title or subject, volume, and page should be given. Every Tuesday and Friday books are brought from and returned to the State Library. When the volumes are received, they are put on the shelf marked "Books from State Library" where members of the laboratory staff are asked to look for them. When the member of the staff has used the volume, it is returned to the shelf marked "Books to be returned to State Library." If a book cannot be obtained from the State Library, the librarian borrows it from the New York Academy of Medicine or from the Army Medical Library.

## MUSEUM

The laboratory museum, which is as yet quite rudimentary, constitutes one of the library collections. It is planned to contain such objects as various types of apparatus and other materials used by different groups, which will show the development of the laboratory activities, and illustrate the work that is being done. In addition to these, the museum contains at present a number of specimens which form the basis of a collection to be used for the study of various pathological processes.

## GENERAL INFORMATION

The research secretary is available for consultation from 10:00 a.m. to 12:00 m. daily, and by appointment.

The library is always open. It is for reference and reading only; the books and periodicals do not circulate.

The material in the library is simply arranged and well indexed so that members of the laboratory staff can find the books or information they want with little or no help.

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# APPENDIX

# LABORATORY STAFF

*Director*

AUGUSTUS WADSWORTH, M.D.

*Assistant Director*

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*Bacteriologist in Charge of the Antitoxin, Serum and Vaccine Laboratories*

MARY B. KIRKBRIDE

*Bacteriologist in Charge of the Diagnostic Laboratories*

RUTH GILBERT, M.D.

*Bacteriologist in Charge of the Branch Laboratory*

H. VIRGINIA LANGWORTHY

*Chemist in Charge of the Laboratories for Sanitary and Analytical Chemistry*

LEONARD M. WACHTER

*Assistant Bacteriologist in Charge of the Department for the Preparation of Media and Glassware*

LURA M. MACK

*Scientific Research Secretary*

MURIEL ALMON

*Librarian*

MARY WILLIAMS

*Pathologist*

LAWRENCE J. EARLY, M.D.

*Chemists*

WALTER S. DAVIS
ANTHONY KOZLOWSKI, PH.D.
FRANK MALTANER
DAVID E. ROELKEY

*Bacteriologists*

VICTOR W. BERGSTROM, M.D.
CLAUS W. JUNGEBLUT, M.D.

*Veterinarian-Bacteriologist*

CHARLES A. GRIFFIN, D.V.M.

*Assistant Pathologists*

James D. McKinnon, M.D.
Edgar R. Maillard, M.D.

*Assistant Chemists*

Rachel F. Brown
Benedict Gordon
Jessie L. Hendry
Leonard W. Hyman
Gordon Kline
Philip P. Murdick
James J. Quigley
Arthur Stull, Ph.D.
Jeanne E. van Amstel, Ph.D.

*Assistant Bacteriologists*

Henry A. Bartels
Marion B. Coleman
Jessie Dow
Jennie G. Drennan, M.D.
William M. Groesbeck
Ella N. Hoppe
Bessie J. Maltaner
Marion C. Morris
Helen Odell
Helen H. Owen
Anna M. Schaible
Grace Sickles
F. Constance Stewart
Mary W. Wheeler

*Assistant Serologists*

Florentine Fitzgerald
Margaret Wemple

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Sophia M. Cohen
Margaret E. Collin
Francis J. Hallinan
Grace V. Maguire
Elizabeth K. Masse
Ruhamah R. Nichols
Anis Rae Peterson
Lucena K. Robinson
Catherine Sickinger
Mildred N. Smith
Albrecht Weber

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Julia M. Coffey
Bernice G. Smith
Elsa Wachter

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Albert Williams
Anna Willig
Margaret Zimmer
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Ella E. Bassett
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Margaret Hawley
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Florence Griner
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Eleanor M. Plum
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James Crone

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William H. Van Wie

*Firemen*

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John J. Finn
David R. Galloway
Clinton Hyde

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Charles Schadler

*Assistant Stablemen*

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Jack Dennick
John Gerard
George McTague

*Laundresses*

Dora Ecker
Edna Wagner

*Cleaners*

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Edythe B. Andrews
Viola Armour
Sarah Baker
Andrew Brew
Vina Brew
Eva Brokaw
Agnes Connell
Lorena Cook
Ursula S. Corcoran
Henry Cramer
Martha Fallon
Gertrude Feiden
Kathryn Fisher
Louis H. Gawne
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Mabelle Griner
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Marion Powers
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Luitgard Rettich
Rose Rizza
Gertrude F. Robinson
Elizabeth Rose
Rose Scheuer
Joseph P. Sheehy
Etta E. Smith
Josephine Smith
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Howard Jacobson
Walter King
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Dennis Lezott
Harry Martin
William Mesick
Henry Sharer
William Sharp
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Gabriel Trombe
Herbert Wigley

# INDEX

# *Sans Tache*

# *Sans Tache*

IN THE "elder days of art" each artist or craftsman enjoyed the privilege of independent creation. He carried through a process of manufacture from beginning to end. The scribe of the days before the printing press was such a craftsman. So was the printer in the days before the machine process. He stood or fell, as a craftsman, by the merit or demerit of his finished product.

Modern machine production has added much to the worker's productivity and to his material welfare; but it has deprived him of the old creative distinctiveness. His work is merged in the work of the team, and lost sight of as something representing him and his personality.

Many hands and minds contribute to the manufacture of a book, in this day of specialization. There are seven distinct major processes in the making of a book: The type must first be set; by the monotype method, there are two processes, the "keyboarding" of the MS and the casting of the type from the perforated paper rolls thus produced. Formulas and other intricate work must be hand-set; then the whole brought together ("composed") in its true order, made into pages and forms. The results must be checked by proof reading at each stage. Then comes the "make-ready" and press-run and finally the binding into volumes.

All of these processes, except that of binding into cloth or leather covers, are carried on under our roof.

The motto of the Waverly Press is *Sans Tache.* Our ideal is to manufacture books *"without blemish"*—worthy books, worthily printed, with worthy typography—books to which we shall be proud to attach our imprint, made by craftsmen who are willing to accept open responsibility for their work, and who are entitled to credit for creditable performance.

The printing craftsman of today is quite as much a craftsman as his predecessor. There is quite as much discrimination between poor work and good. We are of the opinion that the individuality of the worker should not be wholly lost. The members of our staff who have contributed their skill of hand and brain to this volume are:

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