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WAR DEPARTMENT

U.S. Dept. of Army

TECHNICAL MANUAL

METHODS FOR LABORATORY TECHNICIANS

October 17, 1941

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TECHNICAL MANUAL ★ ★

METHODS FOR LABORATORY TECHNICIANS

CHANGES No. 1

WAR DEPARTMENT,
WASHINGTON, June 29, 1942.

TM 8-227, October 17, 1941, is changed as follows:

213. Carbohydrate broth (for fermentation tests).—To 1,000 cc meat extract broth (par. 173), add 0.5 percent of the desired fermentable substance, dissolved in a little hot water. Then add 1 cc of 1.6 percent alcoholic solution of bromcresol purple per liter. Place 5-cc portions in fermentation tubes and autoclave 10 minutes at 7 pounds or by fractional method in Arnold sterilizer.

[A. G. 062.11 (6-2-42).] (C 1, June 29, 1942.)

BY ORDER OF THE SECRETARY OF WAR:

G. C. MARSHALL,
Chief of Staff.

OFFICIAL:

J. A. ULIO,
Major General,
The Adjutant General.

469436°—42

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U. S. GOVERNMENT PRINTING OFFICE: 1942

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TECHNICAL MANUAL

METHODS FOR LABORATORY TECHNICIANS

CHANGES No. 2

WAR DEPARTMENT,
WASHINGTON 25, D. C., 1 April 1944.

TM 8–227, 17 October 1941, is changed as follows:

10. Use.

* * * * * * *

d. Adjustment of focus (Superseded).—The number on the objective indicates its "equivalent focal length" in millimeters. If the 16-mm objective were a simple converging lens, the equivalent focal length would also be its working distance (that is, the distance above the object when the lens is in focus) but, for a compound objective, the working distance is always much less than the equivalent focal length. For example, it will be found that the working distance of the 16-mm objective is about 5 mm, that of the 4-mm objective, less than 1 mm, and that of the 1.9-mm objective, less than 0.2 mm. Most microscopes are equipped with parfocal objectives so that if the 16-mm objective is in focus the others will be approximately in focus when the nosepiece is revolved. To focus the 4-mm or 1.9-mm objective, carefully lower the objective with the coarse adjustment, watching it from the side with the eye on the level of the microscope stage, until the objective nearly touches the coverslip. Then, looking through the ocular, slowly raise the objective until the object can be seen, and bring into sharp focus with the fine adjustment. If necessary, readjust the intensity of the light to give maximal visibility. Never lower the objective with the fine adjustment. The contact cannot be felt if it strikes the slide; if this occurs the slide is liable to be broken, and the lens to be scratched and ruined.

[A. G. 300.7 (22 Mar 44).]

BY ORDER OF THE SECRETARY OF WAR:

G. C. MARSHALL,
Chief of Staff.

OFFICIAL:

J. A. ULIO,
Major General,
The Adjutant General.

582655°—44

U. S. GOVERNMENT PRINTING OFFICE: 1944

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TECHNICAL MANUAL
No. 8-227

WAR DEPARTMENT,
WASHINGTON, October 17, 1941.

METHODS FOR LABORATORY TECHNICIANS

Prepared under direction of
The Surgeon General

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CHAPTER 1

GENERAL

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SECTION I

GENERAL

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Purpose	1
Scope	2

1. Purpose.—This manual is intended primarily for use in training medical laboratory technicians in Army laboratories. It also provides detailed directions for most of the diagnostic clinical laboratory tests required in a hospital, corps area, or field laboratory. Standardization of technical procedure and the use of standard medical supplies and equipment in Army laboratories are encouraged.

2. Scope.—This manual is based upon the fourth (1935) edition of Laboratory Methods of the United States Army, but has been entirely rewritten to meet the different objective. As a training manual for the apprentice technician it has been given detail of technic but abridgment of subject interpretation. Some subjects, not properly approached by the apprentice technician, have been given an entry for general orientation. Due to limitation of space, the less frequently used tests and duplicate or optional tests have been omitted. Where several acceptable tests are available for a single purpose, the one most adaptable to apprentice technician and to Army laboratories has been described. A number of new approved methods have been added. Names of bacteria, protozoa, and insects have been changed to conform to latest accepted nomenclature. Free reference has been made to standard texts on the various subjects. (See app.)

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SECTION II

MEDICAL SUPPLIES

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Laboratory supplies	5

3. Standard.—These are entered in current edition of Medical Department Supply Catalog and stocked by supply depots. This list is designed to provide for all ordinary requirements. Insofar as practicable only standard items should be used, methods being chosen when available which will utilize standard items. Most laboratory supplies are in classes 1 and 4 of this catalog. An allowance table in the Medical Department Supply Catalog estimates the annual average requirement for laboratories serving the various population or bed capacity groups. Special requirements, or lack of requirement, warrant deviation from these estimates.

a. "S. G." items.—These are expensive items which are available for issue only after prior specific authority of The Surgeon General.

b. Expendable items.—These are items such as glass slides which, when they become unserviceable, can be discarded without further accountability. These are indicated by a capital letter "X" in column following the item number. It is customary, however, for these items to be assembled within the laboratory for periodic review by the chief of service to determine rates of losses, avoidable factors of breakage, and replacement requirements.

c. Nonexpendable items.—These are items which remain charged to the responsible or accountable officer until disposed of by memorandum receipt, shipping ticket, or survey by a board of officers. Broken items of this group should never be discarded, but the parts preserved and turned in to the accountable officer for credit.

d. Deteriorating supplies.—These are items such as rubber goods and biologicals, which require more frequent replacement than do the other items. These are indicated in the supply catalog by the figure "(1)" in column after item number.

4. Nonstandard.—These are items not entered in the supply catalog, either because they are replaceable by some standard item or because their use is so limited as to not warrant storage at the supply depots. A few items listed in this manual are nonstandard for the latter reason. Requisitions for nonstandard supplies are required to include exact data as to name, grade, cost, and dealers, and to state that no standard supply item will fill the need.

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5. Laboratory supplies.—Requisitions are submitted periodically by the medical supply officer of a unit upon the basis of estimates made by him or specific requests made to him by the officer in charge of the laboratory.

a. Medical supply depots fill requisitions from stock or by purchase.

b. Corps area laboratories and the Army Medical School supply a number of items, such as specific diagnostic products, so listed in class 1 of supply catalog and some unlisted biological products to meet special needs.

SECTION III

LABORATORY REPORTS

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Purpose	6
Preparation	7
Forms	8

6. Purpose.—Reports of laboratory examinations are made to give useful information for diagnosis, for guidance to treatment, or for epidemiological or sanitary guidance. Those reports which pertain to a hospital patient are filed with and become a part of the clinical record of that patient. Those reports having epidemiological or sanitary reference become a part of the office file of administrative offices. Duplicate reports become the laboratory office file for future reference. It may be stated as an axiom that a laboratory report, as seen on a patient's record chart, reflects the acumen, industry, and neatness of the laboratory examiner: a neat, concise, understandable report reflects careful technic in the examination; an untidy, incomplete, or vague report reflects careless or incomplete technical handling. A technician should not impair the value of an examination on which he has spent hours of careful work and thought, by terminating it in a few minutes with a hasty, careless report.

7. Preparation.—Reports are prepared by the laboratory in duplicate; the original report is forwarded to the ward, office, or officer requesting the examination; the duplicate copy is retained in the laboratory for file. Some reports, such as autopsy protocols and reports of milk and water analysis, call for multiple copies on special forms.

a. Substance of the report is entered by the technician or officer making the examination and should be exact, understandable, neat, and prompt, giving complete identification as to source, date, special circumstances, result of examination, and date of report.

b. Verification of the report is made by the initials of the examining technician or officer and the signature of the laboratory officer.

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c. Reports of laboratory work are strictly confidential; information as to results of tests is to be released only through medical channels. Observe caution in giving out information by telephone.

d. Work notes are kept for all procedures other than those subject to immediate report. These contain a record of the day-by-day observations and procedures and serve as the basis for the final report of the examination. At no time should memory be used for guidance of past findings and events.

e. Monthly and annual reports are prepared by the laboratory officer giving the kinds and numbers of examinations made during the period. These are prepared from the duplicate report forms and from records of each component part of the laboratory on unreported as well as reported undertakings.

8. Forms.—The following Medical Department blank forms are provided and should be used in reporting results of laboratory work:

Blood (W. D., M. D. Form No. 55 L–1).
Blood (Chemistry) (W. D., M. D. Form No. 55 L–2).
Serology (W. D., M. D. Form No. 55 L–3).
Spinal Fluid (W. D., M. D. Form No. 55 L–4).
Urinalysis (W. D., M. D. Form No. 55 L–5).
Urinalysis (Quantitative) (W. D., M. D. Form No. 55 L–6).
Sputum (W. D., M. D. Form No. 55 L–7).
Gastric Analysis (W. D., M. D. Form No. 55 L–8).
Feces (W. D., M. D. Form No. 55 L–9).
Carbohydrate Tolerance (W. D., M. D. Form No. 55 L–10).
Renal Function (Concentration and Dilution) (W. D., M. D. Form No. 55 L–11).
Renal Function (P. S. P.) (W. D., M. D. Form No. 55 L–12).
Renal Function (Urea Clearance) (W. D., M. D. Form No. 55 L–13).
Basal Metabolism (W. D., M. D. Form No. 55 L–14).
Miscellaneous (W. D., M. D. Form No. 55 L–15).
Report of Sanitary Chemical Analysis of Water (W. D., M. D. Form No. 94).
Report of Bacteriological Analysis of Water (W. D., M. D. Form No. 95).
Record of Serological Reactions of Syphilis (W. D., M. D. Form No. 97).

NOTE.—Form No. 55 L–15 is used in reporting most bacteriological examinations and for other tests for which no special form is provided.

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SECTION IV

COMPOUND MICROSCOPE

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Structure	9
Use	10
Care	11

9. Structure.—*a.* A microscope, the working tool of a microbiologist, consists of four groups of parts, each group composed of a number of units:

(1) *Framework.*—(*a*) Base, on which the microscope rests.

(*b*) Handle, by which it is carried and which supports the magnifying and adjusting systems.

(*c*) Stage, a perforated horizontal shelf on which the object rests.

(*d*) Mechanical stage, which moves the object about on the stage.

(2) *Illumination system.*—(*a*) Mirror, which reflects light upward.

(*b*) Condenser, placed just beneath hole in stage.

(*c*) Diaphragm, just beneath condenser, controlled by a small button to open or close it, in controlling the light intensity.

(3) *Magnification system, through which light passes.*—(*a*) Nosepiece, generally triple, to receive the objectives.

(*b*) Objectives, generally three, the main magnifying part, designated according to their focal distance as 16, 4, and 1.9 mm, the latter being the highest power and used in most bacterial studies.

(*c*) Body tube and drawtube, through which the light passes to ocular.

(*d*) Ocular, an additional magnifying piece, of which two are generally furnished, 6.4X giving somewhat less magnification than 10X (number indicates times object is magnified).

(4) *Adjustment system, which moves body tube up or down for focusing of objective to object.*—(*a*) Coarse adjuster gives rapid movement over a wide range and is used to obtain an approximate focus.

(*b*) Fine adjuster gives a very slow movement over a limited range and is used to obtain an exact focus, after prior coarse adjustment.

b. The magnification of any combination of objectives and oculars may be obtained by multiplying the magnification of the objective by that of the ocular. The magnification given by different combinations of objectives and oculars is as follows:

	6.4 X	*10 X*
16 mm (10X)	×64	×100
4 mm (43X)	×275	×430
1.9 mm (95X)	×610	×950

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10. Use.—*a. Adjustment of light.*—A suitable light source, intense for the higher magnification, is placed in front of the microscope; this may be daylight (not sunlight) or a bright artificial light. The mirror is adjusted to direct this light upward through the condenser. Having attained a bright light through condenser, the light may be reduced to the desired intensity by closing the diaphragm.

b. Adjustment of object.—The material to be examined, on a glass slide, is placed on the stage, held in the grip of the mechanical stage, and moved around by it until the desired areas lie beneath the objective.

c. Adjustment of magnification system.—The desired objective is rotated into place at lower end of the body tube. The desired ocular is placed in the upper end of the drawtube. The observer then closely applies an eye to the ocular.

d. Adjustment of focus.—With the coarse adjustment screw, the tube is placed at the proper approximate position. Each objective requires a distance in millimeters between the object and the lower part of the objective, corresponding to the number of that objective (which is its focal distance); the 1.9 objective is placed at about 1.9 mm ($\frac{1}{12}$ inch) above the object. This objective (1.9), but not the 16 and 4, requires that there be a drop of cedarwood oil between object and objective. Having gained this approximate focus, the observer's eye applied to the ocular further guides the coarse adjustment to a more approximate focus, until the microscopic object can be roughly seen. The fine adjustment is then used to give an exact focus, providing a clear image. A readjustment of the light intensity may then be made to give the maximum visibility. In general, it is best to focus upward, for the beginner who focuses downward may force the objective into the object with great force and break it.

11. Care.—*a.* Objective and ocular surfaces may be cleaned by a little breath moisture followed by stroke of lens paper, as in cleaning spectacle glass.

b. Cedarwood oil, used on objective, is to be wiped off after each use, with soft lens paper, avoiding gauze or other scratching agents.

c. Cleaning of entire microscope is to be done frequently, to remove dust, finger marks, oil, grease, or specimen remnants.

d. Cover is to be provided at all times when microscope is not in use.

e. Dried oil may be removed by wiping with lens paper soaked with xylol, wiping away surplus at once with dry lens paper. Caution

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must be used in applying any solvent fluid to the objective; alcohol should never be used.

f. Housing of the binocular tubes should never be removed except by an expert, for slight maladjustment of the contained prisms will distort images.

g. Light machine oil is applied to working parts occasionally.

SECTION V

MISCELLANEOUS PROCEDURES

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Glass cutting	15
Bending of glass tubing	16
Capillary pipettes	17
Ampoules	18
Blowing bulb in glass tubing	19
Trimming damaged glassware	20

12. Acidproof wood stain.

Solution No. 1		*Solution No. 2*	
Copper sulfate	125 gm	Analin oil (fresh and of good quality)	150 gm
Potassium chlorate	125 gm	Hydrochloric acid (concentrated)	180 cc
Water	1,000 cc	Water	1,000 cc

13. Application of wood stain.—The wooden surfaces of the laboratory desks and tables may be proofed against the action of strong acids by the following procedure: The wood must be free from paint, varnish, grease, and chemicals. With a paint brush, apply two coats of solution No. 1 (par. 12) boiling hot, allowing each coat to dry thoroughly before applying the next coat. Then apply two coats of solution No. 2 in the same way. When the wood is completely dried, wash off excess chemicals with hot soapsuds. Finish with raw linseed oil. Polish by rubbing the oil down well with a cloth or sponge. Whenever the tables get dingy, go over them again with a coat of linseed oil and rub smooth.

14. Glass handling.—*a. Purpose.*—Cutting, bending, and simple fusion of glass tubing, preparation of capillary pipettes and vaccine vials, and other simple glass-handling procedures are part of the technical routine of every laboratory. Larger and more complicated glassware repair and manufacture are beyond the capacity of the

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average laboratory, requiring expert glass blowers with special equipment.

b. Equipment.—Soft glass can be handled with the ordinary laboratory equipment, using a blast burner with illuminating gas and foot power or motor-driven air pressure. Some work can be done with the ordinary bunsen burner, with or without a fishtail tip. Glass rods, test tubes, and thick-walled glass tubing (6, 8, and 10 mm) are easily cut, bent, and fused. A small triangular file is used for cutting. The hard glasses such as pyrex have a high melting point and cannot be handled without special high-temperature blowpipes.

15. Glass cutting.—*a. Glass tubing and rods.*—Hold the piece of glass firmly on the top of a laboratory table and nick it in one spot with the edge of a triangular file. Then hold the tubing or rod in both hands with thumbs opposite the nick; exert a slight pull on the tubing and break with a quick snap; a clean-cut, even break should result. If one end of the tubing is too short to handle, the snap may be effected by holding the long end rigidly in one hand and hitting the small end with the file held in the other hand. Finally smooth the surface of the break by melting it in a hot flame; at this time the bore of the opening can be reduced to any desired size by overmelting or increased in size by manipulating with the rounded handle of the file.

b. Test tubes.—Make a deeper file nick than for glass tubing and preferably encircle the tube. A thin tube may be broken at this point by a bimanual snap. Thick tubes require additional aids to complete the break; at one point make the file nick especially deep, then touch the tube firmly at this point with the red-hot file tip; a fracture should then result; if the fracture is not complete, it may be traced around the tube by keeping the red-hot file tip just ahead of the fracture line, on a cold test tube. Trim up same as for tubing.

16. Bending of glass tubing.—Holding both ends, place the tubing in a hot flame so that at least an inch gets hot. Rotate tube while it is heating to make the heat even on all sides. When the glass is red hot and soft, remove from flame and bend to the desired form, keeping it in that position until it has hardened. If a broad bend is desired, as in making a U-bend, several inches should be so heated. If only a slight bend is to be made, an inch of glass will suffice. At first you will have a tendency to overheat the glass and draw the two ends apart, distorting the shape and caliber. Also if you underheat, or put forced pressure on the bending effort, an undesirable collapse of the tubing at the bend will occur. A satisfactory bend retains the same caliber throughout the tubing. If, in working with thin tubing, the collapse at the bend cannot be prevented, there is a method

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available for preventing it: seal one end of the tubing and then heat to melting at the desired point as above; apply the mouth to the open end of the tubing while effecting the bend; make enough air pressure into the tubing to return the collapsed tubing to its proper form. Glass rods may be bent or pressed to any desired form.

17. Capillary pipettes.—Glass tubing is cut to desired lengths, each end fused to smoothness, and put aside for future use. To form these lengths of glass tubing into pipettes, a central area is heated over the hot flame to softness, removed from flame, drawn apart by pulling on the two ends, and held in place until hardened. The size of the resultant capillary tubing will depend on the degree of heat, the rapidity of drawing out, and the extent of the drawing out. The tendency is to make the tube too small by a too rapid separation of a narrow length of heated tubing. Many laboratories keep on hand 8-inch lengths of clean, sterile glass tubing with both ends rounded and plugged with cotton for use in making into pipettes as desired for special purposes.

18. Ampoules.—Heat a clean, sterile, cotton-plugged test tube about 2 inches from mouth, rotate constantly, and then draw the two ends about 2 inches apart, leaving a neck of about 4 mm in diameter. The ampoule is now ready for use and later can be sealed at the constriction.

19. Blowing bulb in glass tubing.—Select a piece of glass tubing of the desired size and of sufficient length to allow for holding one end by hand and the other in the mouth while blowing. Seal one end. Hold the tubing over the flame, rotating with both hands, until the desired portion is heated to the melting point. Immediately place the open end of the tube in your mouth and blow up the bulb to the required size. If the bulb is to be of considerable size, some concentration of glass must be attained before the final blow; this is done by gently approximating the two ends while the middle is soft, giving an occasional slight blow to prevent collapse of melted glass. The trouble encountered may consist of eccentric bulbs, due to uneven heating, or to thin paper-shell bulbs due to overblowing without sufficient glass concentration. A test tube may be similarly handled. A terminal bulb may be made at the end of glass tubing by a one-hand manipulation and blowing.

20. Trimming damaged glassware.—Many pieces of damaged glassware can be saved and rendered satisfactory for further use by examining all glassware at time of washing and picking out selected damaged pieces for repair.

a. Pipettes.—Chipped tips may be smoothed with a file and trimmed to evenness in a flame; pipette with broken tips may be drawn out

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and new end prepared as described for capillary pipettes. It must be recognized that repairs to tip alter to some extent, the volumetric exactness and such repaired pipettes are unsuitable for future exact measurements. Pipettes with chipped or broken mouth parts can be saved by fusing in flame, by evening off with file and smoothing in flame, by fusing mouth part in flame and immediately pressing gently on smooth, flat surface of a solid object, or by cutting off a portion and trimming in flame.

b. Flasks, beakers, and test tubes.—Similarly, many other pieces of glassware with chipped, cracked, or broken lips, mouth parts, or rims can be saved by removing sharp edges with a file, fusing with a flame, and shaping with the rounded tip of a file.

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CHAPTER 2

EXAMINATION OF BLOOD—CLINICAL PROCEDURES

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Platelet count	34
Coagulation time	35
Bleeding time	36
Clot retraction time	37
Prothrombin time (Howell's method)	38
Sedimentation rate	39
Venipuncture	40

21. General.—The routine laboratory work on blood requires very small amounts of blood, which may be obtained from the finger or ear in adults, or from the bottom of the heel in infants. Usually, the ball of the middle or ring finger is used. Some of the more technical tests require larger amounts of blood for which it is necessary to puncture a vein by the method described in detail in paragraph 40.

22. Finger puncture.—*a. Materials.*—(1) Puncture instrument with a sharp-cutting edge (not a round needle or pin) such as automatic blood lancet, Hagedorn needle, or Bard-Parker blade (size 11). This blade may be pushed through a cork and this cork used to stopper the alcohol bottle. Keep all the cutting blades as clean and shiny as possible.

(2) Cotton; alcohol 70 percent or acetone-alcohol, equal parts.

(3) Clean pipettes and chemically clean slides.

b. Procedure.—(1) Rub the finger briskly or place hand in warm water to promote blood flow.

(2) Clean the finger with the preferred sterilizing solution (alcohol or acetone-alcohol) and dry. If the fingertip is wet the blood will not form a round drop.

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(3) Hold the ball of the finger tightly between your thumb and index finger until the skin color is dark red. Puncture the finger with a firm, quick stroke deep enough so the blood will flow immediately. Do not squeeze the finger after the puncture because this forces tissue juices into the cut and dilutes the blood.

(4) Wipe off the first drop with dry cotton.

(5) Collect a large drop before touching a blood pipette or slide to the drop. Fill the pipettes and make films as indicated.

23. Hemoglobin determination.—*a. Materials (Tallquist method).*—(1) Finger-puncture equipment.

(2) A Tallquist scale, which is a paper strip with red bands, each graded to represent hemoglobin content on test paper from 10 to 100 percent.

(3) Absorbent paper (supplied in a book with the scale).

b. Procedure.—(1) Blot a drop of blood with a sheet of the absorbent paper. Set aside for a moment.

(2) Match against the color standard. Use a white background, in daylight if possible. Note the figure of color band similar to color of test spot.

c. Report.—Reports are expressed in percentages, stating the test used, for example, 90 percent (Tallquist). This Tallquist test is commonly used, though very inaccurate and shows only gross changes. The scale is based on 15.8 gm of hemoglobin per 100 cc of blood equals 100 percent.

d. Other methods.—Haden-Hausser, Newcomer, Dare, and Sahli hemoglobinometers, the Sahli-Hellige hemometer, and the various photelometers which may be used at some laboratories will all have printed directions with them. All of them will require practice with that particular instrument to attain uniform results. All of the above methods are more accurate than the Tallquist scale, but require more complicated and expensive equipment.

24. Red blood cell counting.—*a. Materials.*—(1) Lancet for puncture.

(2) Cotton or gauze.

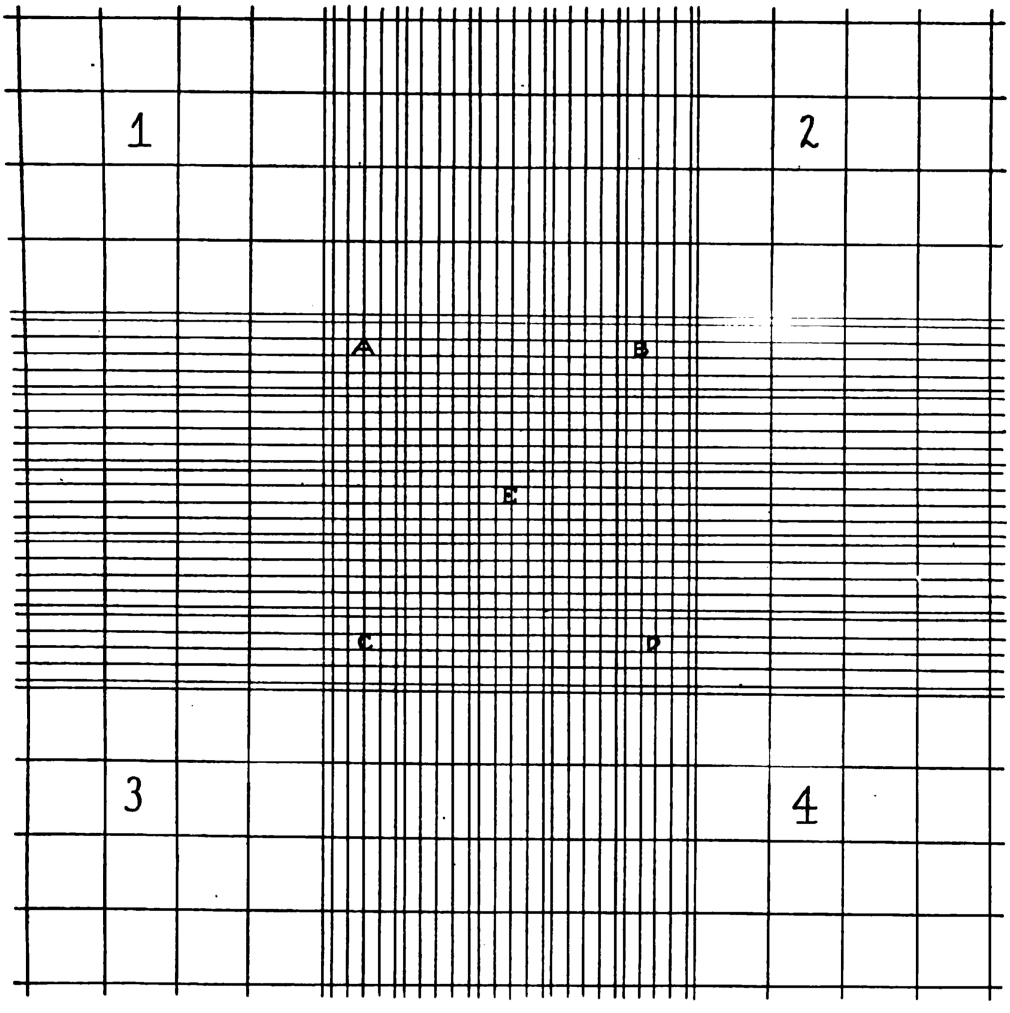
(3) Water, alcohol, and ether for cleaning pipettes.

(4) Microscope.

(5) Diluting pipette for red blood cells. It often has a red bead in the bulb to make it quickly recognizable. The Thoma pipette is marked in graduated lines along the capillary bore. The fifth graduation from the tip is marked 0.5, the tenth 1.0; above the bulb is a line marked 101. In this pipette, if blood is drawn to the 0.5 mark and the diluting fluid to the 101 mark, the dilution is 1 to 200.

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(6) Counting chamber. The Levy chamber with the improved Neubauer ruling is the supply table item of issue. There are other types of ruling and several kinds of chambers, all similarly used. The chamber is a thick glass slide with two central platforms; on the surface of each is engraved a series of rulings. The side plat-



NOTE.—The method of blood counting is thoroughly explained herein. The numbers 1, 2, 3, and 4, and the 16 surrounding squares of each, indicate the parts of the slide used in counting white blood cells. The letters A, B, C, D, and E, and the areas between the double lines, indicate the areas used in counting red blood cells.

FIGURE 1.—Improved Neubauer counting chamber.

forms on which the special cover glass fits are exactly 0.1 mm higher than the central platforms. When the cover slip is in place there is a space 0.1 mm deep, the ruled areas having a surface area of 9 sq mm. The four large corner squares outside the double ruled lines (marked 1, 2, 3, and 4 in fig. 1) are each subdivided into 16 smaller

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squares. The central square is divided by double lines into 25 small squares each of which contains 16 smaller squares, making a total of 400 squares (see fig. 1). Each small square then is 1/400 sq mm.

(7) Diluting fluid—Hayems solution:

Sodium chloride	1.0 gm
Sodium sulfate	5.0 gm
Mercuric chloride	0.5 gm
Distilled water	200 cc

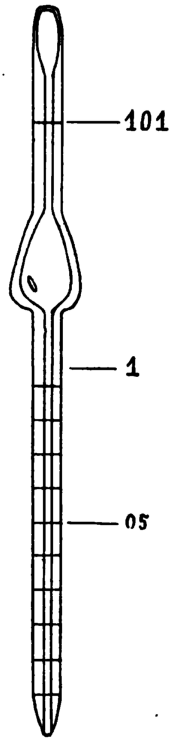


FIGURE 2.—Red blood cell pipette.

b. Counting chamber and pipette cleaning.—All pipettes and counting chambers should be clean and dry before using and should be cleaned immediately after using. Avoid harsh rubbing or strong solutions on the counting chamber.

(1) *Counting chamber.*—Cleanse the surface of the counting chamber with soap and water. Wash with distilled water and air-dry. If needed immediately, dry on soft gauze and lens paper. Cleansing with xylol and other cement solvents must be avoided; alcohol or ether may be used, with care, to remove oil.

(2) *Pipette.*—(*a*) Draw water through pipettes by suction (mouth suction, water pump, or air pump).

(*b*) Draw alcohol through pipettes by suction; this will remove the water.

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(*c*) Draw ether through pipettes; this will remove the alcohol. Continue the suction of air for a few seconds to remove the ether.

(*d*) The small bead in the bulb should then shake about freely, indicating a clean and dry pipette. If pipettes become plugged through neglect, clean capillary bore with a horsehair and soak overnight in dilute nitric acid, then clean as above.

c. Procedure.—(1) Puncture the finger in the usual way.

(2) Draw up blood exactly to the 0.5 mark on the red blood cell pipette. Remove any excess on the outside of the tip by wiping on a piece of gauze.

(3) Draw up diluting fluid exactly to the mark 101, making a dilution of 1 to 200.

(4) Kink the rubber tube at the end of the pipette, hold it against the middle finger with the capillary point on the ball of the thumb, and shake in a figure-of-eight motion for 2 minutes to insure good mixing.

(5) Put the cover slip in place on the counting chamber.

(6) Blow out 3 drops, touch the tip of the pipette to the edge of the platform, and allow a thin layer of fluid to flow under the cover glass. If the fluid flows into the troughs, or there are bubbles under the cover glass, clean the counting chamber and try again.

(7) Allow the cells to settle for 2 minutes.

(8) Examine under the high-dry lens of a microscope.

d. Counting.—Count all the cells in squares A, B, C, D, and E, as illustrated in figure 1. In counting cells in each square (as A in fig. 1) enclosed by double lines, count all cells touching the inner lines on the right and top of the square. Do *not* count any cells touching the lines on the left and bottom of the square. The difference between the number of cells in any two blocks should not be more than 15 cells. If this is the case, the mixing was not complete or the chamber was dirty. From this count calculation is made of the number of cells per cubic millimeter of blood.

e. Calculation example.—(1) *Long method.*—(*a*) Squares A, B, C, D, and E give counts 100, 98, 98, 104, and 100; total 500.

(*b*) Therefore, 80 small squares, which occupy $\frac{5}{25}$ or $\frac{1}{5}$ sq mm, contain 500 cells.

(*c*) One square millimeter would contain $5 \times 500 = 2{,}500$ cells.

(*d*) As this cell layer is 0.1 mm thick, 1 cu mm would contain $10 \times 2{,}500 = 25{,}000$.

(*e*) As the blood was diluted 200 times: $200 \times 25{,}000 = 5{,}000{,}000$ cells per cubic millimeter of blood.

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(*f*) Summary: Count of 80 small squares×5 (for area)×10 (for volume)×200 (for dilution) equals number of cells per cubic millimeter of blood.

(2) *Short method.*—If the dilution was 1:200, the total cells per cubic millimeter may be found by adding four zeros to the total red blood cell count in squares A, B, C, D, and E. For example, 500 with four zeros added will be 5,000,000.

f. Normal red blood cell (erythrocyte) counts.

Men: 4,500,000 to 6,000,000 per cu mm.

Women: 4,000,000 to 5,500,000 per cu mm.

g. Sources of error.—(1) Failure to hit the mark exactly with blood.

(2) Inaccurate dilution—either a bubble in pipette or failure to exactly hit the mark with diluting fluid.

(3) Improper placing of the cover slip on the chamber.

(4) Overfilling the chamber.

(5) Not shaking long enough in the pipette.

(6) Dirty chamber or pipette.

(7) Yeast growing in the diluting fluid.

25. Color index.—*a.* The term "color index" means the amount of hemoglobin in the average red blood cell of the patient compared with the normal amount.

$$\text{Color index} = \frac{\text{hemoglobin percent}}{\text{red blood cells percent}}$$

b. To express the red blood cell count as percent, it is necessary only to multiply the first two figures of the total red blood cell count by two. Example:

Red blood cells, 5,000,000.

Hemoglobin, 100 percent.

$$\text{Color index } \frac{100}{50\times 2} = 1.$$

c. A normal color index ranges from 0.85 to 1.15.

26. White blood cell (leucocyte) counting.—*a. Materials.*—(1) Same as for red blood cell count except for the pipette and the diluting fluid.

(2) White blood cell pipette is similar to the red cell pipette, but has a smaller bulb which contains a small white bead and gives less dilution to the blood. The fifth line on the graduated capillary tube is marked 0.5, the tenth line 1.0, and above the bulb 11.

(3) White blood cell diluting fluid:

Glacial acetic acid	0.5 cc
Distilled water	99.5 cc

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This fluid may be tinted blue, for convenience in identifying it, by addition of a drop of 1 percent gentian violet. This solution should be freshly prepared every 2 weeks.

b. Procedure.—(1) Draw blood to the 0.5 mark.

(2) Draw diluting fluid to the mark 11, making a dilution of 1: 20.

(3) Shake as in red blood cell counting.

(4) Discard 3 or 4 drops and fill the counting chamber.

(5) Allow the cells to settle.

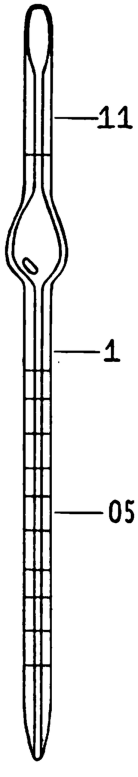


FIGURE 3.—White blood cell pipette.

(6) Examine under the low power of the microscope.

(7) When doing a complete blood count, shake the red blood pipette in one hand, the white in the other. Fill the counting chambers, red on one side and white on the other.

c. Calculation.—The white cells are counted in the four large corner squares labeled 1, 2, 3, and 4 in figure 1. The difference between the largest and smallest number of cells in any two squares should not exceed 10. Each large square contains 16 smaller squares and represents a volume of 0.1 cu mm. The four squares are counted and divided by four to get the average per 0.1 cu mm.

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d. Example of calculation for white blood cells.—(1) *Long method.*

Square 1	34
Square 2	42
Square 3	38
Square 4	36
Total for 0.4 cu mm diluted blood (4 mm squares 0.1 mm thick)	150
Total for 0.1 cu mm diluted blood (150÷4)	37.5
Number of cells in 1 cu mm diluted blood (37.5×10)	375

To obtain the number in 1 cu mm of undiluted blood multiply by 20 (as the dilution is 1 to 20) or (20×375)=7,500. Summary: Count of 4 squares, divided by 4, times 10 (for area) times 10 (for volume) times 20 (for dilution) equals number of cells per cubic millimeter of blood.

(2) *Short method.*—Multiply the total number of cells in 0.4 cu mm by 50; 150×50=7,500 white cells per cu mm.

e. Normal white blood cell (leucocyte) count.—Normally, this count is 5,000 to 10,000 per cu mm.

Many normal persons have variable counts due to activity, time of day, etc. Daily counts on a patient should be done at the same time every day.

In certain cases where the white count is very high, it may be necessary to use a dilution of 1:100, using the red cell pipette, changing the calculation accordingly. In cases where the count is abnormally low, make the dilution 1:10 by drawing blood to the 1.0 mark instead of 0.5.

27. Glassware cleaning (for blood films).—A prerequisite in making a good blood film is to have chemically clean glassware.

a. New slides.—(1) Wash in soapy water and rinse thoroughly with water.

(2) Place slides in a large beaker of 95 percent alcohol.

(3) Polish with a soft lint-free cloth (not gauze).

(4) Flame over a bunsen burner.

(5) Place in box with clean slip of paper between each slide.

b. Dirty slides.—(1) Boil in 5 percent sodium bicarbonate solution.

(2) Scrub with soap and water.

(3) Place in cleaning solution (potassium bichromate-sulfuric acid) for 12 hours.

(4) Then wash as for new slides.

(5) Discard all slides that are badly scratched or discolored.

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c. Cover glasses.—The same as for slides except do not flame. Careful wiping will prevent much breakage. Do not use pressure.

28. Preparation of blood films.—*a. Materials necessary.*—(1) Equipment for finger puncture.

(2) Clean slides free from grease.

b. Procedure.—(1) Puncture the finger.

(2) Place 1 small drop of blood on the end of a slide and place the slide on a flat surface.

(3) Hold a second slide between the thumb and third finger and place one end at a 30° angle on the slide holding the drop of blood.

(4) Pull the upper slide until it touches the drop of blood which then spreads along the narrow end of the top slide (see fig. 4). (The

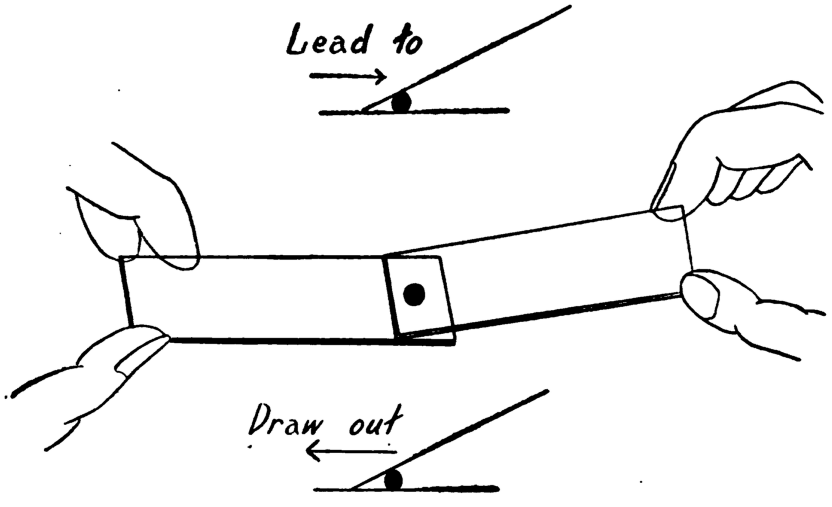


FIGURE 4.—Method of making blood film.

slide held with the left hand is supposed to be laid flat on a smooth surface.)

(5) Push the top slide with a firm, steady motion toward the opposite end of the bottom slide; the slower the movement, the thicker the film; the greater the angle, the thicker the film. Avoid all unnecessary pressure because of the fragility of the cells.

(6) Allow to air-dry. In areas where insects are abundant slides must be protected or they will be ruined. A good film should be smooth and without waves. The edges should be even and the film should not extend to the edges or end of the slide. Labeling may be done by writing on the thicker end of the film with an ordinary lead pencil when the film is dry. The slides should be stained within 24 hours for best results.

29. Blood stains.—*a. Wright's stain.*—This stain is used for routine blood films and for other laboratory purposes. The powdered

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stain is issued from the supply table. The stain solution is prepared by the laboratory as follows:

Wright's stain powder (supply table item)	0.3	gm
Glycerin	3.0	cc
Methyl alcohol, absolute (must be acetone-free)	97.0	cc

Put the powder in a dry mortar; grind with a pestle; add the glycerin and grind together thoroughly. Add the methyl alcohol and mix. Allow to stand overnight in a tightly stoppered flask, then filter and set aside for a few days before use. Age improves the stain. The glycerin may be omitted when air humidity is high.

b. Buffer solution for Wright's stain.

Potassium phosphate (monobasic)	1.63	gm
Sodium phosphate (dibasic)	3.2	gm
Distilled water	1000	cc

c. Wright's stain procedure.—(1) Cover the dried film completely with stain for 1 to 3 minutes. This fixes the blood film.

(2) Add the buffer solution to the stain, drop by drop, until a greenish, metallic scum appears on the top. The stain and buffer should cover the slide, but none should run off. Determine the time for staining by trial with a series of slides. This will usually be about 2 minutes, but is variable with every batch of stain. The color of the cells may be varied by changing the pH of the buffer solution. The granules in the neutrophiles should stain a lilac color, the eosinophils bright red, the basophils deep blue.

(3) Wash with water, continuing washing until the slide is lavender-pink.

(4) Allow the slide to stand on edge until dry.

(5) Apply a drop of immersion oil to slide and examine under the oil-immersion lens.

d. Giemsa's stain.—(1) *Stock solution.*

Giemsa powder (supply table item)	0.5	gm
Glycerin (dissolve powder in glycerin 1 to 2 hours)	33.0	cc
Methyl alcohol, absolute (acetone-free)	33.0	cc

(2) *Dilute stain (ready for use).*—One cc stock solution to 10 cc distilled water.

(3) *Procedure.*—(*a*) Fix film with methyl alcohol 3 to 5 minutes in a Coplin jar.

(*b*) Dry in air.

(*c*) Immerse in dilute stain for 20 to 30 minutes (in a Coplin jar).

(*d*) Wash in distilled water.

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(*e*) Stand on end to dry.

(*f*) Examine under oil immersion.

30. Differential white blood cell count.—*a. Materials.*—(1) Finger-puncture and staining equipment.

(2) Wright's stain.

b. Procedure.—(1) Prepare blood films.

(2) Stain with Wright's stain.

(3) Examine under oil immersion, recording each type of white cell seen. Follow a set pattern in each examination to avoid counting the same cell more than once. Cover the thinner parts of the film until the proper number of cells are counted.

(4) Kolmer and Boerner recommend that the following number of cells be counted, depending on the total white blood count:

Total white count	Number of cells to classify
Under 5,000	50
5,000 to 10,000	100
10,000 to 15,000	200
15,000 to 20,000	300

(5) The usual routine procedure is to count 100 cells, classifying as polys (polymorphonuclears) all cells with multiple nuclei and as lymphs (lymphocytes) all cells with one nucleus. This is a very rough procedure and should be extended to the Schilling count whenever any deviation from the normal is noted. The normal range of count is entered in the Schilling chart. (See fig. 5①.)

31. Characteristics of stained cells.—*a. Red blood cells.*—Normal red blood cells (erythrocytes) are round, nongranular, nonnucleated cells, the centers of which are less intensely colored than the borders. In various diseases the blood may contain erythrocytes showing the following abnormalities:

(1) *Achromia.*—Pale staining erythrocytes; decreased hemoglobin.

(2) *Polychromasia.*—Many of the erythrocytes take a bluish rather than a tan color.

(3) *Anisocytosis.*—A wide variation in the size of the cells.

(4) *Poikilocytosis.*—Many of the red blood cells are not round.

(5) *Macrocytosis.*—The average size of the cells is greater than the normal 7.5 to 8, microns.

(6) *Microcytosis.*—The average size of the cells is smaller than normal.

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(7) *Stippling.*—The cells contain a fine dusting of bluish-black granules, seen in lead poisoning.

(8) *Howell-Jolly bodies.*—The cells contain one or two small, blue-black dots.

Name ..... Rank ..... Ward .....
Hosp. ..... Lab. .....

R. B. C. ..... W. B. C. ..... Hb. .....
Color index ..... Reticulocytes ..... Platelets .....
Clotting time ..... Bleeding time .....
DIFFERENTIAL: Polys. ..... Lymphs. .....

SCHILLING

Count	Baso.	Eosins.	Myelos.	Juven.	Stabs.	Segs.	Lymphs.	Monos.
	0-1	2-4	0	0-1	3-5	58-66	21-25	4-8

R. B. C. morphology .....
.....
.....
Remarks .....
.....

M. C.

Form 55 L-1
MEDICAL DEPARTMENT, U. S. ARMY
(Revised May 31, 1939)

BLOOD

Date .....

16—16973

① Blood count report form.

Name : Ward :

Total Leucocyte Count	Basophiles	Eosinophiles	NEUTROPHILES				Lymphocytes	Monocytes	
			Myelocytes	Juvenile	Staff	Segmented			
									1
									2
									3
									4
									5
									6
									7
									8
									9
									10
% Total									

② Schilling differential count.

FIGURE 5.

(9) *Reticulocytes.*—These red cells have feathery, dark blue, irregular fibers within the cell.

b. White blood cells (leucocytes).—(1) *Lymphocytes.*—These mononuclear cells are sometimes divided for convenience into small,

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medium, and large, but in routine counts are usually classified as small lymphocytes.

(2) *Monocytes.*—See the color plate facing figure 6.

(3) *Granulocytes.*—See the color plate for development and staining reaction of this group of cells.

32. Schilling's nuclear index.—*a. Materials.*—Differential slide equipment.

b. Procedure.—Classify the white cells according to headings shown on the illustration of Schilling's differential count (fig. 5). The stage of maturity, as well as the types of cells, is significant. This is reported by subdividing the neutrophiles into myelocytes, juveniles, staff, and segmented cells, the last being the mature cells. The immaturity of the cells, "shift to the left," indicates an increase in the bone marrow output of new cells. For illustration of cells as they develop from the bone marrow, see the color plate reproduced from Wright's stained cells by Kracke.

33. Reticulocyte counts.—*a. Materials.*—(1) Equipment for finger puncture.

(2) Clean cover slips and slides.

(3) One percent alcoholic solution of the dye, brilliant cresyl blue.

b. Procedure.—There are several other good methods available (see par. 34).

(1) Smear across a slide a few drops of the stain as in preparing a blood film.

(2) Dry in air.

(3) Place a small drop of blood in the center of a cover slip, and place blood side down on the stain.

(4) Let stand 10 minutes.

(5) Examine under oil immersion lens.

(6) Count 1,000 red blood cells, noting the number that have the bluish strands of reticulum. (See color plate.) The thinner the preparation, the easier the count.

(7) Report: percentage of red blood cells which contain reticulum.

(8) Normal counts, 5 to 10 reticulated red cells per 1,000 or 0.5 to 1.0 percent.

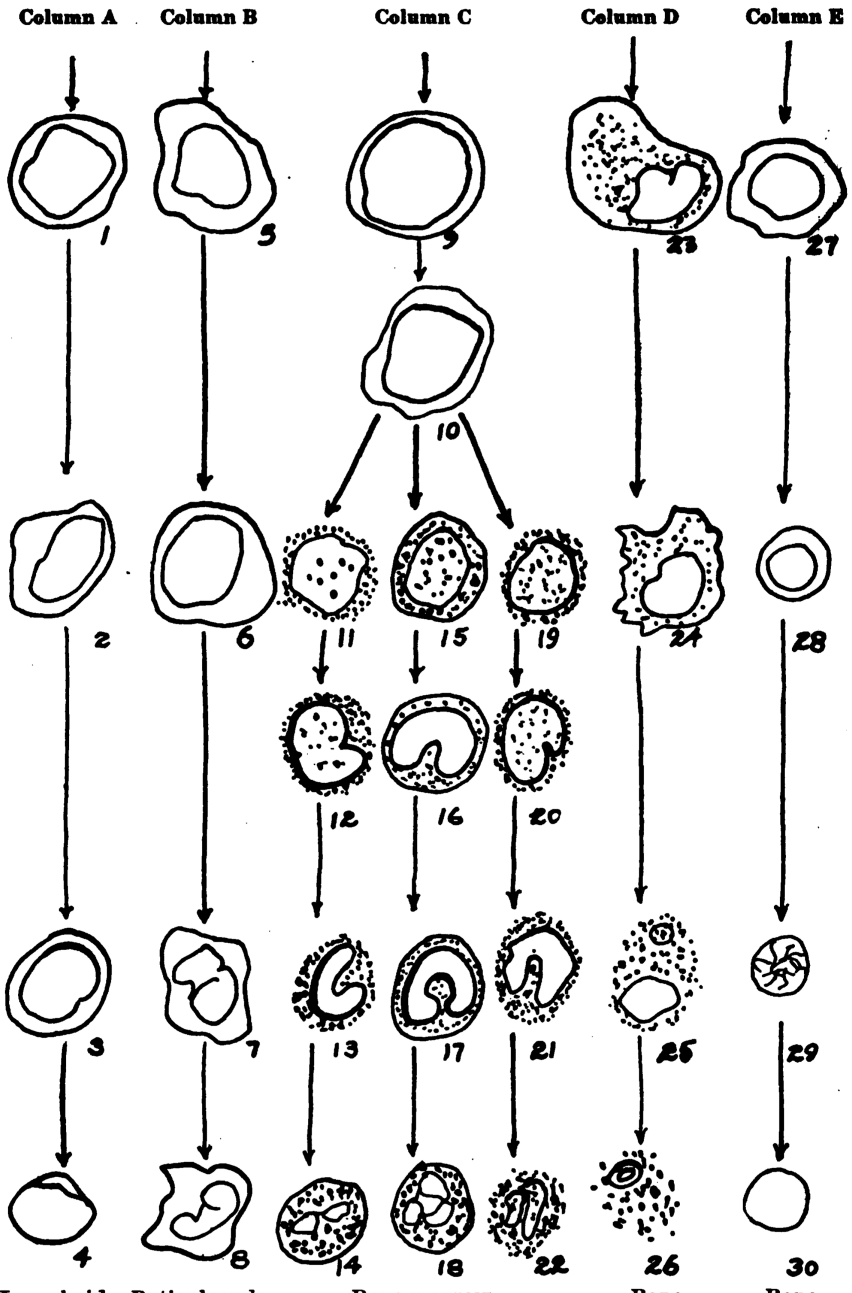
34. Platelet count.—*a. Materials (Olef's method).*—(1) Finger-puncture equipment, red blood cell pipette, slides, cover glasses.

(2) Warm water, soap, alcohol, ether.

(3) Paraffin cup. Prepared by melting the center of a small cube of paraffin with the heated end of a glass rod.

(4) Wooden applicator, tip of which has been dipped in melted paraffin.

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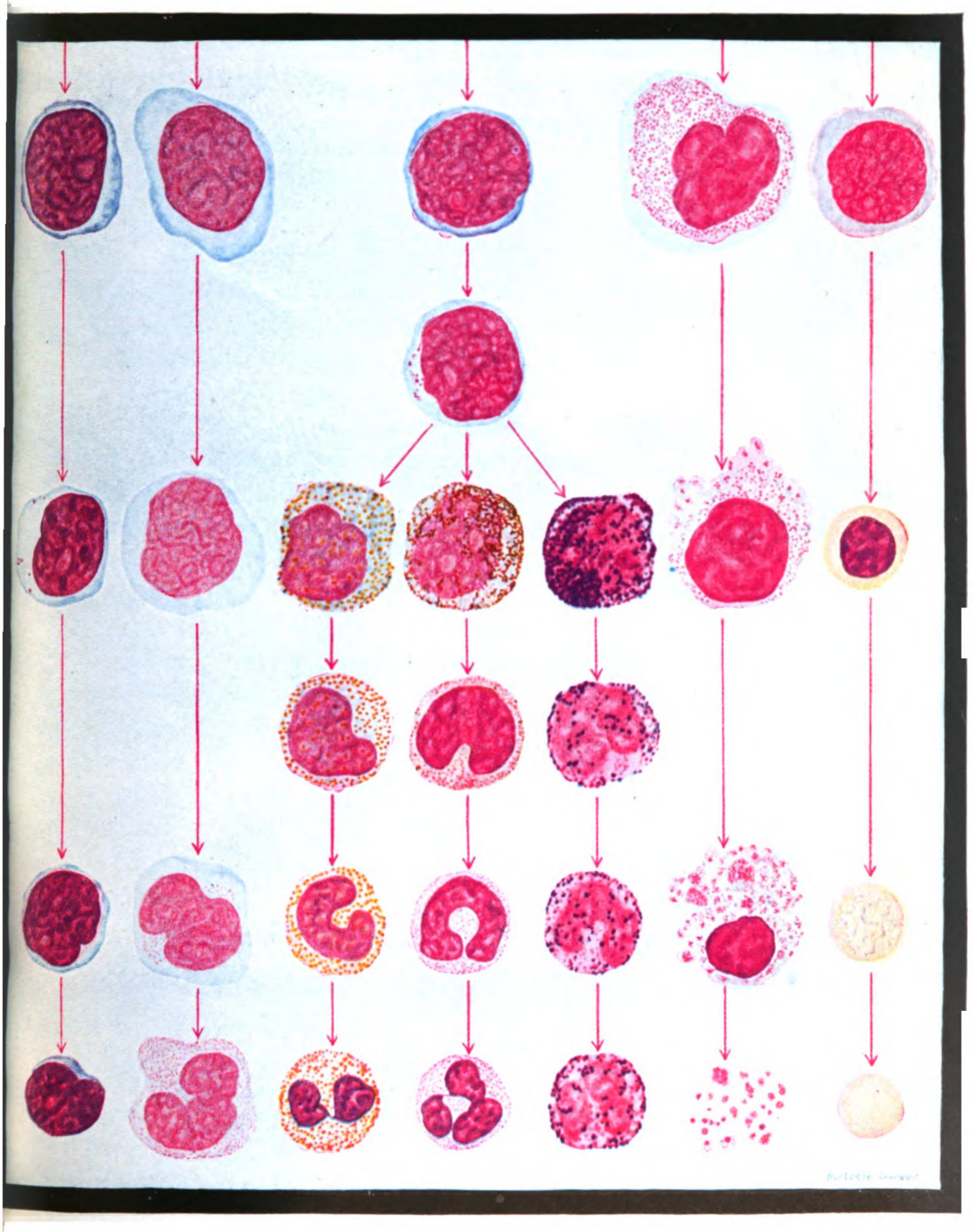


Column A: 1, lymphoblast; 2, large lymphocyte; 3, intermediate lymphocyte; 4, small lymphocyte. *Column B:* 5, monoblast; 6, large monocyte without azure granules; 7, monocyte with a few azure granules; 8, mature monocyte with azure granules. *Column C:* 9, Myeloblast; 10, premyelocyte; 11, eosinophilic myelocyte; 12, juvenile eosinophil; 13, band eosinophil; 14, segmented eosinophil; 15, neutrophilic myelocyte; 16, juvenile neutrophil; 17, band neutrophil; 18, segmented neutrophil; 19, basophilic myelocyte; 20, juvenile basophil; 21, band basophil; 22, segmented basophil. *Column D:* 23, megakaryocyte (bone marrow); 24, later megakaryocyte; 25, megakaryocyte (peripheral blood); 26, thrombocytes (platelets). *Column E:* 27, megaloblast; 28, normoblast; 29, reticulocyte; 30, normocyte (erythrocyte).

FIGURE 6.—Origin and development of blood cells.

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Plate I.



From Roy R. Kracke's Diseases of the Blood and Atlas of Hematology. Copyright, 1941, by J. B. Lippincott Company

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(5) Diluting fluid. This solution must be kept in a cool place and filtered every few days. Formula:

Sodium metaphosphate	1.0 gm
Sodium chloride	.4 gm
Dextrose	.1 gm
sodium bicarbonate	.1 gm
Brilliant cresyl blue	.15 gm
Distilled water	100 cc

This solution preserves both platelets and reticulocytes, and both counts may be done at once.

b. Procedure.—(1) Immerse the patient's hand in warm water.

(2) Wash with soap, water, alcohol, and ether. Make a deep puncture in the finger without squeezing.

(3) Put 5 drops of the diluting fluid into the cup.

(4) Shake the first drop of blood off the finger.

(5) Place a drop of the diluting fluid on the puncture wound, and turn the hand over so that the blood will drop into the paraffin cup. Let enough blood fall into the cup to make the dilution approximately 1:5. This will be 1 or 2 drops.

(6) Stir with the wooden applicator.

(7) Allow to stand 1 to 2 minutes. Stir again, and then transfer a drop of this material to a slide with the applicator. (If several preparations are to be done, a heated glass slide placed on top of the paraffin block will keep the fluid from evaporating.)

(8) Place a cover slip over the drop and allow to stand for 15 minutes.

(9) Examine under oil immersion, counting both platelets and reticulocytes until a total of 1,000 red blood cells have been counted.

(10) Make a red blood cell count in the usual way.

c. Calculation.

$$\text{Number of platelets} \times \frac{\text{red blood cell count}}{1{,}000} = \text{absolute number of platelets per cubic millimeter.}$$

The average number of platelets determined by this method is about 500,000 per cubic millimeter. This is one of the most accurate methods.

35. Coagulation time.—*a. Slide method.*—(1) *Materials.*—Finger-puncture equipment, clean slide, needle, and watch.

(2) *Procedure.*—(*a*) Clean the finger with alcohol and make a puncture.

(*b*) Place a few drops of blood on the slide.

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(*c*) At half-minute intervals, draw a needle slowly through the blood drop. When a fine thread (fibrin) can be picked up by the needle point, coagulation has begun.

(*d*) The time elapsed between the flow of blood and the formation of fibrin is recorded as the coagulation time.

b. Capillary tube method.—Draw out soft glass tubing into a capillary pipette over a wing-top bunsen burner. After finger puncture, fill this tube by touching it to the drop of blood. Then at half-minute intervals, break off a small fragment of the tube. If the fibrin strings out at the broken end, coagulation has begun. The elapsed time is reported as the coagulation time. The normal coagulation time is from 2 to 8 minutes.

36. Bleeding time.—The bleeding time is the time that it takes the blood to stop flowing from a measured cut in the finger or ear.

a. Materials.—Finger-puncture equipment, filter paper, and watch.

b. Procedure.—(1) Puncture the finger or the ear lobe.

(2) Note time when blood begins to flow.

(3) Blot with the filter paper every half minute.

(4) The time between the first drop and the last is the bleeding time.

(5) The normal bleeding time is 1 to 3 minutes. When bleeding continues longer than 10 minutes the bleeding time is seriously prolonged.

37. Clot retraction time.—*a. Materials.*—Equipment for venipuncture, test tube or blood vacuum tube, incubator, and watch.

b. Procedure.—(1) Place 2 cc of blood in a dry test tube.

(2) Incubate at 38° C. for 24 hours.

(3) Observe at the end of each hour for 6 hours and at intervals of 6 to 12 hours thereafter.

(4) Record the elapsed time before the clot retracts.

(5) Under these conditions the clot normally retracts completely within 18 to 24 hours after it is formed. Delay in retraction, or failure of the clot to retract, is usually associated with a decrease in platelets.

38. Prothrombin time (Howell's method).—*a. Materials.*—(1) Equipment for venipuncture.

(2) Two-cc syringe rinsed in physiologic salt solution.

(3) Centrifuge tube containing 0.25 cc of 1 percent sodium oxalate in physiologic salt solution.

(4) Physiologic salt solution.

(5) Five-tenths percent solution of calcium chloride.

(6) Dropper, four small test tubes, and watch.

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b. Procedure.—(1) Take 2 cc of blood from the vein, avoiding suction.

(2) Immediately place the blood in the centrifuge tube containing the sodium oxalate.

(3) Mix the blood and the sodium oxalate by inverting the tube three times.

(4) Centrifugalize for 2 to 3 minutes.

(5) Place 5 drops of the supernatant fluid (plasma) in each of the four small test tubes.

(6) Add calcium chloride solution as follows: 2 drops to tube No. 1; 3 drops to tube No. 2; 4 drops to tube No. 3; 5 drops to tube No. 4. Mix by shaking gently.

(7) Coagulation occurs in all tubes, but at varying times. Invert the tubes to determine the presence of coagulation; the tube which clots first is used in determining the "prothrombin time." This prothrombin time is the interval between the addition of calcium chloride solution to the plasma and the completion of coagulation.

(8) With each unknown, run a known specimen of blood.

(9) The average prothrombin time is 10 minutes.

39. Sedimentation rate.—There are so many methods now in use for performing this test that results have not been comparable. Only one method will be given here with the normals for this method only.

a. Materials.—(1) Venipuncture equipment.

(2) Cutler tube. This is a tube graduated at 1-cc capacity and marked into fifty 1-mm divisions with the zero at the top.

(3) Two-cc sterile syringe.

(4) Three percent sodium citrate solution (sterile).

b. Procedure.—(1) Draw 0.1 cc of 3 percent sodium citrate into the 2-cc syringe.

(2) Draw into the same syringe 0.9 cc of blood from the vein.

(3) Mix and pour into the upright Cutler tube.

(4) Read the height of the blood cell column every 5 minutes for 1 hour, plotting results as a graph. The graph may be constructed by a series of horizontal lines, divided from zero to 40. The vertical lines drawn across the horizontal lines are spaced in minutes, in units of 5, from zero to 60. Both zero points should be in the upper left-hand corner of the graph.

(5) The normal for men is under 8 mm and for women, under 10 mm, in 1 hour, with an almost horizontal line.

c. Modified Cutler method.—The modified Cutler method utilizes materials normally on hand in the laboratory. Select test tubes from

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75- by 10-mm stock. The tubes should be of such caliber that 2 cc of liquid produce a column 50 mm high. Etch the tube at this point. Put exactly 0.2 cc of 3 percent sodium citrate in the tube. Fill to the 50-mm mark with blood. Mix by inverting in order to avoid air bubbles. Set tube in the vertical position. Measure the height of the cell column with a millimeter ruler, and record this height at 10-, 20-, 30-, and 60-minute intervals. Plot on a graph as in *b*(4) above.

Normal for men, 2 to 8 mm in 1 hour; for women, 2 to 10 mm.

40. Venipuncture.—*a. For blood culture.*—(1) *Materials.*—(*a*) Luer syringe, 10-cc, and needle, 20-gage, both sterile.

(*b*) Flask of appropriate culture medium.

(*c*) Tincture of iodine; alcohol, 70 percent; and tourniquet.

(*d*) Cotton or gauze pledgets, sterile.

(*e*) Alcohol lamp if no gas burner is available.

(2) *Procedure.*—(*a*) Thoroughly cleanse the skin over the arm vein and surrounding area with alcohol.

(*b*) Paint with iodine over the median vein and leave on for 2 or 3 minutes.

(*c*) Light alcohol lamp or burner.

(*d*) Unwrap syringe and insert plunger into barrel. Do not touch inside of barrel or shaft of plunger.

(*e*) Remove plug from needle tube and flame mouth of tube.

(*f*) Insert neck of syringe into mouth of tube and tilt tube so that needle will slide down over the neck.

(*g*) Remove syringe and needle and set the needle firmly on the neck, being careful to touch only the hub of the needle.

(*h*) Flame both the needle point and the mouth of the tube that contained the needle. Cover the needle with the flamed tube and set aside while completing the preparation of the arm.

(*i*) Apply tourniquet above the elbow, not too tightly. If the vein does not distend well, have patient clench fist.

(*j*) Sponge off the iodine with alcohol.

(*k*) Puncture the skin with needle *a little to one side of the vein* and parallel to it; then enter the vein from that side, about half an inch above the skin puncture.

(*l*) After securing the desired amount of blood, loosen the tourniquet and have patient open fist.

(*m*) Withdraw needle quickly, then press an alcohol-soaked pledget firmly over the puncture. Have patient hold pledget tightly in place.

(*n*) Open flask and flame mouth thoroughly, holding the syringe near but not in the flame at the same time.

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(*o*) Insert needle into flask and force blood directly into culture medium. Withdraw needle from flask without touching sides of flask with either needle or blood.

(*p*) Flame neck of flask again, replug, and incubate.

b. For other purposes.—In taking the large number of routine blood samples required for other purposes which do not demand sterile blood, it is simpler to use only the sterile needle, as so many syringes are seldom available.

(1) Swab the site with iodine, followed by alcohol or acetone. Acetone alone may be used.

(2) Tighten tourniquet about the upper arm enough to dilate the vein firmly.

(3) Remove the needle from its tube and take the stylet out, being careful to touch only the hub.



FIGURE 7.—Venipuncture (method for holding needle and test tube when collecting blood directly from vein).

(4) Hold the needle tightly between the thumb and index finger at the hub. Place the tube for the blood specimen below the needle, grasping it with the third and fourth fingers, so that the hub of the needle is just within the mouth of the tube (see fig. 7). This is much easier to accomplish if the patient's arm is allowed to hang straight down.

(5) Puncture the skin a little to one side of the vein and parallel to it. If the needle is sharp, this can be done with one quick motion and is not at all painful.

(6) Turn the needle point slightly toward the vein and enter with a quick, short stab. If the point is turned too squarely toward the vein, there is risk of puncturing both walls.

(7) When 10 to 15 cc of blood is collected, loosen the tourniquet.

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(8) Press a pledget of cotton, soaked in alcohol or acetone, over the puncture and withdraw the needle quickly, maintaining the pressure until the bleeding, if any, has stopped.

c. Care of needles and syringes.—(1) As soon as the blood sample is taken, shake as much of the blood from the needle as possible and drop it into a beaker of water to lake the blood. On return to the laboratory, clean thoroughly with cold water and dry by forcing alcohol followed by ether through the bore. Never put a wet needle away, as rust is dangerous.

(2) Replace the stylet, leaving the loop of wire outside the point for the protection of the latter. Slide the needle, point down, into a Wassermann tube, plug tube with cotton, and sterilize by dry heat or in the autoclave.

(3) Sharpening the needles is best done on the finest grade of emery cloth, stretched on a flat surface. Finish on a fine, blue water stone. Even the finest grade of emery or carborundum, if used alone, will leave a slight saw edge that may cause too much pain.

(4) Syringes must never be left with the plunger in the barrel after use, no matter what has been in the syringe. Always wash out the syringe with water immediately after use and leave the plunger out until both it and the barrel have been carefully dried. Once a plunger has been "frozen" in the barrel, it may not be possible to remove it. Often the best way to free it is to force cold water through the neck of the barrel against the head of the plunger, using another syringe with a needle small enough to insert into the neck. Warming the barrel in hot water may loosen it. Soaking in cold water for several days may be necessary. Never use force.

(5) To sterilize, wrap the plunger and barrel separately in gauze, with an outer wrapping of heavy paper. Secure the wrapping with a turn or two of ordinary twine, tied in a slipknot to expedite unwrapping.

*d. **Cautions:*** (1) Certain dangers and discomforts to the patient must be avoided. These are infection, injury to the vein wall, hematoma, and needless pain.

(2) Infection is due to carelessness. Be certain needles and syringes are sterile. Never touch the shaft of the needle to anything that is not sterile before taking the blood.

(3) Injury to the vein wall may cause a clot to form on the wall. This may break free in the blood stream with serious results. Causes: undue trauma or the passing of the stylet through the needle while still in the vein in the attempt to free the needle from clots.

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Never pass the stylet through the needle before withdrawal. If clots plug the needle, withdraw it and try the other arm.

(4) Hematoma (blood tumor) is often very painful and may become infected. Causes: too large a needle in a delicate vein, withdrawal of the needle before tourniquet is loosened, or making insufficient pressure over the puncture after withdrawal.

(5) Needless pain is often due to excess of care and slowness in making the puncture. Dull needles most often cause it. A sharp 17-gage needle causes less pain than a dull 20-gage needle. Remember that those ill enough to be in a hospital may be set back by even a slight painful shock, especially if many blood tests have to be taken. It is always better to make patients lie down or sit in comfort while blood is being taken.

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CHAPTER 3

GENERAL CHEMICAL TECHNIC

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SECTION I

CARE AND USE OF LABORATORY APPARATUS

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41. General.—*a.* Chemical analyses, in general, are of two kinds: qualitative analyses to learn what substances are present in a specimen, or to find out if some particular substance is present, and quantitative analyses to determine the amounts of all or of some of the constituents present. Qualitative analyses tell only if a substance is present; quantitative determination tells how much there is in a particular specimen. The *detection* of sugar or albumin in the urine is an example of the first; the *determination* of blood or urine sugar, actually measuring the amount present, is an example of the second.

b. Quantitative analyses may be made in many ways. The methods most frequently employed are gravimetric, volumetric, colorimetric, and gasometric.

(1) In gravimetric analysis the constituent sought is separated from solution as an insoluble compound of known chemical constitution, dried, and weighed. From the weight of this compound the weight of the desired constituent is computed.

(2) In volumetric, or titrimetric, analysis the compound sought is not weighed but is determined by measuring the volume of a solution of known concentration which reacts with it. Many procedures in clinical chemistry are based on this principle.

(3) Colorimetric methods are also widely used in the clinical laboratory. The substance to be measured is made to react with other

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chemical compounds to produce a colored substance. It is essential that the depth of the color produced be proportional to the concentration of the substance sought for in the original specimen.

(4) Similarly in gasometric methods the particular unknown to be measured is made to react to produce a gas. This may be done direct in some cases; in others it is first necessary to combine the unknown with some other compound. In any event it is necessary that the amount of gas produced be in direct relationship to the amount of unknown originally present. The volume of the gas produced is measured under conditions which permit correction of the volume to standard temperature and pressure, or the volume may be held constant and the pressure exerted by the gas be measured on a manometer. From the volume or the pressure the amount of unknown is determined by computation. Although not an item of standard equipment, the manometric apparatus of Van Slyke and Neill is used in many of the larger laboratories of the army.

c. The reference books listed in the appendix give more detailed descriptions of these analytical methods.

42. Chemical balance.—The balance consists essentially of a horizontal lever with two arms of equal length, and in order to be sensitive and accurate the arms must be of equal length; the point of support must be above the center of gravity; the fulcrum (knife edge) and the knife edges from which the pans are suspended must lie in the same plane and be parallel to one another; and the balance must be level on a vibrationless support.

a. Selection of proper types.—(1) Balances which are most suitable for use in the laboratory are of three kinds:

(*a*) *Fine analytical balances.*—Fine analytical balances are used for gravimetric analyses and for preparing volumetric solutions and other fine weighings. Such a balance should have a capacity from 100 to 200 gm with a sensitivity of 0.1 mg when fully loaded. Some of the newer balances such as the Chainomatic have devices which obviate the use of fractional weights and greatly expedite weighings. Precision in weighing can be insured by the use of a standard set of weights whose accuracy is certified within certain limits by the U. S. Bureau of Standards, or by cheaper sets of ordinary weights on which the corrections have been determined. A standard set of weights may be used to check others, or a set may be calibrated by any one of several methods found in most textbooks on quantitative analysis.

(*b*) *Large balance.*—A large balance with a capacity of 2 to 5 kg and a sensitivity of 0.05 gm or less.

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(*c*) *Trip scales.*—Trip scales to balance tubes for the centrifuge and to weigh materials for the preparation of solutions that do not have to be extremely accurate.

(2) With the introduction of microchemical methods there has been a gradually increasing demand for the microbalance which permits the weighing of minute amounts of material with an accuracy of 0.001 mg. Since but few laboratories are so equipped and few technicians trained in microgravimetric analysis these methods will not be taken up in this manual.

b. Precautions in using.—(1) The balance should rest on a firm support which is practically free from mechanical vibration. Direct sunlight should not fall on it as it will cause irregularities and errors in weighing.

(2) When not in use the balance beam should *always* be raised off the knife edges or these will be injured by jarring. Always lower the beam slowly and carefully. Never permit the beam to rest on the knife edges while weights or substances are being added to or removed from the pans.

(3) The beam may be set swinging by dropping the rider upon it or removing it for an instant or by gently fanning one pan with the hand. Never start motion by touching the pan or by suddenly lowering the beam upon the knife edge.

(4) Make all weighings methodically by trying the weights, one after another in proper order. Before making a weight, see that the balance is properly adjusted. It must be level as shown by the plumb-bob or spirit level in the case. The pointer must rest at the zero mark with the beam raised. The pointer must swing equal distances to either side of the zero when the beam is set in motion and there is no load on the pans. Adjustment of the swings is made by the balancing screw weights on the ends of the beam. If the balance is equipped with pan arrests, these arrests should be so adjusted that the pointer is on the zero mark when the beam is on its knife edge and the arrests are in place.

(5) Final adjustments and final weighings should always be made with the balance case closed to prevent errors arising from air currents. When not in use the balance case should be closed and covered with a rubber cover to prevent attack from fumes and gases.

(6) Substances to be weighed should never be placed directly upon the pans, but upon watch glasses, weighing papers, or other containers. The object to be weighed must be at the same temperature as the air within the balance case. Air currents from a hot body and condensation upon the surface of a cold one introduce serious errors.

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The weight should always be checked carefully; first, by adding up the weights missing from the box (in which every weight should always have its own place); second, by adding up the weights that are on the pan; and last, by adding them up as they are returned to the box.

(7) Never handle weights or place them upon the pans except with the forceps. Always place the substance to be weighed on the left-hand pan and the weights on the right-hand one, except when the method of double or transposition weighing is used.

c. Methods of weighing.—(1) *Ordinary method of direct weighing.*—(*a*) Place the object to be weighed on the left pan. If the container is glass or porcelain and has been recently wiped dry, allow it to stand a few minutes in the balance case before weighing it. With the weight forceps place a weight which is judged to be slightly heavier than the object to be weighed on the right pan. Gently lower the beam a little and note which way the pointer swings; if to the left, the weight is too heavy, if to the right, the object is the heavier. Raise the beam and replace the weight with the next heavier one, if it was found to be too light. Continue the trials until a weight is found that is too heavy, always remembering to have the beam raised whenever anything is added to or removed from the pan.

Replace the weight which is too heavy with the next lighter one and try the fractional weights in order in the same manner until the range covered by the rider is reached. Close the balance case and adjust the rider until the pointer swings equal distances to the right and left.

Record the weight from the empty places in the box and check by counting the weights on the pan. Recheck the weights as they are replaced in the box.

(*b*) In making weighings there is a tendency to make the pointer swing through too great an arc. Because of retardation, which is proportional to the length of the swing, an error is introduced in long swings which becomes inappreciable in short ones. It is easier to read the end points of short swings because the pointer is moving more slowly, and there is also less danger of parallax error in reading short swings. A swing of two scale divisions to right and left is ample.

Instead of trying to get two exactly equal swings it is easier to adjust the rider until the swings are nearly the same, and then estimate from the known sensitivity of the balance how much the rider should be moved to make them equal. Place the rider just a little beyond this point and test the swings again. If the balance

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is adjusted so that the sensitiveness amounts to 5 scale divisions for 1 mg, then a swing that varies less than one-quarter division on the two sides of the center shows that the weight to the nearest 0.1 mg has been found. This method makes for speed and with substances that give up or take on water on standing, a rapid weight is more accurate than one that is made slowly. This method of weighing is to be preferred for most work to the precise method which follows.

(2) *Method of swings.*—(*a*) In this method, which is very precise, the zero reading of the balance, without any load, must be determined first. Set the beam in motion and record the turning points, or extreme positions, of the pointer for an uneven number of swings, usually 5, and take the mean of the readings. The first two swings are inaccurate because of the jar in shutting the balance case, and for other reasons, and are disregarded.

(*b*) For example, suppose the swings are: Left 6.5, right 2.5, left 6.3, right 2.3, left 6.1. The mean of the three left swings would be $\frac{6.5+6.3+6.1}{3}$ or 6.3; of the two right swings $\frac{2.5+2.3}{2}$ or 2.4; and the rest point would be $6.3-2.4$ or 3.9 scale divisions to the left. It is customary to give the minus sign to displacements to the left and the plus sign to those to the right, since these signs indicate whether the observed difference is finally to be subtracted from or added to the weight as given by the weights and rider.

(*c*) It may be simpler to number the divisions from 0 to 20 from left to right and so give the same algebraic sign to all observed readings. In this case, if both balance arms are equal, the zero point would be at 10. The zero reading may change during the course of the day and should be determined before each weighing. When a series of weighings are to be made, determine the zero point at the beginning and at the end of the series and use the mean.

(*d*) Next determine the sensitiveness of the balance for the particular load to be weighed by placing the object on the left pan and the weights on the right until equilibrium is established as nearly as possible. Determine the rest point on the scale in the same manner as in making the zero reading. Add or remove 1 mg by means of the rider and determine the rest point again. The difference between this and the previous point of rest gives the sensitiveness of the balance; that is, the number of scale divisions equal to 1 mg in weight with the particular load in question.

(*e*) Assuming that the first point of rest lies at 4.1 scale divisions to the right with a load of 25.723 gm and the second rest point, with

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a load of 1 mg less, or 25.722 gm, at 1.9 scale divisions to the right, then the sensitiveness of the balance will amount to 4.1−1.9 or 2.2 scale divisions. Since the zero reading was 3.9 scale divisions to the left and the rest point with a load of 25.723 gm was at 4.1 divisions to the right, it follows that the object was heavier than the weights in the pan by an amount sufficient to displace the rest point from 3.9 to the left to 4.1 to the right, or 8 scale divisions to the right. The weight equal to this amount of displacement can be calculated from the sensitivity as determined above. Since 2.2 scale divisions correspond to 1 mg, then 8 scale divisions would be $\frac{8.0}{2.2}$ or 3.64 mg, approximately 3.6 mg. The true weight of the object, therefore, would be 25.723−0.0036 or 25.7194 gm. The weight is expressed only to the fourth decimal place, since most analytical balances will scarcely detect with certainty less than 0.1 mg.

(*f*) For the methods of double or transposition weighing, reduction of weighings in air to "in vacuo," and for the calibration of weights, reference should be made to the various books on quantitative analysis listed in the appendix.

43. Cleaning laboratory glassware.—For ordinary use laboratory glassware can be cleaned in hot, soapy water with the aid of the various types of brushes, then rinsed in hot tap water, then in distilled water and allowed to dry.

Where chemical cleanliness is required one of the more elaborate methods of cleansing must be used.

a. Dichromate cleaning solution.—(1) Pour 1 liter of commercial concentrated sulfuric acid into 35 cc of saturated aqueous solution of sodium dichromate, technical. (The sulfuric acid is item No. 10360 and the sodium dichromate is item No. 14320 in the Medical Department Supply Catalog.)

Caution: Never pour the aqueous solution into the acid. Handle with care; avoid contact with flesh and clothing.

(2) Soak the glassware in this solution for several hours or overnight and then rinse repeatedly in hot tap water until all traces of cleaning solution are removed. Finally rinse in distilled water and allow to dry. In rinsing, note whether the water completely wets all of the interior surface and runs off leaving a thin film. If it collects in drops or patches the glassware is not clean and the process must be repeated. It may be advisable to give a preliminary scrubbing with hot, soapy water, followed by rinsing in tap water before using the cleaning solution. If used hot the dichromate-sulfuric acid solution is more effective.

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b. Cleaning with nitric acid.—Hot nitric acid is very effective in removing oxidizable organic matter. It must be used with due caution. Immerse the glassware in a beaker of concentrated nitric acid and heat it to boiling point under a hood. Pipettes may be placed in an enameled or earthenware tray or in a tall, glass specimen jar and the hot nitric acid poured over them. Rinse thoroughly and dry.

c. Cleaning fermentation tubes.—These and other glassware difficult to clean by ordinary methods may be treated by moistening the inside with ethyl alcohol. Pour off the excess, leaving not more than 2 cc in the tube. Add 10 cc of concentrated nitric acid, place the tubes in a glass or earthenware vessel under a hood, and let stand. A vigorous reaction soon occurs with the evolution of much nitrogen dioxide. When the reaction ceases, wash with water. Do not close the tube.

d. Trisodium phosphate detergent.—A good cleaning agent may be made by dissolving 60 gm of trisodium phosphate ($Na_3PO_4 \cdot 12\ H_2O$) and 30 gm of sodium oleate in 500 cc of water. Soak the glassware in this solution for 15 to 25 minutes, then scrub it with a stiff brush, rinse, and dry. Glassware used in phosphorus determinations should never be cleaned in this solution.

e. Alcoholic sodium hydroxide cleaning solution.—For removing tarry and greasy residues this solution is very effective. Dissolve 120 gm of sodium hydroxide in 120 cc of water, then dilute to 1 liter with 95 percent ethyl alcohol. The solution may be used hot or cold and must be followed by the usual thorough rinsing in tap and distilled waters.

44. Volumetric glassware.—Volumetric flasks, pipettes, and burettes are used for accurate measuring of liquids by volume. For ordinary rough measurement, graduated cylinders may be used. The graduate used in pharmacies has no place in the chemical laboratory.

Manufacturers produce several grades of volumetric glassware of varying accuracy. Catalogs of the laboratory supply firms list burettes, pipettes, and volumetric flasks in four different grades. The least accurate is the "student" or educational grade. Next comes the "retested" grade which is sufficiently accurate for rough quantitative work. The "precision" grade supposedly fulfills the requirements of the U. S. Bureau of Standards, but each item is not tested and certificates of accuracy are not furnished. This grade is suitable for all but the most exacting work. The highest grade of glassware available is "certified." This means that the individual piece has been tested by the U. S. Bureau of Standards and complies with

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its specifications. Each piece is etched with the official certification stamp and a certificate is furnished with it.

As furnished by the Medical Department supply depots, volumetric glassware is supposed to conform to certain specifications, as a rule corresponding to the retested grade. For most clinical purposes such as gastric analyses, urine sugar, many blood chemistry determinations, etc., the accuracy is sufficient. In case of doubt, however, the glassware should be calibrated by the methods given below. This is especially necessary when preparing volumetric solutions for the standardization of other solutions, in preparing all standards for blood chemistry, and in making such solutions as normal sodium hydroxide for use in preparing salvarsan for intravenous injection.

a. Volumetric flasks.—(1) For general use in the preparation of accurate solutions measuring flasks are a necessity. Convenient sizes are 1,000-cc, 500-cc, 250-cc, 100-cc, 50-cc, and 25-cc. More rarely, the 10-cc size is needed.

(2) The level at which the upper surface of the liquid stands must be in the neck of the flask and the neck should be narrow to insure accurate readings. Most flasks are calibrated to contain a definite amount and have only one mark etched on the neck. Some may have two marks, the lower one to contain, the upper to deliver, the specified volume of solution. The calibration mark of the manufacturer may be accepted as correct in the better quality flasks, but for the highest degree of accuracy it may be necessary to check the mark and recalibrate the flasks. This is done as follows:

Cleanse and dry the flask, then weigh it on a balance of appropriate size and sensitivity, taking into consideration the fact that the water to be introduced should be weighed to 1 part per 1,000; a 10-cc flask must be weighed to 0.010 gm, a liter flask to 1 gm. Instead of weighing the flask, it may be counterpoised with shot. Calculate the weight of water necessary to fill the flask to the mark at the proper temperature from table I and place this weight on the balance pan. Fill the flask with distilled water, which has been freshly boiled and cooled, until balance is attained, making sure that no drops of water adhere to the neck of the flask. Mark the lowest point of the meniscus with a wax pencil sharpened to a chisel edge, then etch in the mark or make a scratch mark with a diamond pencil or sharp file.

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TABLE I.—*Apparent weights and volumes of water weighed in air*

[For use in calibration of volumetric apparatus]

Temperature ° C.	Weight of 1 cc	Volume of 1 gm	Temperature ° C.	Weight of 1 cc	Volume of 1 gm
15	0. 9979	1. 0021	23	0. 9966	1. 0034
16	. 9978	1. 0022	24	. 9964	1. 0036
17	. 9977	1. 0023	25	. 9961	1. 0039
18	. 9975	1. 0025	26	. 9959	1. 0041
19	. 9973	1. 0027	27	. 9956	1. 0044
20	. 9972	1. 0028	28	. 9954	1. 0046
21	. 9970	1. 0030	29	. 9951	1. 0049
22	. 9968	1. 0032	30	. 9948	1. 0052

NOTE.—The figures in this table, from Landolt and Börnstein's "Tabellen," are based on the weights of water per cubic centimeter which must be weighed into a glass vessel under ordinary conditions in order to indicate the mark to which the vessel must be filled with freshly boiled and cooled distilled water so that it will contain the desired volume at 20° C. Corrections for the buoyant effect of air upon vessel and weights are included.

b. Pipettes.—Pipettes are tubes adapted for delivering smaller fixed quantities of liquid than measuring flasks. A pipette for delivering a fixed volume is called a transfer pipette while one for delivering varying quantities is known as a measuring pipette. Pipettes must be narrow at each end and the graduation at the top should be at a sufficient distance to safeguard against sucking liquid into the mouth. As with flasks, it may be desirable to have two graduations on transfer pipettes, one to mark the point to which the pipette must be filled to deliver the indicated volume and the other to mark the total content.

(1) *Calibration of pipettes for delivery.*—(*a*) Pipettes are calibrated by weighing the water which they deliver into a weighing bottle which contains a layer of paraffin oil a few millimeters thick to prevent evaporation. If the mark is not accurate another mark is then made with a wax pencil and tested. This procedure is repeated until the mark is located correctly. The mark is then etched in. The weight of 1 cc of water at any temperature from 15° to 30° C. may be found in table I.

(*b*) If an uncalibrated pipette is to be marked, two preliminary marks separated by a definite distance, 50 mm, are made on the stem by a chisel-edged wax pencil, and the water delivered from each is weighed. From the difference, the weight of water contained in each millimeter length of the stem is calculated, and from this the number of millimeters from either preliminary mark to the correct mark. The latter is located at the calibrated level on the stem, is tested by weighing the water delivered from it, and finally etched in.

Example: The weights of the water delivered at 20° C. by a 10-cc

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pipette from two preliminary marks 50 mm apart are 9.900 and 10.275 gm, respectively. Hence the weight of a column of water between marks is 0.375 gm or $\frac{0.375}{50} = 0.0075$ gm per millimeter of column length. The weight of 10 cc of water at 20° C. is 9.972 gm or 0.072 gm more than that delivered from the lower preliminary mark. Hence the correct mark is $\frac{0.072}{0.0075} = 9.6$ mm above the lower mark.

(2) *Calibration of pipettes to contain.*—The weight of water required to fill the dry, clean pipette is measured. One may either weigh the pipette empty and full or may fill it with water from a weighing bottle, which is weighed before and after. Other details are the same as for delivery pipettes.

(3) *Use of pipettes.*—(*a*) The Ostwald-Folin type of pipette with a relatively large bulb and short delivery tip is usually calibrated by the manufacturer to deliver the fixed quantity by blowing out the last few drops remaining in the delivery tip. They are marked "TD" (to deliver) and are etched with a single or double broad band near the top. With these pipettes, so marked, the universal practice for all sizes should be to blow out the last few drops.

(*b*) The volumetric or transfer pipette with the bulb in the middle is also marked "TD." Some, however, and also some Ostwald type pipettes are marked "TC" (to contain). With those marked "TD", the practice should be to permit free outflow until the liquid level approaches the lower part of the bulb, then to restrain the outflow with the finger tip, at the same time touching the tip at a slight angle from the vertical, to the wet sidewall of the receiving vessel. When as much of the liquid has run out as will do so, remove the finger from the upper end and hold the pipette tip against the wall of the receiver for a few seconds to make sure that no more of the solution will flow out. *Never blow out this type of pipette.*

(*c*) Pipettes marked "TC" must be rinsed out thoroughly with the diluting solution used in the particular analysis.

(*d*) Measuring pipettes delivering varying quantities between marks are used more frequently in serology and bacteriology than in chemistry. The outflow should be controlled by the finger and should be slow enough to permit the walls to drain. Frequently the final graduation to the tip is not accurate and this portion should not be used unless absolutely necessary.

(*e*) To insure the greatest accuracy in the use of all kinds of pipettes, after filling and before the fluid level is adjusted to the mark, the wet part of the delivery end should be wiped with a clean, absorbent cloth to remove the excess.

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c. Burettes.—(1) Burettes differ mainly from measuring pipettes by being open to atmospheric pressure at the top while in use. The outflow at the lower end is started, regulated, and arrested by means of a stopcock or a suitable clamp on an attached rubber tube.

(2) To calibrate a burette of 25- or 50-cc capacity, fill the clean burette with water to a point slightly above the zero mark. Run out the water slowly until the bottom of the meniscus just touches the mark. Wait a minute for complete drainage of the walls to take place. Adjust the meniscus again to the zero point if necessary. Remove excess water from the tip of the burette. Then run a 2-cc portion into a weighing bottle containing a little liquid petrolatum, bottle and petrolatum having first been weighed. After delivering the 2-cc portion, touch the drop adhering to the tip to the surface of the petrolatum carefully so as to remove it. Weigh the bottle and contents. Continue with 2-cc portions over the entire range of the burette, then take the temperature of the water.

Multiply the weight in grams of each portion of water by the volume of 1 gram at the observed temperature, as taken from table I, in order to calculate the actual volume delivered. From the actual volume delivered, make a table of corrections. For ordinary work such as measuring the acidity of gastric contents, the calibration of burettes as made by the manufacturer is sufficiently accurate. For more exact volumetric work the best grade of standardized burette must be used or it must be calibrated in the laboratory.

(3) The glass stopcocks of burettes require especial care. They must be kept clean and lubricated to prevent "freezing" and leakage. Various lubricants are given in section IV. A minimum of lubricant should always be used and the old removed before applying fresh.

(4) Alkali solutions of greater strength than 0.1 N should not be allowed to stand in burettes overnight, otherwise the glass stopcock will "freeze."

(5) Table II gives the correction to be added per liter to the observed volume of water or standard normal solution to give the volume at the standard temperature of 20° C. By subtracting the corrections from the volume desired at 20° C., the volume that must be measured out at other temperatures in order to give that desired at the designated temperature will be obtained.

Example: A liter flask is calibrated to hold exactly that volume at 20° C. A normal solution of NaOH is prepared at 25° C. The table shows that the solution at 25° C. would occupy at 20° C. exactly 1.52 cc less, so in order to make the solution exactly normal, 1.52 cc of water should be added.

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TABLE II.—*Temperature correction per 1,000 cc for normal volumetric solutions for reduction of volume to standard temperature of 20° C.*

Temperature	Water and 0.1 N solution	1 N HCl	1 N H_2SO_4	1 N $H_2C_2O_4$	1 N KOH and NaOH	1 N Na_2CO_3
15	+0.77	+0.97	+1.30	+1.05	+1.33	+1.29
16	+0.64	+0.79	+1.06	+0.85	+1.08	+1.05
17	+0.49	+0.61	+0.81	+0.65	+0.82	+0.80
18	+0.34	+0.41	+0.55	+0.44	+0.55	+0.54
19	+0.17	+0.21	+0.28	+0.23	+0.28	+0.27
20	0	0	0	0	0	0
21	−0.19	−0.22	−0.28	−0.24	−0.29	−0.28
22	−0.39	−0.44	−0.56	−0.49	−0.59	−0.56
23	−0.60	−0.67	−0.85	−0.75	−0.90	−0.85
24	−0.81	−0.91	−1.15	−1.02	−1.21	−1.15
25	−1.04	−1.17	−1.46	−1.29	−1.52	−1.46
26	−1.28	−1.43	−1.78	−1.57	−1.84	−1.77
27	−1.53	−1.70	−2.11	−1.85	−2.17	−2.09
28	−1.80	−1.98	−2.45	−2.04	−2.50	−2.41
29	−2.05	−2.26	−2.79	−2.44	−2.84	−2.75
30	−2.33	−2.55	−3.13	−2.77	−3.19	−3.09

NOTE.—The figures for water and 0.1 N solutions are applicable to all solutions 0.1 N or weaker and also to 1 N solutions of sodium chloride.

Example: In a titration 46.15 cc of N HCl were used at a temperature of 23° C. At the standard temperature of 20° C., this amount of solution would occupy a volume of—

$$46.15-\frac{46.15\times0.67}{1,000}=46.12.$$

A 0.1 N solution of silver nitrate is prepared at 27° C. From the table, the volume when reduced to 20° C. would be 1,000−1.53 or 998.47 cc. The solution is, therefore, stronger than 0.1 N for it contains as much $AgNO_3$ as there should be in a full liter at standard temperature. Each cubic centimeter of this solution is equivalent to 1.00153 cc of a 0.1 N solution of silver nitrate prepared at the standard temperature of 20° C., and this number is the *factor* for the solution. If the actual number of cc of solution used in a titration be multiplied by this factor, the result will be the number of cubic centimeters of an exactly 0.1 N solution.

d. Microburettes.—Burettes with a capacity of 0.1 cc to 3 cc, so constructed that the error of delivery does not exceed 0.001 of the capacity, are microburettes. The construction, internal diameters, outlets, and stopcocks must be quite different from those described for standard burettes. When the total volume delivered from a measuring apparatus is very small, accurate measurement demands that the fluid be delivered in minute drops, and the amount adherent to the tip of the burette must be insignificant. Both objects are attained to a considerable degree by using a delivery tip drawn out to a fine point. Such tips, however, if of glass, are too flexible and fragile. By substituting small-gage hypodermic needles, drops of the order of 0.00015 cc can be removed. This principle is used in the Shohl burette described below. Stopcocks provide only limited

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control of the flow. By the introduction of thumbscrews working on inelastic reservoirs, finer adjustment and control may be obtained.

(1) *Bang's microburette.*—(*a*) The form of microburette in most common use is that introduced by Bang. This burette is of 3-cc capacity and about 4-mm inner diameter. The bulb reservoir serves to hold a convenient supply of solution, with which the burette tube is refilled after each titration. The sealed tube at the bottom of the burette may be fitted into a stand, so that the apparatus is a conveniently movable unit. In the use of this burette, delivery must be retarded so that the surface of the liquid in the burette falls no faster than 0.5 cm per second. Error due to incomplete drainage is relatively larger in a microburette than in a larger one because the ratio of surface to volume is greater in the smaller tube.

(*b*) Calibration of microburettes can be done by the weighing of 0.2-cc portions of water delivered into a weighing bottle containing a layer of oil. There is no difficulty in making the weighings accurate to within less than 1 mg, which is as exact as anyone can read the level of the meniscus in the burette.

(2) *Shohl tip for microburettes and pipettes.*—This tip can be attached either to microburettes or to stopcock pipettes of the Van Slyke-Neill type. To the delivery end of such a burette or pipette a glass Luer adapter is sealed. The ground end fits snugly into a Luer hypodermic needle of 18- to 23-gage which is cut off horizontally and ground on a stone. Needles of platinum or other noncorrosive metal are desirable. Tips of this type deliver extremely minute drops.

e. Graduated cylinders.—(1) Measuring cylinders are made of glass tubing of relatively large diameter, the bottom being closed by a heavy base which is fused on and provides a steady support. Various sizes from 10 cc to 2,000 cc are made, the graduated subdivisions varying with the size. They are extremely useful for measuring volumes when great accuracy is not required. Some are made for special purposes with most exact graduations, but as a rule the ordinary cylinder is not accurate enough for fine analytical work.

(2) Whenever the volume to be measured is only 10 percent or less of the capacity of a cylinder, the next smaller size should be used, according to the methods of use recommended by the Bureau of Standards.

45. Filters and filtration.—The purpose of filtration is to separate a solid from the liquid in which it is suspended, consequently the pores of the paper or the interstices between the matted fibers

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of other filtering materials must be smaller than the particles which are to be retained. Since precipitates vary greatly with respect to size, proper selection of the paper or other agent is essential.

a. Paper filters.—(1) The desirable qualities in filter papers for quantitative work are strength, uniform texture, proper porosity, and low ash. For qualitative work strength and proper porosity are required. Select the paper best suited to the particular work to be done. All catalogs of laboratory supply houses give the characteristics of the various filter papers handled by them and in each package of Whatman filter papers is a user's guide indicating the correct paper for each purpose.

(2) An example of improper selection is the use of ordinary paper for filtering trichloracetic-acid precipitated blood serum in calcium determinations, since ordinary papers contain considerable calcium which is dissolved out by the acid. In this case only an acid-washed paper is suitable. On the other hand a blood filtrate for nitrogen determinations should not be filtered through an acid-washed paper. In the manufacture of these papers the acid is neutralized with ammonia and some ammonium salts remain in the paper. The blood filtrate dissolves this out, giving a falsely high reading for nitrogen.

(3) The simplest way to fold a circular-cut paper is to crease it across one diameter and then, without opening the paper, make a second fold at right angles to the first one. It is important that the paper fit tightly against the walls of the funnel over the entire extent. The paper should be of such size that its upper edge is below the rim of the funnel, especially when precipitates are to be washed.

(4) Although the method just described is the easiest and quickest for folding a paper, considerable time in filtration may be saved by folding the paper so that the liquid passes through it more rapidly. The following method accomplishes this purpose:

(*a*) Fold the paper evenly across one diameter.

(*b*) Open up the paper and make a second fold at right angles to the first, creasing the paper on the same side.

(*c*) Open and turn the paper over, then make a third fold exactly bisecting two of the quarters, creasing on the opposite side from the first two creases.

(*d*) Make a fourth fold at right angles to the last one. The paper is now divided into eighths, each segment 45° of the circle, with two creases on one side and two on the other.

(*e*) Fold again on the same side as the last, dividing two opposite eighths equally.

(*f*) Make a final fold at right angles to the previous one, and on the same side. In making the folds be sure all creases pass through the

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same center point and protect this point with a finger as the creases are made to prevent tearing it.

(*g*) As the paper is picked up it will tend to shape itself into a cone. Adjust three of the folds, reserving the fourth one to make the paper fit the funnel exactly. This folding gives alternate triple and single thicknesses, with half of the funnel area covered singly. It makes for very rapid filtration and easy washing of precipitates.

b. Asbestos filters.—In analyses where precipitates must be ignited and in the chemical examination of water, asbestos filters in Gooch filtering crucibles are very useful. They are difficult to prepare properly. The following method is quite satisfactory.

Cut some long-fibered, soft asbestos, of the nonhydrated variety, into pieces 0.5 cm long, and digest in hydrochloric acid upon a water bath for an hour. This will separate good asbestos into very small fibers. Collect the mass upon a filter plate and wash with water. For preparation of a Gooch filter, shake some of the material with water in a flask so that a thin suspension is formed. Pour enough asbestos into the crucible to produce a layer of 1 to 2 mm thickness, place a small filter plate upon this layer, and pour a little more of the asbestos suspension into the crucible. Wash the mat with water until no asbestos fibers run through. Dry the crucible at proper temperature and weigh. Repeat the drying and weighing until a constant weight is obtained. It is advisable then to run about 500 cc or more water through the crucible (to be sure that no asbestos fibers run through) and again dry and weigh. If the weight is constant the crucible is ready for use.

c. Fritted glass filters.—(1) These filters are made by fusing a disk of sintered glass in a funnel of proper size. They come in many shapes and sizes, and the fritted glass disks are of numerous grades of porosity from extremely fine to coarse. In making the disks, powdered glass is sifted to a uniform degree of fineness, pressed into a disk, and heated just enough to cause the individual grains of glass to adhere to each other.

(2) Fritted glass filters may be used for filtering anything except hydrofluoric acid solutions and hot, strong alkali solutions. They are especially useful where filtration by suction is employed.

46. Funnels.—In filtration, a funnel of proper size and shape is essential. For blood filtrates, a funnel large enough to hold all of the mixture should be used. In gravimetric analyses where the precipitate is to be collected, washed, ignited, and weighed, a small funnel, just large enough to conveniently hold all of the precipitate, is best. In all instances, the proper funnel size is important.

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a. Glass funnels.—The ordinary glass funnel should have an internal angle of 60°, a long stem with or without a slight constriction in the neck, and the stem end should be ground at an angle. Such a funnel, fitted with a properly folded filter paper, will give very rapid filtration. The stem should fill with a column of filtrate, the weight of this column exerting a suction pull. The end of the stem should touch the wet sidewall of the receiving vessel to increase the pull, but should not be below the level of the filtrate in the receiver.

b. Büchner funnels.—For filtration by suction, the Büchner funnel is widely used. It is made of porcelain with a perforated disk to support a filter paper. A paper of proper size and type is placed in the bottom of the funnel, wetted with a few drops of the solution to be filtered, and pressed into place. The stem end of the funnel, fitted with a rubber cork or rubber tubing collar to make an airtight joint, is fixed in a filtering flask, slight suction started, and then the mixture to be filtered is poured into the funnel. The suction is then increased to the desired rate. On such a funnel precipitates can be partially or completely dried by aspiration.

SECTION II

INDICATORS

Paragraph
General considerations 47
Preparation of indicator solutions 48
Tables of useful indicators 49

47. General considerations.—*a.* Indicators, especially those used in acidimetry and alkalimetry, are dyestuffs which are of one color in acid solution and another in dilute alkali. The change is due to a rearrangement within the molecule with a consequent change in the color. It should be remembered that when the completion of a reaction is ascertained by means of an indicator, or by means of any other final color change, it is essential that one solution be added to the other in all cases in the same order, otherwise the results obtained will not correspond exactly.

b. Solutions should also be of fair concentration and of approximately equivalent strength. Undue dilution causes some indicators to undergo hydrolytic dissociation and this prolongs the end reaction. Dilution in any case tends to prevent a sharp end point. In any titration only a small amount of indicator should be used.

c. There are many indicators used for various purposes. Those generally useful will be considered in this section, while others for specific determinations will be described in later chapters.

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48. Preparation of indicator solutions.—*a. Methyl orange (tropeolin D).*—(1) In alkaline solution its color is yellow, but the color changes to red on the addition of a mineral acid. This change of color is not produced by carbonic or other feeble acids. Hence this indicator may be used for the titration of the more powerful mineral acids in the presence of carbonic acid and the feebler organic acids. The presence of much water causes the red color of a faintly acid solution of methyl orange to become yellow, probably due to hydrolytic dissociation.

(2) The indicator solution generally used is prepared by dissolving 0.1 gm in 100 cc of distilled water. One drop of this solution is used for each 20 cc of solution to be titrated.

(3) A somewhat more sensitive solution, but one in which it requires more experience to detect the color change, is prepared by dissolving 0.02 gm in 100 cc of hot water, allowing the solution to cool, and filtering out any deposited m-sulfonic acid. This more dilute indicator is to be preferred for very exact titrations with very dilute acids and alkalies.

b. Methyl red.—(1) This indicator is of value in titrating weak organic bases and ammonia. The aqueous solution is orange colored, but if a few drops are added to 50 to 100 cc of water, the latter is colored a pale yellow. The addition of a drop of 0.1 N HCl at once turns the liquid a violet red without passing through any intermediate shade and by the addition of a drop of ammonia the solution becomes nearly colorless again. Methyl red is not very sensitive to carbonic acid, but more so than methyl orange, so that it is less suitable for the titration of carbonates. The chief advantage of this indicator lies in its sharp color change from a very pale yellow to a violet red even in titrating ammonia.

(2) To prepare the indicator solution, dissolve 0.02 gm of the free acid in 100 cc of hot water, allow to cool, and filter. Add 2 to 3 drops of this solution to every 100 cc of the solution to be titrated.

c. Phenolphthalein.—(1) The solution of this indicator in alcohol is colorless. When a few drops are added to the solution of an alkali, the liquid assumes an intense red color; this color is readily destroyed by the addition of an excess of mineral or organic acid. Phenolphthalein is suitable for the titration of organic and inorganic acids and of strong bases but it cannot be used in the presence of carbonic acid or of ammonium salts; it is therefore not suitable for titrating ammonia by an acid.

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(2) The presence of free carbon dioxide in ordinary distilled water causes a slight error, which should be allowed for, particularly if 0.1 N or 0.01 N acid solutions are used.

(3) Concentrated solutions of alkaline hydroxides do not give a red color with this indicator; such solutions should therefore be diluted with water before titration.

(4) The stock solution is prepared by dissolving 1 gm of pure phenolphthalein in 60 cc of absolute alcohol, adding 40 cc of water, and then filtering if necessary. From this a 0.5 percent solution in 50 percent alcohol may be made.

(5) The 1 percent and 0.5 percent solutions are used in the proportion of 1 drop to 10 cc or more of solution. A more sensitive indicator is prepared by diluting the 1 percent stock solution 10 times with 50 percent alcohol. One to four drops of this 0.1 percent indicator is used for each 100 cc of solution. The stronger indicator solutions are used in titrating gastric contents for total acidity while the weaker is used for the exact titration of strong acids and bases. *titration.*

49. Tables of useful indicators.—*a. Ordinary indicators for titration.*

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TABLE III.—*Indicators*

Common name	Chemical name	pH range	Color		Preparation of indicator solution	Principal uses in titration
			Acid	Alkaline		
Töpfer's reagent	p-Dimethylaminoazobenzene.	2.9 to 4.	Red	Yellow	0.5 percent alcoholic.	Free HCl in gastric contents.
Methyl orange	Sodium dimethylaminoazobenzene sulfonate.	3.1 to 4.4.	do	do	0.02 percent aqueous.	Mineral acids and strong bases. Mineral acids in presence of carbonic acid.
Congo red	Sodium tetrazodiphenylnaphthionate.	3 to 5	Blue	Red	0.5 percent alcoholic.	Titration of weak bases and ammonia by mineral acids.
Methyl red	o-Carboxybenzene azodimethylaniline.	4.2 to 6.3.	Red	Yellow	0.02 percent aqueous.	Titration of weak bases and ammonia.
Alizarin red	Sodium alizarin monosulfonate.	4 to 5	Yellow	Purple	1 percent aqueous.	Total acidity of gastric contents except combined HCl.
Nitrazine paper	Sodium dinitrophenylazonaphthol disulfonate.	4.5 to 7.5.	do	Blue	Paper or solution	Approximate pH of urine.
Litmus paper		4.5 to 8.3.	Red	do	Paper	Reaction of urine, reaction of various solutions.
Phenolphthalein		8.3 to 10.	Colorless.	Red	0.1 percent in 50 percent alcohol.	Titration of strong acids and bases. Organic acids by strong bases.

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b. Indicators of Clark and Lubs (supplemented by Cohen).—To make the 0.04 percent indicator solution for use, mix 0.1 gm of the dry compound in a mortar with the number of cubic centimeters of 0.01 N sodium hydroxide shown in the table. Dilute to 250 cc with water. Use 5 drops of indicator for 10 cc of solution. These indicators are used for titrating to a definite pH and for preparing standards for the colorimetric determination of pH.

TABLE IV.—*Indicators of Clark and Lubs*

Name	pH range	Color change acid-alkaline	Cc 0.01 N NaOH
Thymol blue (acid)	1. 2–2. 8	Red to yellow	21. 5
Brom phenol blue	3. 0–4. 6	Yellow to blue	14. 9
Brom cresol green	3. 8–5. 4	do	14. 3
Chlor phenol red	4. 8–6. 4	Yellow to red	23. 6
Brom cresol purple	5. 2–6. 8	Yellow to purple	18. 5
Brom thymol blue	6. 0–7. 6	Yellow to blue	16. 0
Phenol red	6. 8–8. 4	Yellow to red	28. 2
Cresol red	7. 2–8. 8	do	26. 2
Meta cresol purple	7. 4–9. 0	Yellow to purple	26. 2
Thymol blue (alkaline)	8. 0–9. 6	Yellow to blue	21. 5

SECTION III

VOLUMETRIC SOLUTIONS

Paragraph
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Preparation of standard solutions 51

50. Definitions.—*a. Molar solution.*—A molar solution of any chemical compound is a solution of such concentration that 1 liter contains 1 gram-molecule of the compound. The molecular weight in grams of the compound is dissolved in water or other solvent and made up to 1 liter. For example, the molecular weight of sodium chloride is 58.454; a molar solution would contain 58.454 gm of sodium chloride in 1 liter of solution; the molecular weight of sulfuric acid is 98.076, so a molar solution would contain that weight in grams per liter of solution.

b. Normal solution.—By a normal solution of any compound is meant one which contains one gram-equivalent of the active reagent in 1 liter of solution. By gram-equivalent is meant the amount of substance equivalent to 1 gram-atom (1.008 gm) of hydrogen. A milli-equivalent is the thousandth part of a gram-equivalent. Stated differently, a normal solution is one which contains 1 gram-atom

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(1.008 gm) of reacting hydrogen per liter of solution, or which can quantitatively replace or react, directly or indirectly, with an equal volume of such a solution. Of the various types of normal volumetric solutions the ones most commonly used in clinical laboratories fall into the following classes:

(1) *Normal acid solutions.*—In acid-alkali titration, a normal acid solution contains per liter the amount of acid that has 1 gm-atom of hydrogen replaceable by alkali at the pH used as the end point in titration; for example, 1 molecule of HCl contains 1 atom of replaceable hydrogen, therefore a liter of a normal solution of this acid contains 1 gm-molecule or 36.465 gm of HCl. One molecule of sulfuric acid, H_2SO_4, contains, however, for titration to ordinary end points, 2 atoms of replaceable hydrogen; therefore a liter of normal solution of this acid contains ½ gm-molecule, or 0.5 × 98.076 or 49.038 gm of H_2SO_4.

(2) *Normal alkali solutions.*—A normal alkali solution is one which will neutralize, volume for volume, a normal acid solution. A normal solution of sodium hydroxide, NaOH, is molar, but one of barium hydroxide, $Ba(OH)_2$, is half molar. Such solutions are normal in hydroxyl; that is, they contain one gram-equivalent of OH in each liter of solution.

(3) *Normal reducing solutions.*—A normal reducing solution is one which contains in 1 liter 1 gm-atom of oxidizable hydrogen or its equivalent in other reducing substances. Oxalic acid, $H_2C_2O_4$, has 2 hydrogen atoms, both of which are titratable with alkali and both of which are oxidizable by permanganate. Hence a normal solution of oxalic acid, whether for acidimetry or for oxidation by permanganate is half molar.

(4) *Normal oxidizing solutions.*—A normal oxidizing solution is one a liter of which will oxidize 1 gm-atom of hydrogen, or its equivalent of other reducing substances. In the reaction—

$$2KMnO_4 + 5H_2C_2O_4 + 3H_2SO_4 = K_2SO_4 + 2MnSO_4 + 10CO_2 + 8H_2O$$

each molecule of permanganate oxidizes 5 of the hydrogen atoms of the oxalic acid. Hence a normal solution of permanganate is only one-fifth molar.

c. Acidimetry and alkalimetry.—Acidimetry and alkalimetry cover the analysis of acids and bases. In order to determine the amount of acid present, an alkaline solution of known strength is required; and conversely, in the analysis of a base an acid solution is required. In both cases the end point of the reaction is determined with the help of a suitable indicator.

d. Oxidation and reduction.—(1) In oxidation and reduction

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methods the substance analyzed is either oxidized or reduced by means of the solution with which the titration is made. When hydrogen is oxidized by oxygen it is changed from the neutral condition to that of a positive valence, or polarity, of one, and the oxygen is reduced from the neutral condition to a negative valence, or polarity, of two. In this, and all other cases, the equivalent weight of the element used in an oxidation-reduction reaction is the atomic weight divided by the change in polarity. When the atom of any complex molecule is subjected to a change in polarity, oxidized or reduced, the equivalent weight of the molecule is the gram-molecular weight divided by the change in polarity of the oxidized or reduced element. If more than one atom of the reactive element is present in the molecule, the molecular weight is divided by the total change in polarity; that is, by the change in polarity multiplied by the number of atoms undergoing such change.

(2) As an example, potassium dichromate is often used as an oxidizing agent. In it each chromium atom has a polarity of +6 and by reduction two trivalent chromic ions are formed. There is a loss in polarity of three charges for each chromium atom and a normal solution of potassium dichromate, $K_2Cr_2O_7$, will contain one-sixth of a mole.

(3) In a like manner, the equivalent weight of a reducing agent is determined by the gain in polarity which the oxidizing element experiences. Ferrous salts are oxidized to ferric salts and the iron is changed from +2 to +3 in polarity.

$$Cr_2O_7^{--}+6Fe^{++}+14H^+=2Cr^{+++}+6Fe^{+++}+7H_2O$$
$$Cr_2O_7^{--}+6I^-+14H^+=2Cr^{+++}+3I_2+7H_2O$$

e. Precipitation and substitution reactions.—In precipitation and substitution reactions, accurate determinations are made by precipitating or substituting a final product by means of a normal solution. The classical example is the extensively used method for testing silver alloys and this depends upon the precipitation of silver as the chloride from nitric acid, common salt solution of known concentration being used as the precipitant.

51. Preparation of standard solutions.—*a. Normal hydrochloric acid* (HCl=36.465).—(1) *Constant boiling mixture method (Hulett and Bonner).*—(*a*) This method, which is capable of accuracy to 1 part in 10,000, depends on the fact that when hydrochloric acid solution is distilled, the concentration of acid in the undistilled portion approaches a constant concentration of 20.22 percent of HCl by weight after distillation at 760 mm of mercury pressure. If boiling is continued, water and hydrochloric acid distill off in constant

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proportions identical with those in the residual undistilled fraction.

(*b*) To concentrated hydrochloric acid, specific gravity 1.2, add an equal volume of water. Bring the solution to a density of 1.096 at 25° C. by the addition of more water or acid. Distill away three-fourths of the mixture at a rate of 3 or 4 cc per minute. Save this distillate as the starting point for a second lot, adjusting it to a specific gravity of 1.096 at 25° C. The remaining one-fourth has, within 1 part in 10,000, the composition given in table V. Of this quarter, all but the last 50 to 60 cc is distilled, and the distillate used to prepare standard solutions. The barometer is recorded at the time of distillation. In glass-stoppered bottles such solutions keep indefinitely.

(*c*) A 0.1 N standard solution is prepared by diluting the proper weight, not volume, of the acid, estimated from table V, to volume with water. Exactly 16.4 cc (approximately 18 gm) of the acid are measured into a 50-cc flask. More acid is added or withdrawn by means of a medicine dropper with a fine drawn-out tip, until the exact weight as given in the table is obtained. The acid is then diluted with water, rinsed into a 1 liter volumetric flask which has been calibrated, and diluted to volume. Further standardization is unnecessary. In fact, acid prepared in this manner can be relied upon for the standardization of alkali and other reagents. In this and all other volumetric solutions, final adjustment of the volume in the flask must be made at 20° C. since the flasks are calibrated at that temperature. Use of distilled water at 20° C. facilitates this adjustment.

TABLE V.—*Constant boiling hydrochloric acid (Hulett and Bonner)*

(Values for pressures 730 to 770 mm corrected by Foulk and Hollingsworth; values for pressures 620 to 660 mm added by Bonner and Branting.)

Barometric pressure at time of distillation (mm Hg)	HCl concentration by weight (per cent)	Solution required to make 1 liter of 0.1 N HCl (gm)
620	20. 560	17. 719
630	20. 532	17. 743
640	20. 504	17. 767
650	20. 471	17. 800
660	20. 438	17. 824
730	20. 293	17. 956
740	20. 269	17. 977
750	20. 245	17. 998
760	20. 221	18. 019
770	20. 197	18. 041

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(2) *Standardization of other acids by comparison with standard HCl.*—Hydrochloric acid standards prepared by weight from Hulett and Bonner acid, as described above, may be used to standardize any other acid solution by the following procedure.

(*a*) With a pipette measure 20 or 25 cc of standard hydrochloric acid into a flask and titrate from a burette with alkali of approximately the same normality. With the same pipette, the same volume of the other acid is measured into a flask and titrated, using the same quantity of the same indicator, with the same alkali from the same burette, which is filled exactly to the zero point at the beginning of each titration.

(*b*) The normality factor of the unknown acid is calculated as—

$$\text{Normality factor} = \frac{\text{cc of alkali used for unknown acid}}{\text{cc of alkali used for standard HCl}}$$

This procedure eliminates errors of calibration of different pieces of apparatus and those from deterioration or inaccurate standardization of alkali.

(3) *By standardization.*—Normal hydrochloric acid contains in 1,000 cc, exactly 36.465 gm of HCl. Dilute pure, concentrated hydrochloric acid with 11 volumes of water. In this way a solution is obtained that is slightly more than normal in strength. To obtain an exactly normal solution, it may be titrated against a weighed amount of chemically pure sodium carbonate, and from the results obtained the volume of water to be added computed, or it may be standardized gravimetrically with calcite (Iceland spar) as follows:

(*a*) To a weighed crystal of Iceland spar in a beaker, add a known volume, 50 to 100 cc, of the acid to be standardized. Cover the beaker with a watch glass and set aside until the action of the acid on the crystal has completely ceased. Remove the crystal from the solution, wash in distilled water, dry and reweigh. The loss of weight is due to the solution of the carbonate under the influence of the acid.

(*b*) Then $\frac{20 \times \text{gm of Iceland spar dissolved}}{\text{cc of acid used}} = \text{factor for 1 N acid.}$

If the acid is made up somewhat stronger than 1 N, and a given volume of it is diluted to factor times that volume, the diluted acid will have a normality of exactly 1.0000.

b. Normal sulfuric acid ($H_2SO_4 = 98.076$).—This solution is half-molar, and therefore contains 49.038 gm in 1,000 cc. Add slowly, with constant stirring, 30 cc of sulfuric acid to 1,020 cc of distilled water and allow to cool to 25° C. Ascertain its exact strength by titration against freshly standardized normal sodium hydroxide,

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using methyl orange indicator, and adjust to exact normality or to a known strength of approximate normality. The strength may also be ascertained and adjusted after titrating an accurately weighed amount of reagent anhydrous sodium carbonate. It may also be standardized gravimetrically in the same manner as hydrochloric acid.

c. Normal oxalic acid ($(COOH)_2 \cdot 2H_2O = 126.068$).—This solution is a half-molar one. Dissolve 64.5 gm of reagent oxalic acid in sufficient distilled water to measure 1,000 cc. Ascertain its exact strength by titration against freshly standardized normal sodium hydroxide, using phenolphthalein indicator, and adjust to exact normality or to a known strength of approximate normality.

d. Normal sodium hydroxide ($NaOH = 40.005$).—(1) Dissolve 100 to 500 gm of the purest sodium hydroxide obtainable in an equal amount of water in a pyrex flask. The solution becomes very hot. Cool to room temperature and transfer to a paraffin-lined bottle or cylinder, stoppered with a paraffined stopper. In such a concentrated solution carbonate is almost insoluble and will settle out in a few days. The clear concentrated solution contains about 70 gm of NaOH per 100 cc.

(2) Prepare the normal solution by diluting 60 cc of the clear concentrated solution to 1 liter with distilled water. Standardize the hydroxide solution as follows: Place 25 or 50 cc of standardized normal hydrochloric acid in a flask, add a drop or two of methyl orange or phenolphthalein indicator and run in the alkali solution from a burette until the end point is reached. It is best to run the alkali into the acid since the alkali has less opportunity to absorb CO_2 from the air if in a burette and also because the change in the indicator is more definite.

(3) Calculate the normality factor of the hydroxide by the formula—

$$\text{Factor} \frac{\text{cc of 1 N HCl}}{\text{cc of NaOH}}$$

If the normality factor is greater than 1, indicating that the solution is too strong, the concentration of that remaining can be reduced to exactly 1 N by diluting the total volume to the figure obtained by multiplying the cubic centimeters remaining by the normality factor. For example, the normality factor is found to be 1.020 and there are 950 cc of hydroxide solution left from the titration. Multiplying 950 by 1.020 gives 969, the volume to which the hydroxide should be diluted. This is done by adding 19 cc of water. Again titrate and calculate the normality factor.

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NOTE.—Solutions of alkali hydroxides absorb carbon dioxide when exposed to the atmosphere. They should therefore be preserved in pyrex bottles with well-fitting rubber stoppers provided with tubes filled with a mixture of sodium hydroxide and lime (soda-lime tubes), so that air in entering the container must pass through these tubes, which will absorb the carbon dioxide.

e. Normal sodium carbonate (Na_2CO_3=106.004).—This may be prepared directly from anhydrous sodium carbonate of the best quality (American Chemical Society specifications or Standard of Murray) by weighing out slightly more than one-half the molecular weight, dissolving it in distilled water and making up the volume to 1 liter. Ordinarily 53.5 gm will give a solution but slightly stronger than normal.

Standardize by titrating 50 cc of the carbonate solution with 0.1 N HCl, using methyl orange as an indicator.

f. Potassium permanganate 0.1 N ($KMnO_4$=158.026).—A liter of this solution should contain $\frac{1}{50}$ gm molecule, or 3.1605 gm of potassium permanganate. The solution can be made accurately by weight from the pure crystals. However, it tends to become weaker after standing because of reduction by organic substances in the distilled water with which it is prepared. Hence, it is desirable to let it stand for a week before it is used, and then to standardize it against sodium oxalate in the following manner:

(1) Dissolve 0.25 to 0.3 gm of pure sodium oxalate in 200 to 250 cc of hot water in a 400-cc beaker. The water should be at a temperature of 80 to 90° C., since the titration must be completed at a temperature above 60° C. Add 10 cc of sulfuric acid (1 part of concentrated acid plus 1 part of water) and titrate at once with the 0.1 N $KMnO_4$ solution, stirring the liquid vigorously and continuously. A thermometer makes a convenient stirring rod in this instance. The permanganate must not be added more rapidly than 10 to 15 cc per minute, and the last 0.5 to 1 cc should be added dropwise, allowing each drop to be decolorized before the next is added. The excess of permanganate necessary to produce the end-point color should be estimated by matching the end-point color in another beaker containing the same amount of hot water and sulfuric acid as in the titration. Deduct this amount from the titration.

(2) Calculate the normality of the permanganate by the following formula:

$$\text{Normality} = \frac{s}{n \times 0.067}$$

in which s=gm of sodium oxalate taken and n=cc of $KMnO_4$ solution used, and 0.067 is the milli-equivalent of the oxalate.

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(3) If the solution is near normality it is generally used as it is and the 0.1 N equivalent estimated by multiplying the cc used in any titration by the factor determined in the standardization.

g. Sodium thiosulfate 0.1 N ($Na_2S_2O_3 \cdot 5H_2O = 248.194$).—Dissolve 24.82 gm of crystalline sodium thiosulfate in water and dilute to 1 liter to make a 0.1 N solution. To standardize this solution, dissolve 1 to 2 gm of pure potassium iodide in a minimum of water in a flask, acidify with 5 cc of dilute hydrochloric acid (1 to 5), and add 20 to 25 cc of 0.1 N potassium permanganate solution which liberates an equivalent amount of free iodine from the iodide. This solution is at once titrated with the thiosulfate solution, using starch indicator.

$$\frac{\text{Cc of 0.1 N potassium permanganate}}{\text{Cc of thiosulfate}} = \text{factor for 0.1 N thiosulfate.}$$

h. Iodine solution 0.1 N (1=126.92).—For each liter of solution desired weigh 13 gm of iodine, instead of the 12.69 gm actually required, into a weighing bottle. Dissolve about 30 gm of pure KI in about 250 cc of water. Transfer the iodine from the weighing bottle to a 1-liter volumetric flask, washing out any adhering iodine crystals with the iodide solution. Mix the contents of the flask until the iodine is completely dissolved and then dilute to volume. Standardize by titrating 20 cc with 0.1 N thiosulfate solution, using starch indicator.

$$\frac{\text{Cc of 0.1 N thiosulfate}}{\text{Cc of iodine solution}} = \text{factor for 0.1 N iodine.}$$

i. Starch indicator solution.—(1) Make a 1 percent solution by dissolving 1 gm of high-grade soluble starch in cold water and dilute to 100 cc. Starch solutions become moldy and deteriorate after a few days. This deterioration may be prevented or greatly retarded by adding 1 gm of salicylic acid per liter to the water used in preparing the solution.

(2) Another method of preparing starch solution which avoids deterioration to a great extent utilizes zinc chloride as a preservative. Triturate 6 gm of soluble starch with cold water to a thin paste and rinse into a liter of boiling water. Add to this a solution of 6 gm of zinc chloride in 50 cc of water. Distribute in small bottles, tightly corked.

(3) A clear, sensitive solution can readily and easily be prepared by extracting with water the well-known breakfast food, puffed rice.

j. Potassium dichromate 0.1 N ($K_2Cr_2O_7 = 294.212$).—Dissolve about 5 gm of potassium dichromate in 1,000 cc of distilled water;

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transfer exactly 25 cc of this solution to a 500-cc glass-stoppered flask, add 2 gm of KI, free from iodate, and dilute with 200 cc of distilled water. Add 5 cc of hydrochloric acid, allow to stand 10 minutes in a dark place, and titrate the liberated iodine with 0.1 N thiosulfate using starch solution as an indicator. Adjust to exact normality or to a known strength of approximate normality.

$$\frac{\text{Cc of 0.1 N thiosulfate}}{\text{Cc of dichromate}} = \text{factor for 0.1 N dichromate.}$$

NOTE.—Reference should be made to the section "Volumetric Solutions" in the U. S. Pharmacopoeia, to Van Nostrand's "Chemical Annual," or to "The Handbook of Chemistry and Physics" of the Chemical Rubber Co., etc., for tables of equivalents of N and 0.1 N solutions.

Standardization of acids and bases, etc., may be most accurately done by means of samples of benzoic acid, sodium oxalate, arsenic trioxide, and acid potassium phthalate secured from the Bureau of Standards, Washington, D. C., at small cost. Instructions for use accompany all standard samples.

SECTION IV

USEFUL LABORATORY ARTS AND RECIPES

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52. Stopcock lubricants.—*a. Glycerol.*—For sealing ground glass joints and stopcocks to prevent leakage of ethyl ether, petroleum ether, or any other fluid insoluble in it, glycerol (glycerin) is very satisfactory. It prevents sticking as well.

b. Stopcock grease.—Melt 2 parts of paraffin and 4 parts of vaseline together, then add slowly 1 part of pure gum rubber cut into small pieces. Stir while heating until a smooth paste is formed. Be careful not to burn the rubber.

c. Stopcock lubricants.—(1) Mix together 500 gm of petrolatum and 50 gm of raw crepe rubber. Keep in an oven at 125° to 150° C. for several days until the paste is smooth.

(2) Mix 2 parts of ordinary rubber cement and 1 part of vaseline. Heat on a water bath until the solvent from the rubber cement is driven off.

53. Cements and adhesives.—*a. Acid-resisting cement.*—Mix sodium silicate and asbestos powder to a thin paste. If allowed to dry for a day this cement will resist the strongest acids.

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b. Vacuum wax.—For ordinary vacuum seals and for vacuum distillations where the temperature does not get too high, an excellent wax may be made by melting together equal parts of beeswax and rosin. This wax is pliable and is easily removed by using hot water.

54. Label protection.—*a. Collodion for labels.*—Soak 3 to 4 gm of pyroxylin in 25 cc of absolute ethyl alcohol, then add 75 cc of ether.

b. Vinylite lacquer.—Dissolve 20 gm of vinyl acetate polymer (Vinylite A) in 100 cc of solvent made by mixing 75 cc of toluene and 25 cc of 95 percent alcohol. This lacquer gives a transparent, colorless film which resists most reagents well.

55. Cleaning fluid.—To remove grease spots from cloth or leather use a mixture of carbon tetrachloride, 80 cc, ligroin, 16 cc, and tertiary amyl alcohol, 4 cc.

56. Iodine stains.—To remove iodine stains from clothing, wash thoroughly with a 10 percent solution of sodium thiosulfate in water, then rinse with clear water.

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CHAPTER 4

EXAMINATION OF URINE

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SECTION I

COLLECTION AND PRESERVATION OF SPECIMENS

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57. Samples.—*a. Single samples.*—These are used as a matter of convenience but only for qualitative examination. Abnormal findings in such a specimen should be checked by the examination of a 24-hour specimen. Of the single samples, one passed 3 hours after a meal is best. The single sample voided on arising is least likely to yield pathological findings, yet it may be at times the most desirable for checking the presence of pus and mucus.

b. Day and night samples.—In certain conditions and for some tests it may be desirable to collect the day and night urines separately. Usually all urine voided from 8 AM to 8 PM is collected as the day sample and that from 8 PM to 8 AM as the night sample.

c. Twenty-four-hour sample.—This sample is preferred for routine examination and is absolutely essential for accurate study of kidney conditions. No quantitative test should be done except on a representative portion of a 24-hour sample. For convenience the collection is usually started in the morning at 7 AM. The patient voids and this voiding is discarded. All urine passed subsequently is saved. At 7 AM the following morning the patient voids and this urine is added to the sample. The volume is noted, the total specimen is well mixed, and 120 to 240 cc sent to the laboratory for analysis.

58. Preservatives.—*a. Cold.*—If a refrigerator is available, samples may be kept in it until examined. Samples for pregnancy tests must be so preserved. Avoid freezing.

b. Chemical preservatives.—(1) *Toluene.*—This is the best chemical

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preservative. Sufficient amount should be added to form a thin layer on the surface.

(2) *Camphor.*—A small piece sufficient to give a saturated solution is used.

(3) *Formaldehyde.*—Two drops of the ordinary commercial solution to an ounce of urine are sufficient. If in excess it interferes with albumin, sugar, and indican tests. It is most satisfactory for the preservation of formed elements.

SECTION II

PHYSICAL EXAMINATION

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59. Color.—Various shades of yellow and amber are normal, the darker shades being associated with increased concentration and high specific gravities, the lighter with dilution and low specific gravities. Increased amounts of the normal urinary pigments (urochrome, urobilin, and uroerythrin), the presence of abnormal pigments, and the renal elimination of drugs may produce a variety of colors. Color is usually recorded as straw, yellow, amber, etc., indicating the shade as light, medium, or dark; that is, light straw, dark amber, etc.

60. Appearance.—Normal, freshly passed urine is clear and sparkling. Concentrated urines, on cooling, may develop a white, pink, or reddish sediment. Pus or blood, if present, produce a cloudy or smoky appearance. During the change to alkalinity in older specimens phosphates may separate out. Marked cloudiness in a freshly passed specimen is significant of a pathological condition. Appearance is usually described as clear, slightly cloudy, very cloudy; the sediment as slight, moderate, or heavy, and its color white, pink, reddish, etc.

61. Reaction.—Determine whether the reaction is acid, alkaline, or neutral by the use of blue and red litmus paper. Freshly voided normal urine is usually acid, averaging about pH 6.0. Properly preserved 24-hour samples are also slightly acid. The diet is the most important factor in modifying the reaction of the urine. (See also par. 81 for the use of nitrazine paper (phenaphthazine) for determining pH.)

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62. Specific gravity.—This is most conveniently estimated with the urinometer, a special type of hydrometer. Each urinometer is calibrated to give readings at a definite temperature, usually 25° C. (77° F.); this calibration is marked on the spindle. If the temperature of the urine is above that at which the urinometer is calibrated, a correction of 0.001 must be added for each 3° C. above the standard temperature and similarly deducted for each 3° C. below this calibration temperature. For example, if the urinometer, calibrated at 25° C., reads 1.018 in a urine at a temperature of 31° C., then 0.002 must be added, giving the corrected specific gravity of 1.020. Normally the specific gravity varies between 1.015 and 1.030; pathologically it may range from 1.000 to 1.060.

63. Quantity.—Normally 1,200 to 1,500 cc are passed in 24 hours. In diabetes mellitus and diabetes insipidus the quantity is much increased. In water loss due to diarrhoea, excessive vomiting, or profuse sweating the amount is decreased. Normally, the day volume exceeds the night volume, being three to four times as much.

64. Total solids.—The amount of solids excreted in the urine may be roughly calculated by means of Long's coefficient, 2.6. The last two figures of the specific gravity taken at 25° C. multiplied by this coefficient give the number of grams of total solids in 1,000 cc of urine.

SECTION III

ROUTINE CHEMICAL EXAMINATION

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Blood	72

65. Albumin.—All normal urines contain some albumin, but the amount is so small it escapes detection by the methods generally used. The principles employed are either coagulation by heat or precipitation by chemical agents. No test is absolutely satisfactory due to interference of other substances precipitated with the albumin. Mucin is a common source of error. When present, it can be removed by adding acetic acid and filtering. All tests for albumin require that the urine be absolutely clear; cloudy samples should be cleared by filtration or centrifugalization, otherwise reactions due to small amounts of albumin will be masked in the general turbidity.

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a. Heat and acetic acid test.—(1) *Reagent.—Acetic acid*, 5 percent.

(2) *Procedure.*—Fill a test tube two-thirds full of clear urine. Heat the upper portion gently to boiling in an open flame. Precipitates forming at this point may be due to albumin or phosphates. Add 3 to 5 drops of 5 percent acetic acid solution, a drop at a time.

(3) *Results.*—If the precipitate dissolves, it is due to phosphates. If due to albumin, the precipitate will become heavier and more flocculent. Compare the cloudiness produced with the unheated urine in the lower portion of the tube.

b. Nitric acid and magnesium sulfate ring test (Roberts').—(1) *Reagent.*—To 5 volumes of saturated aqueous solution of magnesium sulfate, U. S. P., add 1 volume of concentrated nitric acid.

(2) *Procedure.*—(*a*) Place a few cubic centimeters of reagent in a test tube, tilt the tube, and introduce the urine sample with a pipette, allowing it to flow gently down the side of the tube so as to overlay the reagent without mixing. If albumin is present, a fluffy, white ring of precipitated albumin forms at the juncture line.

(*b*) Another method uses a pipette made of glass tubing with an inside diameter of about 5 mm. Place a few cubic centimeters of Roberts' reagent in a test tube. With the pipette take up a small column of urine, about 1 cm long, wipe excess urine from the outside, then place it in the test tube carefully, holding the finger firmly over the upper end until the other end touches the bottom of the tube. Release the finger pressure gradually, allowing the reagent to rise in the pipette, forming a clear, distinct layer with the urine. After standing for a minute or two, read against a dark background.

(3) *Results.*—A white ring at the junction of the liquids indicates albumin, the thickness and density of the ring showing the amount. No confusing colored rings due to indican, iodides, bile pigments, or the oxidation products of organic constituents are formed as is frequently the case when nitric acid alone (Heller's test) is used. A white ring or cloudiness may form above the contact zone, due to urates or mucus, but such rings are less sharp, broader, and lie above the albumin ring when both are present.

c. Sulfosalicylic acid test (Exton).—(1) *Reagent.*—Dissolve 200 gm of crystalline sodium sulfate ($Na_2SO_4 \cdot 10H_2O$) in 800 cc of water with the aid of heat. Cool to 35° C. and add 50 gm of sulfosalicylic acid. Dissolve and dilute to 1 liter.

(2) *Procedure.*—Mix equal volumes of clear urine and reagent in a test tube. Warm the mixture gently; do not boil.

(3) *Results.*—If cloudiness does not develop in the cold, albumin is absent. If cloudiness appears and persists or increases on gently heating, albumin is present.

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d. Osgood-Haskins' test.—(1) *Reagents.*—(*a*) *Acetic acid*, 50 percent solution.

(*b*) *Sodium chloride*, saturated aqueous solution, 30 percent.

(2) *Procedure.*—To 5 volumes of urine in a test tube, add 1 volume of the 50 percent acetic acid, followed by 3 volumes of the saturated sodium chloride solution. Heat the mixture gradually to boiling.

(3) *Results.*—A precipitate appearing upon the addition of the acid indicates bile salts, urates, or resin acids, etc., whereas a precipitate appearing after the addition of the salt solution suggests Bence-Jones protein, or globulin in excess of 0.38 gm per liter. As the temperature is raised the precipitate of Bence-Jones protein, if present, will go back into solution; if albumin or globulin are present a precipitate will form. This test has the advantage of indicating the presence of Bence-Jones protein as well as albumin and globulin.

66. Glucose (Benedict's test).—*a. Reagent* (*Benedict's qualitative solution*).

Cupric sulfate crystals ($CuSO_4 \cdot 5H_2O$) U.S.P	17.3 gm
Sodium carbonate, monohydrated U. S. P	117.0 gm
(or sodium carbonate, anhydrous U.S.P. 100 gm)	
Sodium citrate, U. S. P	173.0 gm

Dissolve the copper sulfate in about 100 cc of water. Dissolve the carbonate and citrate in 700 cc of water, with the aid of heat, if necessary. Cool to room temperature and pour in the copper solution slowly with constant stirring. When completely mixed make up the volume to 1,000 cc.

b. Procedure.—Place 5 cc of reagent in a test tube. Add 8 drops (0.5 cc), not more, of urine. Boil vigorously over an open flame for 1 to 2 minutes, then allow to cool spontaneously. Do not hasten cooling by immersion in cold water. If a large number of tests are to be run, the tubes may be placed in a boiling water bath, or a beaker of boiling water, and heated for 5 minutes, then allowed to cool.

c. Results.—In the presence of glucose the entire solution will be filled with a bulky, colloidal precipitate which may be greenish yellow, yellow, or red in color, depending on the amount of glucose present. In the presence of over 0.2 to 0.3 percent of glucose the precipitate will form quickly. If no glucose is present the solution will remain perfectly clear or will show a faint turbidity due to precipitated urates.

67. Indican (Obermayer's test).—This test depends on the decomposition of indican and the subsequent oxidation of the liberated indoxyl to indigo blue, at times to indigo red.

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a. Reagents.—(1) *Obermayer's reagent.*—Add 2 gm of ferric chloride to 1,000 cc of concentrated hydrochloric acid (sp. gr. 1.19 or 23.5° Baumé).

(2) *Chloroform.*

b. Procedure.—To 5 cc of urine in a test tube add an equal amount of reagent and 1 to 2 cc of chloroform. Mix by inverting 10 times. Allow the chloroform to settle and examine its color.

c. Results.—(1) A pale blue to deep blue to violet color indicates the presence of indican, the intensity of the color being proportional to its concentration. If the oxidation is slow, a red color due to the formation of indigo red may appear. Iodides may give a red-violet color due to the liberation of iodine. The addition of a few drops of a concentrated solution or a small crystal of sodium thiosulfate will discharge this color. Thymol may produce a violet color; the thiosulfate will destroy this also. Bile pigments interfere with the test and must be removed by adding one-fifth volume of 10 percent calcium or barium chloride solution and filtering.

(2) Urotropin (hexamethylene tetramine) and formaldehyde prevent the appearance of the indigo blue even when indican is present in large amounts.

(3) Report the test as negative, slight excess, or large excess. Normal urines may give a faint blue.

68. Acetone.—*a. Sodium nitroprusside test (Lange's).*—(1) *Reagents.*—(*a*) *Acetic acid, glacial*, 99 percent.

(*b*) *Ammonium hydroxide*, 28 percent, stronger ammonia water.

(*c*) *Sodium nitroprusside*, freshly prepared saturated solution. Dissolve several crystals in 1 to 2 cc of water by gentle heat, having a slight excess of undissolved crystals remaining.

(2) *Procedure.*—Place 5 cc of filtered urine in a test tube, add 0.5 cc of glacial acetic acid and 0.5 cc of the freshly prepared sodium nitroprusside solution and mix. Tilt the tube and carefully overlay the mixture with 1 to 2 cc of strong ammonia water.

(3) *Results.*—A purple or purplish-red ring forms at the contact zone in a few minutes if acetone is present. The ring tends to be more purple or violet in low concentrations, more red-purple in high. Amorphous urates may give a brown or orange ring if present in large amount.

b. Rantzman modification.—Aqueous solutions of sodium nitroprusside decompose rapidly and hence must be freshly prepared. This modification gives a reagent which keeps fairly well.

(1) *Reagent.*—Dissolve 37.5 gm ammonium nitrate crystals and 2.5 gm sodium nitroprusside in distilled water and make up to 100 cc.

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In a brown glass-stoppered bottle this reagent will keep for 2 months.

(2) *Procedure.*—To 3 cc of urine in a test tube add 1 cc of the reagent. Mix and overlay with strong ammonia water.

(3) *Result.*—If acetone is present a sharply defined purple or burgundy-red ring appears at the contact zone. The smaller the amount of acetone present, the longer it takes the ring to appear.

c. Ross modification of Rothera's test.—(1) *Reagent.*—Mix 1 part of powdered sodium nitroprusside and 100 parts of powdered ammonium sulfate.

(2) *Procedure.*—Place 1 gm of the dry powdered reagent in a test tube and add 5 cc of clear urine. Mix until the powder is dissolved, then overlay with strong ammonia water.

(3) *Result.*—A red-purple permanganate color indicates the presence of acetone.

69. Aceto-acetic acid (diacetic acid) (ferric chloride test—Gerhardt).—*a. Reagent.—Ferric chloride*, 10 percent aqueous solution.

b. Procedure.—To 5 cc of urine in a test tube, add the ferric chloride reagent drop by drop until no more phosphates precipitate. Filter and add more ferric chloride solution.

c. Result.—If aceto-acetic acid is present a bordeaux-red color develops. A similar color is produced by phenols, coal tar antipyretics, bicarbonates, salicylates, etc.

70. Bile pigments.—*a. Rosenbach's modification of Gmelin's test.*—(1) *Reagent.—Nitric acid*, concentrated, containing nitrous acid. This is concentrated nitric acid which is slightly yellow, due to the presence of nitrous acid. Colorless nitric acid will become yellow by exposing it to sunlight in a plain glass container for several days. To hasten this change, heat colorless acid in a beaker with a small sliver of pine wood (piece of match stick) until fumes appear.

(2) *Procedure.*—Filter 10 to 20 cc of urine, acidified with 1 or 2 drops of dilute hydrochloric acid, through a small, heavy filter paper. Introduce 1 drop of the nitric acid into the apex of the paper, then unfold it.

(3) *Result.*—A play of colors appears in the order of green, blue, violet, red, and reddish yellow, the last nearest the center of the paper.

b. Hammarsten's test for bilirubin.—(1) *Reagents.*—(*a*) *Barium chloride*, 10 percent aqueous solution.

(*b*) *Ethyl alcohol*, absolute.

(*c*) *Nitric acid, 1:4.*—Dilute 1 part of concentrated acid with 3 parts of water.

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(*d*) *Hydrochloric acid, 1:4.*—Dilute 1 part of concentrated acid with 3 parts of water.

(*e*) *Stock reagent.*—Mix 1 part of the dilute nitric acid and 19 parts of the dilute hydrochloric acid.

(*f*) *Test reagent.*—To 1 part of stock reagent, add 4 parts of the absolute ethyl alcohol.

(2) *Methods.*—(*a*) *With whole urine.*—To 2 cc of test reagent in a test tube, add a few drops of urine.

(*b*) *With urinary precipitate.*—To 5 cc of acid urine (acidify if necessary) add 5 cc of 10 percent barium chloride solution. Mix well and centrifugalize. Decant and discard the supernatant. Mix the precipitate with 2 cc of the test reagent and centrifugalize.

(3) *Result.*—A green color is produced if bile pigments were present. This test is sensitive to 1 part of bile pigment in 1,000,000 parts of urine.

c. Huppert-Nakayama test.—(1) *Reagents.*—(*a*) *Barium chloride,* 5 percent solution.

(*b*) *Nakayama reagent.*—Dissolve 0.4 gm ferric chloride in a mixture of 99 cc of 95 percent ethyl alcohol and 1 cc of concentrated hydrochloric acid.

(*c*) Nitric acid, concentrated.

(2) *Procedure.*—To 5 cc of urine add 5 cc of the barium chloride solution. Mix thoroughly and centrifugalize. Pour off the supernatant fluid. To the sediment add 2 cc of the Nakayama reagent, mix, and bring to a boil.

(3) *Result.*—A brilliant, deep-green color develops if bilirubin is present. On adding a few drops of nitric acid the color changes to violet or red.

71. Urobilinogen.—*a. General.*—Bilirubin excreted into the intestine in the bile is decomposed with the production first of the colorless compound urobilinogen, and then urobilin. Normally these compounds are partially absorbed from the intestine, carried to the liver, and reconverted into bilirubin. Some part of the urobilinogen normally finds its way into the general circulation and is excreted in the urine. In disturbances of liver function and in certain toxemias, infectious diseases, and hemolytic conditions larger amounts may get into the general circulation and appear in the urine.

b. Ehrlich aldehyde test (modification of Wallace and Diamond).—This test is roughly quantitative.

(1) *Reagent.*—Dissolve 2 gm of paradimethylaminobenzaldehyde in 100 cc of 20 percent (by volume) hydrochloric acid.

(2) *Procedure.*—To 10 cc of bile-free, undiluted urine at room

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temperature, or warmed to 21° to 22° C., add 1 cc of the reagent; allow to stand for 3 minutes. If a deep cherry-red color appears, proceed with the test using 10-cc portions of dilutions of the urine. Make 1:10, 1:20, 1:50, 1:100, and 1:200 dilutions with tap water at room temperature. Add to the 10-cc portions 1 cc of reagent, let stand 3 to 5 minutes, not longer, and read.

(3) *Results.*—Express the result in terms of the highest dilution giving a faint but definite pink or cherry color; that is, positive in 1:20 dilution. Normally this is at the 1:20 dilution. Any greater dilution yielding a definite pink color indicates a pathological amount of urobilinogen. A daily estimation showing positive in greater and greater dilution is especially significant.

72. Blood.—The detection of traces of blood requires microscopic examination of the urinary sediment for red blood cells and chemical examination for hemoglobin. See paragraphs 73 to 76, inclusive, and chapter 5 for the methods.

SECTION IV

MICROSCOPIC EXAMINATION OF URINARY SEDIMENTS

	Paragraph
Preparation of specimen	73
Examination of drop	74
Recording of findings	75
Character of urinary sediments	76

73. Preparation of specimen.—*a.* Urines should be examined within a few hours after voiding, unless kept at a low temperature or preserved with a chemical. Alkaline specimens should be examined as soon as possible. If large amounts of suspended amorphous phosphates in an alkaline urine obscure the field, the specimen should be cleared with dilute acetic acid and reexamined. Heavy urate sediments in an acid urine may be dissolved by gently warming the specimen.

b. The sediment for examination is usually secured by centrifugalizing a 15-cc portion of the urine at a relatively low speed (1,000 to 2,000 rpm), for 5 minutes. The supernatant urine is poured off as completely as possible and the sediment mixed with the urine remaining by tapping the end of the tube with the finger. A drop of the mixed sediment is removed to a clean glass slide by means of a pipette, or a drop may be poured directly onto the slide from the centrifuge tube.

c. If a centrifuge is not available, sedimentation in long, conical urine test glasses may be done. This is not as satisfactory as centrif-

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ugalization because of the longer time required and because the various constituents, due to differences in specific gravity, settle out at different rates, giving a less homogeneous sediment. A drop of sediment collected in this manner must be transferred to the slide by means of a pipette.

74. Examination of drop.—Examination is usually made with a low-power objective (16 mm) and a moderately high-power eyepiece (10X). The high-power objective (4 mm) is needed occasionally for detailed examination of casts and to distinguish between pus cells and red blood cells. For low-power examination it is not necessary to use a cover slip; it may be desirable to use one when the higher power objective is used. The substage condenser should be lowered and the light cut down by the iris diaphragm until the objects stand out clearly.

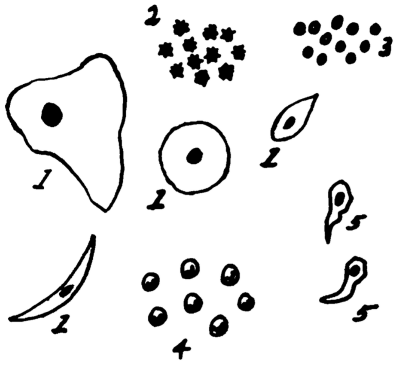
75. Recording of findings.—The frequency of occurrence of the various objects observed should be noted as well as their mere presence. The terminology used may be: occasional, few, many, very many, etc. A uniform technic of examination and of reporting should be followed so that the results of different examinations may be comparable. The same amount of urine should be centrifugalized at the same speed for the same length of time in each case. The supernatant should be poured off to the same degree of completeness, and approximately the same thickness of drop examined.

76. Character of urinary sediments.—There are two classes of sediments: unorganized chemical compounds; and organized, formed, morphological entities. The latter are by far the more important as the mere presence of some or a superabundance of others indicates pathology somewhere in the urinary tract provided the specimen has not been contaminated.

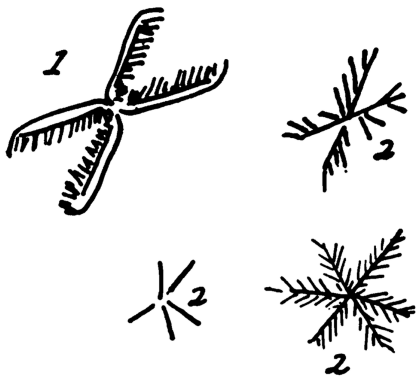
a. Unorganized sediment.—(1) Only rarely has the unorganized sediment clinical significance. Its presence and character depends on metabolic activities of the body which are influenced by innumerable factors, or upon fermentation and decomposition processes occurring in the bladder or in the container after voiding. Unorganized sediments appear as crystals of definite structure that can be readily identified morphologically or as amorphous deposits having a granular, structureless appearance; these latter can be partially identified by solubility or microchemical tests conducted on the slide while under microscopic observation.

(2) In acid urines one may find amorphous, pinkish sediments of urates; brownish, wedge-like, "whetstone," or dumbbell crystals of uric acid; small dumbbell or square "envelope" crystals of calcium

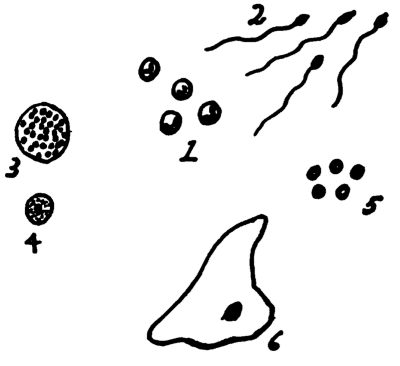
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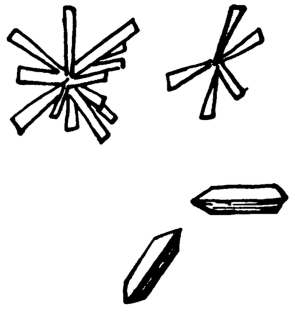
1, 5, EPITHELIAL CELLS
2, 3, ERYTHROCYTES
4, LEUCOCYTES



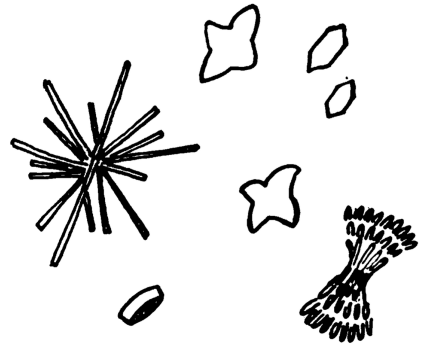
1 & 2, TRIPLE PHOSPHATE CRYSTALS



1, LEUCOCYTES; 2, SPERMATOZOA;
3, 4, 6, EPITHELIAL CELLS;
5, ERYTHROCYTES



NEUTRAL CALCIUM PHOSPHATE CRYSTALS



URIC ACID CRYSTALS. (YELLOW TO GREENISH YELLOW).



URIC ACID CRYSTALS (YELLOW TO GREENISH YELLOW).

FIGURE 8.—Urinary sediments (unorganized).

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oxalate; refractile, colorless, six-sided plates of cystine; yellowish, small spheroids of leucine; fine needles of tyrosine; and brownish needles or prisms of hippuric acid.

(3) In neutral urines those already mentioned may occur and in addition slender, pyramidal crystals of neutral calcium phosphate united at their apices to form rosettes.

(4) Alkaline urine may contain white amorphous phosphate deposits; "coffin lid" or feathery crystals of ammonium magnesium phosphate (triple phosphates); spheres or dumbbells or amorphous deposits of calcium carbonate; and dark yellow to brown "cockle-burr" crystals of ammonium urate.

b. Organized sediments.—(1) *Casts.*—As the name suggests these are molded in the tubules of the kidney and are composed of an albuminous material and various types of cells. They vary greatly in size but in almost all instances their sides are parallel and ends rounded or broken off squarely. They may be straight or curved, long or short, but the diameter is usually uniform throughout the length. Casts have been classified according to their microscopical characteristics as hyaline, granular, epithelial, blood, pus, fatty, and waxy. The finding of casts in the urine is very important for their presence usually indicates some form of kidney disorder, especially if albumin is also present.

(*a*) Hyaline casts are found more frequently than any others. They are composed of colorless, homogeneous, slightly refractile material; are usually narrow; cellular elements may be attached to the surface.

(*b*) Granular casts are usually short and thick. The basic hyaline substance is filled with granules which may be fine or coarse.

(*c*) Epithelial casts bear on their surface cells from the lining of the kidney tubules. The basic material is hyaline, either granular or nongranular.

(*d*) Blood casts are made up of red blood cells on a fibrin or hyaline base.

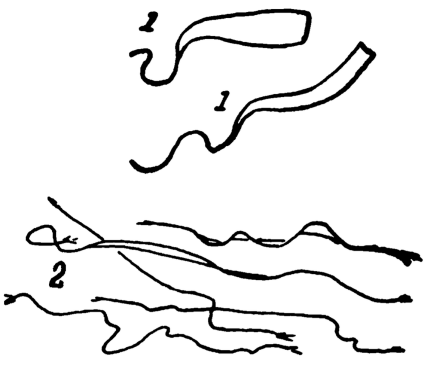
(*e*) Pus casts are quite rare. They are covered with pus cells on a hyaline base.

(*f*) Fatty casts may be produced by the deposition of fat globules or fatty acid crystals on a hyaline cast.

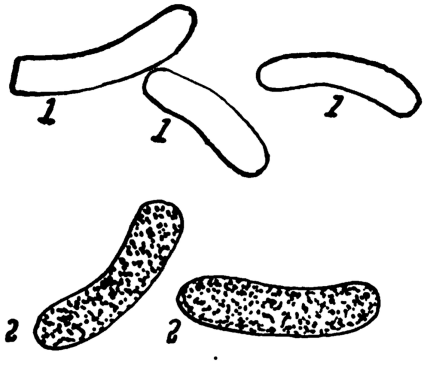
(*g*) Waxy casts are made up of a basic substance similar to that of hyaline casts. As a rule they are longer, larger, and more refractile, with sharper outlines and sometimes of a yellowish color.

(2) *Cylindroids and pseudo-casts.*—Mucus threads, with or without deposits of unorganized sediment about them, may simulate true

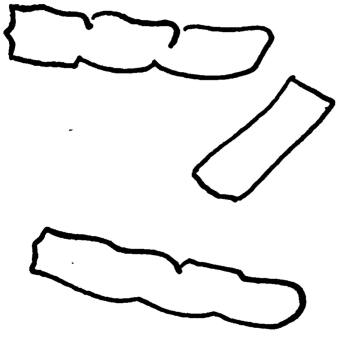
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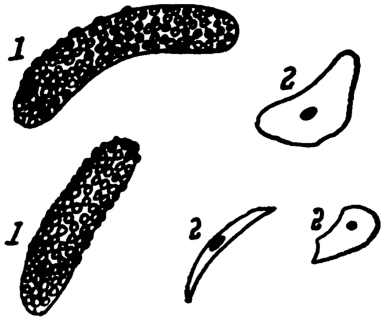
1, CYLINDROIDS
2, MUCUS THREADS



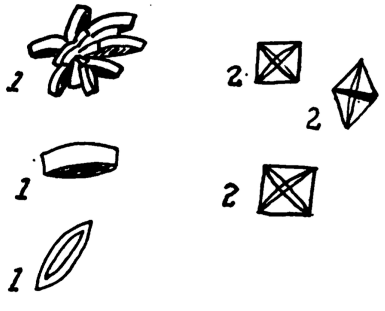
1, HYALINE CASTS
2, GRANULAR CASTS



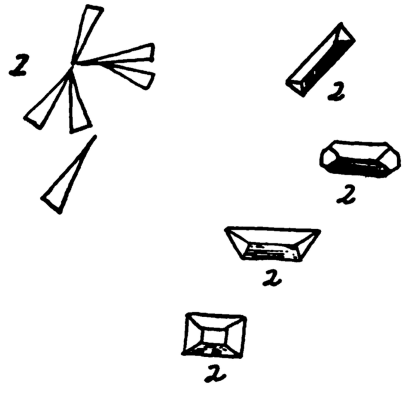
WAXY CASTS



1, BLOODY CASTS
2, SQUAMOUS EPITH. CELLS



1, URIC ACID (YELLOW) CRYSTALS
2, CALCIUM OXALATE CRYSTALS



1, CALCIUM PHOSPHATE CRYSTALS
2, TRIPLE PHOSPHATE CRYSTALS

FIGURE 9.—Urinary sediments (organized).

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casts. They are usually rough-edged, larger, tapering, or frayed at the ends. Cylindroids are often pale, ribbon-like structures, too long to be casts, with variable widths and small diameters.

(3) *Leucocytes or pus cells.*—These are round, mono- or poly-nucleated structures, ordinarily colorless. Adding a drop of dilute acetic acid to the sediment brings out the nucleus. They may be scattered about the field singly or occur in clumps.

(4) *Erythrocytes or red blood cells.*—In fresh urine these appear as biconcave disks becoming compact and crenated in concentrated acid urines, and swollen, colorless, disintegrating, faint shadows in dilute alkaline urine. Where less than 12 red blood cells are present per high-power field, the benzidine test is likely to be negative.

(5) *Epithelial cells.*—A few squamous cells are usually present. In certain pathological conditions they are greatly increased in number and since the different parts of the urinary tract are lined with different types of epithelium, the number and type of cells present may be of considerable diagnostic value.

(6) *Spermatozoa.*—These are readily identified by the characteristic oval head and long tail. The illustrations in this section give a general idea of the appearance of the more usually encountered unorganized and organized sediments.

SECTION V

QUANTITATIVE CHEMICAL EXAMINATION

	Paragraph
Albumin	77
Urea nitrogen (urea)	78
Ammonia	79
Glucose (Benedict method)	80
Hydrogen ion concentration or pH	81
Ethyl alcohol	82
Sulfonamides	83

77. Albumin.—*a. Method adopted by the Committee on Urinary Impairments of the Association of Life Insurance Directors of America.*—(1) *Reagents.*—(*a*) *Sulfosalicylic acid.*—A 3 percent solution in distilled water.

(*b*) *Permanent standards.*—Dissolve 20 gm of purest sheet gelatin in 120 to 140 cc of distilled water at 45° to 55° C. and make up to 200 cc. Add half of the white of an egg and stir it in well. Heat on a water bath for at least 30 minutes after a temperature of 90° C. has been attained. Filter hot through a Whatman No. 4 paper, yielding a perfectly clear, slightly yellow solution. Immediately before use

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add 0.3 cc of formalin (40 percent formaldehyde solution) to each 100 cc of gelatin solution. Formazin, the material to be suspended in the gelatin, is made up as follows: Dissolve 2.5 gm of urotropin (hexamethylene tetramine) in 25 cc of distilled water at room temperature. Add this to 25 cc of 1 percent hydrazine sulfate solution also at room temperature. Mix, stopper, and allow to stand at least 15 hours. Suspend the white amorphous precipitate uniformly by gently inverting the flask several times. Add 14.5 cc of the formazin suspension to 100 cc of the 10 percent gelatin solution, to which the correct amount of formalin has been added, at 45° to 55° C. and mix thoroughly. This produces a turbidity equivalent to that made by an albumin solution of 0.1 percent, or 100 mg in 100 cc, when precipitated by three volumes of 3 percent sulfosalicylic acid. Dilute the stock suspension according to table VI to make the other standards required.

TABLE VI.—*Permanent albumin standards*

Stock formazin suspension equivalent to 100 mg albumin per 100 cc	10 percent clarified gelatin	Value of standard made	
Cc	*Cc*	*Percent*	*Mg/100 cc*
25.0	26	0.05	50
20.0	30	.04	40
15.0	35	.03	30
10.0	40	.02	20
5.0	45	.01	10
2.5	55	.005	5

Pour each standard into a test tube of the same dimensions as those used in making the test with urine. Seal the tubes with waxed stoppers and allow to cool to room temperature. Keep in a well-lighted room. In extremely hot weather, keep in a cool place. If in time they become greenish, exposure to sunlight will bleach them. There is no appreciable change in turbidimetric value in 6 to 8 months and only a slight change in a year. It is best to replace them after 8 months.

(2) *Procedure.*—Pipette 2.5 cc of urine, cleared by filtration or centrifugalization, into a test tube graduated at 10 cc and add 3 percent sulfosalicylic acid to the 10-cc mark. Invert several times, allow to stand for 10 minutes, and compare the turbidity with that of the permanent standards. Record the value of the standard most closely matched as the albumin content of the urine.

b. Sedimentation method of Shevky and Stafford.—(1) *Reagents.*—Tsuchiya's solution is used as the precipitant. Mix 15 gm of phos-

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photungstic acid, 50 cc of concentrated hydrochloric acid, and 1,000 cc of 95 percent ethyl alcohol.

(2) *Procedure.*—The first step is the dilution of the urine. Nephritic urines are usually diluted ten-fold. In urines with very scanty protein content a lesser dilution or none at all will give more exact results. Occasionally a urine is encountered with more than 2.8 percent of protein which is the maximum that can be determined with a ten-fold dilution. In such a case a fresh sample is diluted twenty-fold and the determination repeated. Of the diluted urine, 4 cc are measured into a special graduated centrifuge tube (Shevky-Stafford or McKay tube; A. H. Thomas Co. No. 3007-A; Eimer and Amend No. 18368; Fisher Scientific Co. 5-663), the 4-cc mark on the tube itself serving for the measurement. Tsuchiya's reagent is added to the 6.5-cc mark. Mix the contents well by inverting the tube several times, allow to stand exactly 10 minutes, and centrifugalize for exactly 10 minutes at 1,800 rpm. The volume of precipitate is read on the scale in hundredths of a cubic centimeter.

(3) *Calculation.*—Grams of protein per liter of urine=cc of precipitate×7.2×dilution, where dilution indicates the number of times the urine was diluted before the sample was measured into the tube.

78. Urea nitrogen (urea).—*a. Aeration method (Van Slyke and Cullen).*—(1) *Reagents.*—(*a*) *Urease paper.*—Transfer 30 gm of jackbean meal to a 200-cc flask, add 100 cc of dilute ethyl alcohol (30 cc of 95 percent ethyl alcohol diluted to 100 cc) and 1 cc of the buffer described in (*b*) below. Stopper and shake vigorously for 15 minutes. Transfer to centrifuge tubes, close the mouths of the tubes with tinfoil, and centrifugalize for 30 minutes. Transfer the supernatant to a flat-bottomed dish and take up at once on strips of filter paper such as is used in the preparation of amboceptor paper (Schleicher and Schull's No. 597). Suspend the papers from a wire or rack by means of paper clips and allow to dry overnight in an incubator at 37.5° C. As soon as dry cut into pieces about 1 by 2.5 cm and preserve in wide-mouthed dark glass bottles. Urease so prepared will retain its activity for at least 6 months.

(*b*) *Acetate buffer.*—Dissolve 15 gm of crystallized sodium acetate in a 100-cc volumetric flask with 50 to 75 cc of water. Add 1 cc of glacial acetic acid, dilute to volume, and mix.

(*c*) *Potassium carbonate.*—Use the dry, granulated salt or a saturated solution made by dissolving 90 gm in 100 cc of distilled water. Sodium carbonate may be used instead.

(*d*) *Sulfuric acid, 0.02 N.*—One cc reacts with exactly 0.28016 mg nitrogen, or 0.60053 mg urea.

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(*e*) *Sodium hydroxide, 0.02 N.*—Dilute 20 cc of tenth normal solution (ch. 5) to 100 cc with distilled water. Check the 0.02 N sulfuric acid against this alkali.

(*f*) *Caprylic alcohol.*

(*g*) *Methyl red or alizarin indicator solution.*

(2) *Procedure.*—(*a*) Prepare five aeration tubes, 200 by 25 mm, with inlet and outlet tubes, two-holed rubber stoppers, and rubber tubing connections for aeration as shown in figure 10.

(*b*) Mark the tubes *1*, *A*, *B*, *C*, and *D* in the order in which the stream of air will pass through. In tube *1* place 20 cc of dilute sul-

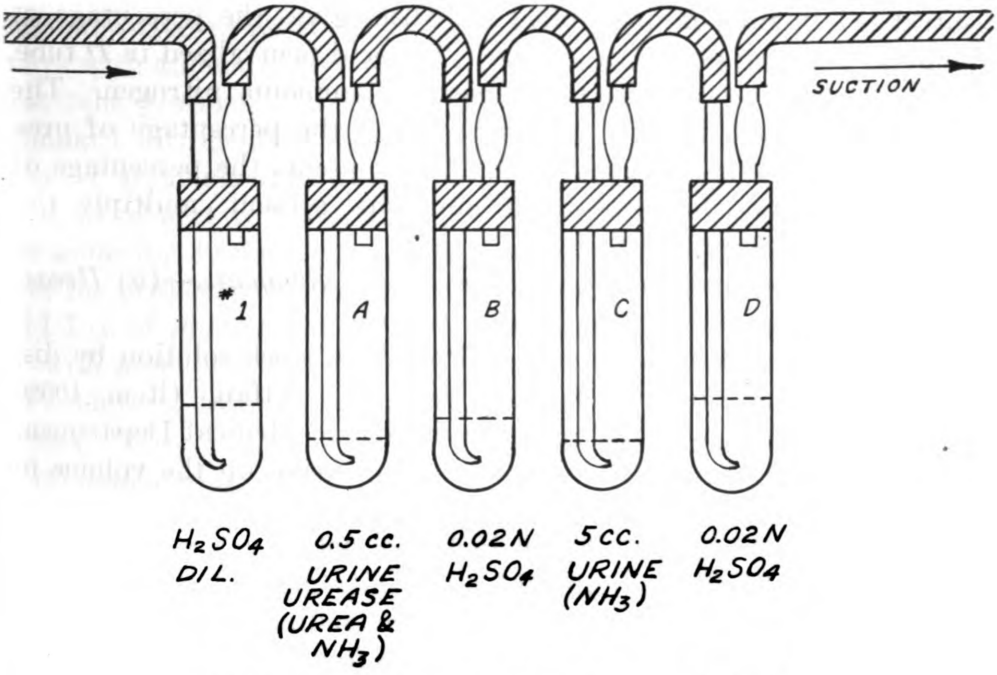


FIGURE 10.—Aeration apparatus (for urea nitrogen).

furic acid to remove ammonia from the incoming air; in *A*, 10 cc of ammonia-free water, 2 drops of the acetate buffer and a piece of urease paper; in *B* and *D* place 25 cc of 0.02 N sulfuric acid and 5 drops of indicator; in *C* place 5 cc of urine and 5 drops of caprylic alcohol. Insert stoppers tightly making sure that the inlet tubes reach nearly to the bottom of each tube. Remove the stopper from *A* and add 5 drops of caprylic alcohol and then exactly 0.5 cc of urine, holding the tip of the pipette down near the liquid already in the tube. Stopper quickly. Allow to stand 20 minutes or longer, occasionally shaking tube *A* to free the urease from the paper. Attach the train to a suction pump and draw air slowly through all the tubes for 1 minute, to collect in the acid any free ammonia in *A* and *C*. Shut off the suction and

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add to *A* and *C* 10 cc of the potassium carbonate solution or about 5 gm of the salt in powdered form. Stopper quickly and begin suction slowly, gradually increasing to a moderate rate. Aerate for at least 30 minutes; 1 hour may be required if too small a stream of air is used. The proper rate of speed should be determined and a safe margin used in all runs.

(*c*) When the aeration is complete, titrate the acid remaining unneutralized in tubes *B* and *D* with 0.02 N sodium hydroxide. Be sure to turn off the suction before disconnecting the tubes (center one first) to prevent back suction.

(3) *Calculation.*—The number of cubic centimeters of 0.02 N acid neutralized in tube *B*, multiplied by 0.056, gives the percentage of ammonia nitrogen plus urea nitrogen; the acid neutralized in *D* tube, multiplied by 0.0056, gives the percent of ammonia nitrogen. The difference between the two (*B* minus *D*) is the percentage of urea nitrogen; this figure multiplied by 2.14 represents the percentage of urea. To obtain the percent of ammonia present, multiply the percentage for *D* by 1.1216.

b. Colorimetric method with permutit.—(1) *Reagents.*—(*a*) *Urease paper.*—Same as in method given in *a* above.

(*b*) *Standard nitrogen solution.*—Prepare a stock solution by dissolving 4.716 gm of pure, dry ammonium sulfate (item 10680 ammonium sulfate ACS, for nitrogen standard—Medical Department Supply Catalog) in 0.2 N sulfuric acid and make up the volume to 1,000 cc. This solution contains 1 mg nitrogen per cc. For use, dilute 10 cc of this stock solution to 100 cc with 0.2 N sulfuric acid. One cc of this dilution contains 0.1 mg of nitrogen.

(*c*) *Nessler's solution.*—Dissolve 22.5 gm iodine in 20 cc of water containing 30 gm of potassium iodide. After solution is complete, add 30 gm of metallic mercury and shake vigorously so as to break up the mercury into globules, cooling the mixture from time to time by immersing the flask in cold water. Continue until the supernatant has lost all color due to iodine. Decant the supernatant from the excess mercury and test for free iodine by adding a drop or two of the solution to 1 cc of a 1 percent starch solution. If the starch test for iodine is negative, add a few drops of an iodine solution of the same concentration as that above, until a faint excess of iodine can be detected by the starch test. Dilute the double iodide solution to 200 cc with distilled water and mix well. Prepare accurately a 10 percent sodium hydroxide solution from a saturated solution of sodium hydroxide which has been allowed to stand until all carbonates have settled out. To 975 cc of this 10 percent hydroxide solu-

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tion, add the entire solution of potassium mercuric iodide prepared above. Mix thoroughly and allow to settle.

(*d*) *Acetate buffer.*—Same as in method given in *a* above.

(*e*) *Permutit.*

(*f*) *Gum acacia, 5 percent solution.*—This solution may be preserved by adding 3 cc of the potassium mercuric iodide solution made for Nessler's solution to each 100 cc of gum solution. Keep the gum solution in a tall cylinder where the precipitate which forms can settle out.

(2) *Procedure.*—Shake out 3 to 5 cc of urine in a small flask with about 1 gm of permutit to remove ammonia. Place 1 cc of this ammonia-free urine in a 500-cc volumetric flask and dilute to the mark with ammonia-free water. Place 5 cc of this dilution in a large test tube graduated at 25 cc. In a similar tube place 1 cc of the standard nitrogen solution containing 0.1 mg of nitrogen, and add 4 cc of ammonia-free water. To both standard and unknown add 2 drops of acetate buffer and a piece of urease paper. Stopper and set aside for 30 minutes at room temperature, shaking frequently to free the urease from the paper. At the end of this digestion period, add 1 cc of gum acacia solution to each tube and dilute to about 20 cc with ammonia-free water. Then to each tube add 2 cc of Nessler's solution, dilute to the 25-cc mark, and compare in the colorimeter. (See ch. 6.)

(3) *Calculation.*

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{mg N in standard used} \times \frac{\text{dilution}}{\text{cc of dilution taken}} \times 100 = \text{milligrams urea nitrogen per 100 cc urine.}$$

Following the procedure above, using 5 cc of a 1:500 dilution of urine, and 1 cc of standard containing 0.1 mg of N, the calculation is—

$$\frac{S}{U} \times 1{,}000 = \text{mg N/100 cc urine}$$

Multiplying the nitrogen figure by 2.14 gives the milligrams of urea per 100 cc of urine.

79. Ammonia (as determined in par. 78*a*).—Multiply the cubic centimeter of 0.02 N acid neutralized in tube *D* by 0.0056 to obtain the percentage of ammonia nitrogen, and this figure by 1.1216 to obtain the percentage of ammonia.

80. Glucose (Benedict method).—*a. Reagents.*—(1) *Sodium carbonate*, crystals or monohydrated salt.

(2) *Pumice* or *talc.*

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(3) *Benedict's reagent, quantitative.*

Copper sulfate, U. S. P. crystals ($CuSO_4 \cdot 5H_2O$)	18.0 gm
Sodium carbonate, monohydrated ($Na_2CO_3 \cdot H_2O$) (74 gm of anhydrous Na_2CO_3 or 200 gm of the crystalline salt, $Na_2CO_3 \cdot 10H_2O$, may be used.)	87.0 gm
Sodium or potassium citrate	200.0 gm
Potassium thiocyanate	125.0 gm
Potassium ferrocyanide, 5 percent solution	5.0 cc
Distilled water to make	1000.0 cc

With the aid of heat, dissolve the carbonate, citrate, and thiocyanate in enough water to make about 800 cc of solution. Filter if necessary. Dissolve the copper sulfate separately in about 100 cc of water, and pour this solution slowly into the first one, with constant stirring. Add the ferrocyanide solution, cool to room temperature, and make up to 1,000 cc in a volumetric flask. Of the various salts only the copper sulfate must be weighed with extreme accuracy. Exactly 25 cc of this reagent are reduced by 50 mg of glucose.

b. Procedure.—Dilute 10 cc of clear urine to 100 cc with water, unless the sugar content is known to be low. Fill a 50-cc burette with this diluted urine. Measure exactly 25 cc of the Benedict's reagent into a porcelain evaporating dish, add about 15 gm of crystalline sodium carbonate (half that amount of the monohydrated salt, or 6 gm of the anhydrous), and a small amount of pumice or talc. Heat to boiling over a free flame and keep the mixture boiling vigorously during the entire titration. As soon as the carbonate is completely dissolved add the diluted urine from the burette, rapidly at first, until a chalk-white precipitate forms and the blue color begins to fade perceptibly. It is then run in a few drops at a time until the last trace of blue disappears from the solution. Half-minute intervals must be allowed to elapse between additions of urine in the final steps of the titration. Water may be added if the mixture becomes too concentrated. The end point must be determined while the solution is still hot; upon cooling the solution tends to regain a bluish-green tint. With urine, the color at the end point tends to be a slight yellowish, or yellowish green due to urinary pigments.

c. Calculation.—When the urine is diluted 1:10, the following formula applies:

$$\frac{0.050}{N} \times 1{,}000 = \text{percent of glucose in original sample}$$

where N is the number of cubic centimeters of diluted urine required to reduce 25 cc of the reagent.

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In general, $\frac{0.050}{X} \times 100 =$ percent glucose, where X = number of cubic centimeters of *undiluted* urine required for the reduction.

81. Hydrogen ion concentration or pH.—There are numerous methods for determining the hydrogen ion concentration (or pH) of the urine, but the simplest method satisfactory for clinical purposes is that involving the use of nitrazine paper (phenaphthazine).

a. Reagent.—Nitrazine paper, Squibb.

b. Procedure.—With a clean glass rod transfer a drop of urine to the surface of the paper strip and spread evenly by stroking or leave a small drop on the paper. After 1 minute compare with the color chart furnished. The paper may be dipped into the urine three consecutive times and the excess shaken off. Compare after 1 minute.

c. Results.—The color comparison chart reads from pH 4.5 to 7.5 in 0.5 divisions. It is possible to interpolate between these divisions by estimating the color half way between them. Report the pH as read.

82. Ethyl alcohol.—See chapter 6 for the determination of ethyl alcohol in urine and blood.

83. Sulfonamides.—See chapter 6 for the determination of the various sulfonamides in urine and blood.

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CHAPTER 5

EXAMINATION OF GASTRIC CONTENTS

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SECTION I

GENERAL

	Paragraph
General	84

84. General.—*a.* The examination of the gastric contents yields information as to the secretory, digestive, and motor functions of the stomach and indirectly aids in the diagnosis of conditions that affect the stomach secondarily, such as the demonstration of absence of acidity in pernicious anemia.

b. The routine examination is concerned chiefly with the secretory response of the stomach to a test meal or to the stimulation supplied by drugs such as histamine. In the course of a complete examination, pathological conditions such as hemorrhage, new growth, or parasites may be disclosed. It is well to keep in mind that the gastric contents are not only supplied by the activity of the stomach, but include a variety of materials swallowed and others that are regurgitated from the small intestines. Pus, blood, bacteria, tissue fragments, etc., from the nose, mouth, pharynx, and esophagus find their way into the stomach; similar materials and secretions and excretions of the liver, gallbladder, and duodenum may be regurgitated through the pylorus, and with reversal of peristalsis, the fecal contents of the jejunum and ileum may likewise gain entrance.

c. The fundus glands of the stomach secrete hydrochloric acid, and the ferments pepsin and rennin which are activated only in the presence of free hydrochloric acid. Lipase is also secreted but it has little activity except on previously emulsified fats such as milk or egg yolk. The pyloric glands contribute an albuminous secretion. The goblet cells, distributed along the entire surface epithelium and along the ducts of the fundus and pyloric glands, add mucus.

d. The hydrochloric acid combines loosely with protein food; this is designated "combined HCl." When all the protein has been con-

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verted to acid metaprotein, the additional acid secreted remains uncombined and is designated "free HCl." The pepsin activated by it continues the digestive process. In pathological conditions there may be an absence of hydrochloric acid designated achlorhydria; this is also occasionally seen in normals in the first specimens of the fractional analysis. The absence of "free HCl" does not necessarily mean that no acid is being produced; it may be secreted and neutralized by an excessive regurgitation of the alkaline duodenal juice; this physiological regurgitation is a normal process and may reduce an acidity of 0.4 percent to as low as 0.15 percent.

SECTION II

PHYSICAL EXAMINATION

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85. Macroscopic.—*a. Amount.*—The capacity of the average adult stomach is 1,500 to 1,600 cc. The fasting residuum varies between 20 and 100 cc, usually less than 75 cc with an average of 50 cc. An increase in the residuum may be due to hypersecretion, retention, swallowing of saliva, or duodenal regurgitation. Ordinarily these may be differentiated by noting retained food particles from the previous day's meal in retention cases, or by finding increased amounts of bile and trypsin in regurgitant cases. In true hypersecretion, food particles, increased amount of bile, and duodenal ferments are absent and the juice may show no abnormality except some increased acidity. The amount of juice recovered in 1 hour after the Ewald type of test meal varies from 50 to 100 cc. Larger amounts, 200 to 300 cc, are found in hypersecretion and hypomotility and excessive amounts, 500 to 600 cc, in dilatation accompanying pyloric stenosis. Amounts less than 20 cc may be due to incomplete removal, hypermotility or incomplete closure of the pylorus due to chronic inflammation or scarring.

b. Emptying time.—The normal stomach empties itself completely in 7 hours at the longest, but the time may vary somewhat with the diet ingested. After the ordinary mixed meal it is emptied in 4 to 7 hours, after the Ewald type, in 1 to 3 hours. Large quantities of juice obtained after the normal time for emptying indicate a pathological basis.

c. Color.—(1) The gastric juice, as obtained from the fasting stomach, is usually clear, colorless, and easily filtered. Over 50 percent of residuums are faintly bile-tinged, appearing green or yellow due

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to regurgitation of bile incidental to straining during tube passage.

(2) A bright red color is an indication of fresh bleeding; dark brown or black blood is encountered in older bleeding, the blood having been acted on by the hydrochloric acid, producing a "coffee-ground" appearance. Blood in small amounts is especially characteristic of carcinoma; in larger amounts, of esophageal varices or ulcers, and in massive amounts, up to 1,000 to 2,000 cc, of ruptured varices or aneurysm or sudden ulceration into a large gastric vessel. The presence of blood, if gross, is easily detected, although tomatoes, grape skins, etc., may give confusing reddish or dark flecking. Occasionally it will be necessary to differentiate gastric from pulmonary hemorrhage; in the former the vomitus is usually acid, the color is dark red or brown, and clots are present; in the latter the expectoration is usually alkaline, brighter in color, frothy, and usually mixed with mucus. In all cases it is wise to confirm the gross impression by performing a chemical test for blood.

d. Layering.—The residuum from a fasting stomach normally shows no sediment. After the Ewald test diet the 1-hour specimen forms two layers, the lower consisting of food residue, the upper of clear or slightly cloudy grayish-white or faintly yellow fluid. Ordinarily the proportion of solid to fluid is 1 to 1 or 1 to 2; larger proportions of solids suggest delayed emptying or hyposecretion; larger proportions of fluid indicate hypersecretion. The condition of the food particles gives a rough indication of the digestive efficiency. Normally the breadstuffs forming the bulk of the Ewald type meal are acted on by the ptyalin of the saliva and appear in the lower layer as a pureed mass; in hyperacidity this enzyme is quickly destroyed so that the breadstuff fragments appear incompletely digested. In cases of pyloric stenosis three layers may be noted—at the bottom, fine, starch-like material, midway, a thick layer of cloudy fluid, and on top a foamy layer indicating gaseous fermentation.

e. Odor.—The residuum of the normal fasting stomach is practically odorless. The normal 1-hour contents after the Ewald test meal yield a sweet odor of moist bread. Sour odors, due to acetic acid formation in the course of carbohydrate fermentation, are noted in the contents after longer retention. Rancid odors of butyric acid accompanying lactic acid formation and protein decomposition occur in retention usually with decreased or absent hydrochloric acid. Lactic acid itself is odorless. Putrid odors may accompany carcinomatous ulceration of the stomach due to the discharge into the stomach of the contents of ruptured abscesses of the surrounding tissues. A fecal odor accompanies the discharge of the contents of the small bowel into the

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stomach during the reversal of peristalsis accompanying intestinal obstruction.

86. Microscopic.—This portion of a complete examination does not offer much information as a rule. Occasionally it offers unsuspected clues such as the finding of ova of intestinal parasites. The examination can be applied to vomitus. It is best done on the unfiltered residuum of the fasting stomach, or the unfiltered specimens obtained in the later stages of the fractional removal. If the residue remaining on the filter paper is examined, mix the sediment with a drop of a 1 to 4 dilution of Gram's iodine solution. This will stain tissue cells yellow, yeast cells yellow, starch cells blue or red, and leave the fat globules uncolored. In examining, lower the condenser of the substage to reduce the illumination. If gastric washings are examined, allow them to stand in a conical tube and examine the sediment.

a. Tissue fragments.—If gross particles of tissue are noted, they should be fixed in formaldehyde and prepared for histological examination. Pieces of mucosa may be scraped off during passage of the stomach tube, or portions of carcinomatous tissue may break off into the gastric contents.

b. Cells.—A few epithelial cells may be normally found. Groups of these cells entangled in mucus are seen in chronic gastritis. Red blood cells are usually degenerated; if normal cells are recognizable they usually are due to the trauma of the tube passage. In the ulceration of carcinoma many pus cells may be found; usually only the nuclei can be identified. A drop of dilute methylene blue or eosin solution will bring out the nuclei. Cells deeply stained by bile probably originate from the bile passages or gall bladder.

c. Yeasts and sarcinae.—These stain yellow and brown with iodine solution. If present in considerable numbers they indicate retention and fermentation. Budding forms of yeast ordinarily are not seen.

d. Food remnants.—If recognizable in the residuum of the fasting stomach these remnants indicate retention. After the Ewald meal, starch granules are numerous; a dilute iodine solution will stain them blue or red, depending on their stage of digestion. Striated muscle fibers and connective tissue retain their identity. Fat globules, unstained by the iodine solution, may be stained by running under the cover slip a drop of Sudan III; neutral fat globules stain red or yellow.

e. Bacteria.—Normally only a few bacteria swallowed with nasopharyngeal mucus are present in the residuum. Large numbers of the pyogenic variety occur in infective gastritis. The Boas-Oppler bacilli when present indicate gastric stagnation and are frequently associated with advanced gastric carcinoma. They belong to the lactic acid

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producing group. They are long rods, nonspore-forming, Gram-positive, growing in long jointed chains or clumps, and when present lactic acid is detectable in the gastric juice. To aid in bacterial differentiation a Gram stain is done on a heat-fixed film of the gastric contents.

f. Flagellates.—These may be found in the early anacid stage of gastric carcinoma before lactic acid production is marked; they originate in the intestines.

g. Crystals.—Crystals of fatty acids, bile acids, cholesterol, calcium oxalate, etc., may be found. They have no significance.

h. Animal parasites and ova.—These may be found, although it is questionable if they are regurgitated from the small bowel into the stomach. They are more likely to be due to contamination of the food by faulty personal hygiene.

SECTION III

TEST MEALS

Paragraph
Retention or motor meal 87
Functional meals 88
Histamine test meal 89

87. Retention or motor meal.—It is the purpose of such meals to secure information regarding gastric motility or retention. The meal contains 4 ounces of boiled string beans and 4 ounces of rice and is substituted for the regular evening meal on the day preceding that on which the gastric test is to be made. Instead of employing a special meal, the regular supper can be supplemented by adding 4 stewed prunes or 30 raisins, or raspberry preserve. The idea is to include articles of diet leaving residues that can be readily recognized in the gastric residuum of the fasting stomach the following morning.

88. Functional meals.—*a. Ewald type.*—The purpose of any functional test meal is to stimulate gastric secretion, and to estimate the functional response of the gastric mucosa by the determination in samples of the gastric contents of the production of acid and gastric ferments. A variety of test meals have been prepared. The Ewald meal or some modification is the common test meal and is used in both the older Töpfer single-sample method and the newer Rehfuss fractional method of gastric analysis. It consists of two slices of toasted bread (35 gm) and 8 ounces (250 cc) of water or tea without milk or sugar. Water is to be preferred if tests for the detection of occult blood are to be used, as the tannic and gallic acids of tea interfere with blood tests. The use of toast introduces some lactic acid into the stomach, so that if a test for lactic acid in the contents is particularly desired, a modified Ewald meal or a Boas meal, con-

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sisting of oatmeal gruel containing no lactic acid, is used. The gruel is prepared by boiling down to 500 cc 1 liter of water to which 2 tablespoonfuls of oatmeal are added and straining this concentrate through coarse muslin. The Mayo Clinic modification of the Ewald meal consists of eight arrowroot cookies and 400 cc of water; it contains no lactic acid.

b. Clear fluid type.—(1) This type of test meal, or rather test drink, is employed chiefly in Europe. Bergheim, in this country, showed that ordinary tap water produced as much stimulation as the Ewald type meal. The Bergheim water meal consists of 400 cc of ordinary tap water at room temperature. The alcohol test meal consists of 300 cc of 5 percent pure ethyl alcohol solution (15 cc of absolute alcohol brought up to 300 cc by adding water). The caffeine test meal consists of 400 cc of water containing 0.2 gm of pure caffeine. Usually these clear fluid meals are colored by adding 3 drops of 2 percent solution of methylene blue (methylthionine chloride, U. S. P.), which aids in following the propulsion of the meal out of the stomach. As the stomach empties, the blue color diminishes with each subsequent specimen examined in the fractional method, until colorless specimens are obtained, indicating complete emptying of the stomach. It also helps in recognizing the regurgitation of alkaline bile or colorless duodenal contents by the change of the blue to a characteristic greenish hue. The clear fluid meals permit more accurate determination of food residue, blood, lactic acid, and bile.

(2) The Ewald type is the most satisfactory for routine examination by the Töpfer method. The clear fluid meals, when used in single extraction methods, leave the stomach too quickly, the stomach being empty after 45 minutes. In the fractional method of gastric analysis the clear fluid meals are advantageous in that they can be readily extracted through the small tubes employed; the gastric contents are clear and titrations can be more easily read and no preliminary filtration of the contents is necessary.

89. Histamine test meal.—Various pharmacodynamic substances have been employed hypodermatically to stimulate the gastric mucosa; these include histamine, acetylcholin, insulin, pilocarpine, and eserin. Of these, histamine is commonly used. It appears to have the advantages of being standardizable, of imposing a maximal load on the gastric function, and of yielding pure gastric juice. When injected subcutaneously it acts as a chemical secretagogue stimulating the stomach mucosa via the blood stream. When its dosage is properly limited, it produces no effect appreciable to the patient, except occasionally a transitory increase in the pulse rate, a lowering of blood pressure, and flushing of the face.

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a. Dosage.—Histamine hydrochloride (b-iminazolethylamine) is used in dosage of 0.01 mg per kilogram of body weight, or 0.1 mg for each 10 kilos. A solution of the drug is prepared so that each cubic centimeter equals 1.0 mg per cc. Some use a standard dose for all patients, varying from 0.25 to 1 mg of the hydrochloride or phosphate.

b. Procedure with patient fasting.—The patient is examined in the basal state, in bed, after a 12-hour fast. The stomach or duodenal tube is introduced into the stomach shortly after the patient awakes. The fasting residuum is withdrawn, using a 50-cc Luer syringe. The histamine is then injected subcutaneously. The total gastric secretion is then aspirated at successive 15-minute periods until the flow of gastric juice subsides (usually 1 hour). Measure each specimen as to volume and determine its titratable acidity. The greatest volume of juice secreted in a 15-minute period and the highest acidity obtained in any 15-minute specimen are regarded as the indexes of secretory capability of the stomach.

c. Procedure with functional test meal.—Usually the histamine test is conducted in conjunction with a functional meal. The method in such cases is to have the patient in the basal state and fasting overnight. Shortly after awakening, the duodenal tube is swallowed, arresting the tube at the stomach mark and holding it in place by an adhesive plaster strip. The fasting residuum is aspirated. With the tube in place, a water or Ewald test meal is given. Fractions of the gastric contents are removed, every 15 minutes for 45 minutes when the Ewald is used, for 30 minutes when using the water meal. If no free acid is found in these specimens, using Töpfer's reagent as a qualitative test, inject subcutaneously the proper dose of histamine and collect three or more 15-minute specimens. On completion of the test the total and free acidity are determined for each specimen.

SECTION IV

CHEMICAL ANALYSIS

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90. General.—Two main methods are employed:

a. The method of Töpfer in which a test meal of the Ewald type is given as breakfast, and a single withdrawal of all the contents is made

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1 hour after the ingestion of the test meal. These contents are then examined chemically by Töpfer's method.

b. The fractional method of Rehfuss, in which a retention meal is given for supper the previous evening, and, after a 12-hour fast, the fasting residuum is aspirated, the tube left in place, a test meal of Ewald or clear fluid type given, and sample specimens withdrawn every 15 minutes for 1 or more hours until the stomach is empty. Each fraction is examined for total and free acidity; other tests may be made when indicated. The fractional method follows the cycle of gastric digestion and secretion, allowing the plotting of curves which permit better interpretation, especially of acid secretion.

91. Method of Töpfer.—Measure and record the volume of the sample. Strain through cheesecloth and place 10 cc of the coarsely filtered fluid in each of three beakers or porcelain dishes labeled No. 1, No. 2, and No. 3. If sufficient contents are not obtained, use 5 cc and calculate accordingly. Normally 50 to 100 cc are recovered. In hypersecretion or defective motility, 200 to 300 cc may be recovered. Excessive volumes of 500 to 600 cc indicate gastric dilatation due to pyloric stenosis, benign or malignant.

a. Reagents.—(1) *Phenolphthalein indicator.*—Dissolve .05 gm of phenolphthalein in 100 cc of 50 percent ethyl alcohol.

(2) *Töpfer's reagent.*—Dissolve 0.5 gm of p-dimethylaminoazobenzene in 100 cc of 95 percent ethyl alcohol.

(3) *Alizarin red indicator.*—Dissolve 1 gm of sodium alizarin monosulfonate in 100 cc of water.

(4) *Sodium hydroxide 0.1 N solution.*

b. Procedure.—(1) *Total acidity.*—This includes free hydrochloric, combined hydrochloric, organic acids, and acid salts. To the 10-cc sample of gastric contents in beaker No. 1, add 1 drop of phenolphthalein indicator (which is colorless in the presence of acid). Add 0.1 N sodium hydroxide solution from a burette until a faint pink is produced that persists for 2 minutes. The number of cubic centimeters used is multiplied by 10 to obtain the number of cubic centimeters of 0.1 N hydroxide necessary to neutralize 100 cc of gastric fluid. The value obtained expresses the total acidity. This can be converted into terms of hydrochloric acid by multiplying by 0.00365, which is the equivalent value of 1 cc of 0.1 N sodium hydroxide in grams of hydrochloric acid.

(2) *Free hydrochloric acid.*—This is hydrochloric acid not combined with protein material. To sample No. 2 add 2 to 4 drops of Töpfer's reagent and titrate with 0.1 N sodium hydroxide until the initial red color becomes salmon pink. If there is an initial yellow

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color on adding the indicator no free acid is present. The number of cubic centimeters of sodium hydroxide solution used, multiplied by 10, gives the value for 100 cc of the gastric juice. Töpfer's reagent occasionally gives a red color in the absence of HCl due to large increase in the organic acids, especially when lactic acid is over 1 percent and albumoses are present. In case the amount of gastric juice is small, this same specimen may be used to determine the total acidity. After the end point is reached for free HCl add 2 drops of phenolphthalein indicator and continue the titration with 0.1 N sodium hydroxide until the persistent pink end point of total acidity is reached. The number of cubic centimeters of hydroxide used in the determination of the free HCl, plus the additional cubic centimeters necessary to complete the titration with phenolphthalein, is multiplied by 10, giving the value of the total acidity.

(3) *Free acidity.*—This includes hydrochloric acid in the free state, organic acids and acid salts, but does not include the combined hydrochloric acid. To sample No. 3 add 1 to 3 drops of sodium alizarin sulfonate solution. Titrate with 0.1 N sodium hydroxide solution. As the hydroxide is added the initial tinge of yellow changes to red. The end point is indicated by a distinct violet color. The number of cubic centimeters of hydroxide used, multiplied by 10, gives the free acidity value. Töpfer states that alizarin is sensitive to all acidity except combined HCl.

(4) *Combined hydrochloric acid.*—This value is obtained by subtracting the value obtained for free acidity from that of the total acidity. Cases are seen where there is no free hydrochloric but much combined acid, indicating that acid has been secreted but has combined with the food protein.

(5) *Organic acids and acid salts.*—This value is computed by subtracting the value of free HCl from that of the free acidity, the remainder expressing this value.

c. Results.—Total acidity has a wide range normally, being equivalent to 75 to 100 cc of 0.1 N sodium hydroxide per 100 cc of gastric contents. Of this approximately 50 percent is due to free hydrochloric acid, 25 percent to combined hydrochloric, and 25 percent to organic acids and acid salts. In pathological conditions wide variations may occur from low or absent total acidity to very high acidity with correspondingly high free hydrochloric acid.

92. Fractional method of Rehfuss.—*a. General.*—(1) The patient is instructed to eat, with the evening meal on the day preceding the test, some food article which will leave an easily indentifiable residue if there is gastric retention. Such articles as several

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prunes, 2 dozen raisins in rice pudding, or raspberry preserve, answer this purpose. Nothing is to be taken by mouth after 9 PM. The following morning with the patient in the basal state the test is begun.

(2) A narrow, flexible gastroduodenal tube of the Rehfuss type is swallowed for 20 to 22 inches from the lip margin; check its position by aspirating with a 50-cc Luer syringe until free withdrawal of contents is obtained. When the tube is in the stomach, withdraw by aspiration all the contents, measure the volume, and save the specimen for examination. Maintain the position of the tube by strapping it to the angle of the mouth with adhesive; between withdrawal of the fractions of gastric juice, keep its free end clamped.

(3) An Ewald type meal is given, or a simple water meal, which is easier to swallow past the tube; the tube remains "in situ" until the test is completed. Record the time at which the last of the meal is swallowed. Any saliva that forms is to be expectorated to avoid its diluting effect on the gastric contents.

(4) Samples of about 5 to 10 cc are withdrawn at exactly 15-minute intervals, terminating each withdrawal by forcing a few cubic centimeters of air down the tube to free it for the next withdrawal. To insure a mixed sample of juice the contents may be aspirated and forced back several times before a sample is taken. Each sample is kept separate and marked with the time taken or the number of the specimen.

(5) In the definite-period test the fractions are withdrawn every 15 minutes for 2 hours. The last specimen should represent all the gastric juice remaining in the stomach at 2 hours. To insure complete emptying, the patient turns from side to side and from back to stomach, aspiration being made with the patient in these various positions. By forcing a few cubic centimeters of air down the tube, gurgling sounds will be heard with a stethoscope if there is some juice remaining. Another method is to withdraw all the juice possible and then irrigate the stomach through the tube with 200 cc of water, observing the presence or absence of food particles in the washings.

(6) In the total secretion test, the 15-minute fractions are continued until the stomach is completely empty in order to judge the motility of the stomach. In hypomotility the test is thus prolonged for 3 hours or more.

(7) The usual physical examinations are made of each fractional sample. Chemical examination is confined to total acidity and free hydrochloric acid as a rule because the individual samples are limited in amount. Strain the fasting sample and each fraction separately through gauze or cheesecloth.

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b. Reagents.—The same as in Töpfer's method except that 0.01 N sodium hydroxide is used instead of 0.1 N.

c. Procedure.—(1) *Total acidity.*—One cubic centimeter of the filtrate and 15 cc of water are placed in a porcelain evaporating dish. One drop of phenolphthalein solution is added, and a titration is made, using 0.01 N sodium hydroxide until a faint pink lasting for 2 minutes indicates the end point. Calculation: The number of cubic centimeters of 0.01 N sodium hydroxide required to neutralize 1 cc of the sample, multiplied by 10, gives the number of cubic centimeters of 0.1 N hydroxide needed to neutralize 100 cc of the gastric contents.

(2) *Free hydrochloric acid.*—(*a*) *Töpfer's method.*—Place 1 cc of the strained specimen and 15 cc of water in a porcelain evaporating dish. Add 1 to 2 drops of Töpfer's reagent; if free HCl is present a red or orange color develops. Titrate with 0.01 N sodium hydroxide solution until an orange-yellow color appears (end color is more definitely yellow than orange). The calculation is the same as in total acidity.

(*b*) *Sahli method.*—This requires more time but gives a sharper end point. It is based on the liberation of iodine from the reagent employed, in the presence of free HCl. The iodine is titrated with sodium thiosulfate, using a starch indicator.

Place 1 cc of the strained sample and 10 cc of water in a porcelain evaporating dish. One cubic centimeter of Sahli's reagent (a mixture of equal parts of a 48 percent solution of potassium iodide and an 8 percent solution of potassium iodate) is added; allow the mixture to stand 5 minutes. Titrate with 0.01 N sodium thiosulfate until only a faint yellow of the liberated iodine remains. Add 0.5 cc of 1 percent soluble starch solution; the mixture turns blue; continue titration until the blue disappears.

The total number of cubic centimeters of 0.01 N sodium thiosulfate used in the titration of 1 cc of gastric specimen is equivalent to the number of cubic centimeters of 0.01 N sodium hydroxide necessary to neutralize the free HCl in 1 cc of gastric contents. This value multiplied by 10 represents the number of cubic centimeters of 0.1 N sodium hydroxide necessary to neutralize 100 cc of stomach contents.

d. Results.—It is customary to plot the results of the acidity determinations as a curve on special forms provided for the purpose or on the reverse of the regular form. The graph is made by plotting the cubic centimeters of 0.1 N sodium hydroxide required to neutralize 100 cc of gastric contents against the time in minutes or hours. For examples of normal and abnormal graphs, see the reference books listed in the appendix.

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93. Lactic acid.—Lactic acid is a product of carbohydrate fermentation by bacteria. In health it is not present at the height of digestion. Small amounts may be introduced with the food and may be found in the first fractional specimens. It is most often present with stagnation of the gastric contents associated with deficient HCl. The combination of lactic acid, hypochlorhydria, and decreased motility occurs most often in gastric carcinoma.

a. Phenol-ferric chloride test (Uffelman).—(1) *Reagent.*—Add 10 percent ferric chloride solution to a 1 percent aqueous phenol solution until an amethyst color develops.

(2) *Procedure.*—To 5 cc of reagent add 5 cc of strained gastric juice. To another 5-cc portion add a few drops of dilute hydrochloric acid as a control.

(3) *Result.*—Lactic acid produces a canary-yellow color. The reagent will detect 0.01 percent of lactic acid. Hydrochloric acid discharges the amethyst color, leaving the solution colorless. If the gastric juice contains much free HCl the value of the test is decreased. Other organic acids give results similar to lactic.

b. Mercuric chloride-ferric chloride test (MacLean).—(1) *Reagent.*—Dissolve 5 gm of ferric chloride in a mixture of 100 cc of saturated aqueous solution of mercuric chloride and 1.5 cc of concentrated hydrochloric acid.

(2) *Procedure.*—Place 5 cc of water in a test tube as a control. In another tube place 5 cc of gastric contents. To each add 5 drops of reagent.

(3) *Result.*—A reddish color indicates the presence of lactic acid.

c. Ether-ferric chloride test (Strauss).—In this test the lactic acid is extracted from the gastric contents with ether, making a more satisfactory test since hydrochloric acid, protein digestion products, and other disturbing factors are eliminated.

(1) *Reagents.*—(*a*) *Ether.*

(*b*) *Ferric chloride*, 10 percent aqueous solution.

(2) *Procedure.*—Place 5 cc of strained gastric contents in a small separatory funnel. Add 20 cc of ether and shake thoroughly. Let stand until the ether layer has separated, then run out the layer of gastric juice and all but the final 5 cc of ether. To this add 20 cc of distilled water and 2 drops of the ferric chloride solution. Shake the mixture gently.

(3) *Result.*—When lactic acid is present in a concentration of 0.05 percent a slight greenish color develops. If the concentration is 0.1 percent, the color is an intense yellow. The color is due to ferric lactate.

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94. Occult blood (benzidine test).—This test is a very sensitive one provided the reagents are satisfactory. Different lots of benzidine vary greatly in sensitivity, and hydrogen peroxide solution rapidly loses its strength. For this reason it is always advisable to set up a positive control using water with an extremely minute amount of blood added, such as would adhere to the tip of an applicator.

a. Reagents.—(1) *Benzidine crystals.*—Prepare a saturated solution in glacial acetic acid. If kept in a brown bottle in a dark place this solution will keep fairly well. Many prefer to make the solution just before use by adding the amount of crystals picked up on the point of a knife blade to 5 cc of glacial acetic acid and warming gently to effect solution.

(2) *Acetic acid, glacial.*

(3) *Hydrogen peroxide.*—The usual solution contains 3 percent H_2O_2. Test the peroxide before use by adding a few drops of potassium dichromate solution and a few drops of concentrated sulfuric acid. If the peroxide is still active, a blue color will develop.

b. Procedure for gastric contents.—(1) *Direct test.*—To 3 cc of the saturated solution of benzidine in glacial acetic acid add 2 cc of the gastric contents and mix thoroughly. Add 1 cc of hydrogen peroxide solution.

(2) *Confirmatory test.*—If fat is present, make the gastric contents slightly alkaline with sodium carbonate or sodium hydroxide solution. Extract in a separatory funnel with an equal amount of ether. Discard the ether extract. Make the residue acid with acetic acid and extract with ether. Evaporate the ether extract to dryness, using a water bath which has been heated to boiling and the flame then turned off. Add 1 cc of water, stir to dissolve the residue, then add a few drops of benzidine solution and a drop or two of hydrogen peroxide.

(3) *Results.*—If blood is present a green to deep blue color, depending on the amount of blood, will form on adding the peroxide, in either test. Too much benzidine solution or too much peroxide interferes with the delicacy and accuracy of the test.

c. Procedure for feces.—(1) *Regular test.*—Make a thin fecal suspension in about 5 cc of water. Shake with 5 cc of ether to remove fat. Discard the ether extract. Acidify the residue with acetic acid and again extract with 5 cc of ether. Evaporate the ether extract and test as above.

(2) *Slide test.*—Smear a little of the feces on a microscopic slide. Pour over it the reagent made by dissolving a knife-tip of benzidine in 2 cc of glacial acetic acid, to which is then added 1 to 1.5 cc of peroxide solution.

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(3) *Results.*—A green to deep blue color shows the presence of blood. With the slide test, the smear turns blue without any misleading green tints from the fluid.

d. Procedure for urine.—(1) *Direct test.*—To 2 cc of urine, or to the centrifugalized urinary sediment mixed with 2 cc of water, add 3 cc of benzidine solution and 1 cc of peroxide solution.

(2) *Confirmatory test.*—Add a drop or two of glacial acetic acid to 10 cc of urine. Extract with 5 cc of ether. Evaporate the ether extract and test as shown under the confirmatory test for gastric contents.

(3) *Results.*—A green to deep blue color indicates the presence of blood.

NOTE.—The benzidine test is sensitive to 1 part of blood in 3,000,000.

e. Modified procedure for above tests.—A modification of the tests above consists of wetting a filter paper, suspended by a clip, with the gastric contents, fecal suspension, or urine sediment. When partly dry, allow a few drops of the reagent prepared as for the slide test with feces, to flow across the material on the filter paper. The blue color of a positive test shows up clearly and promptly.

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CHAPTER 6

BLOOD CHEMISTRY

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SECTION I

COLORIMETRY

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95. General.—*a.* Nearly all methods of blood analysis in common use depend upon the production of colored compounds and subsequent comparison of the intensity of color produced by the blood constituent with that of a known standard. This process is known as colorimetry and the instrument used for the comparison is a colorimeter.

b. Colorimetry is based upon Beer's law which states that light in passing through a colored solution is absorbed in direct proportion to the concentration of colored substance. The intensity of observed color is therefore directly proportional to the concentration of the colored substance and inversely proportional to the thickness of layer or depth of the column of solution traversed by the beam of light.

96. Colorimeter.—Visual colorimeters vary greatly in principle and design. The one furnished in the military service as a standard item is of the Duboscq type. Photoelectric colorimeters of many types are also in use. They vary a great deal in price, complexity, and accuracy. The better ones are accurate research instruments; the poorer ones offer little, if any, advantage over the usual type of instrument.

a. Standardization.—The colorimeter must be standardized before use and by the person using it. Frequent checks must be made to be sure that the instrument remains in proper adjustment throughout the day. To standardize, first see that the plungers are clean and are screwed firmly into their sockets. Place the empty, dry cups in their holders, being sure to use the same cup on the same side always, since the thickness of the glass cup bottoms varies. Run the carriers up until the plunger just rests in contact with the bottom of its cup, then adjust the verniers as may be necessary to read

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zero. With the cups still in position adjust the mirrors (or, in some types, the light source) so that the amount and quality of light are equal in both halves of the field.

b. Care and cleaning.—(1) When beginning work, or when changing from one colored solution to another, rinse the cups carefully, first with distilled water, then with a small amount of the solution to be used in the respective cups. The plungers should be wiped with a damp cloth, then with a dry one.

(2) After use, rinse and dry plungers and cups. Never fill the cups so full that they will overflow. Keep all solutions and reagents from contact with the mirrors; Nessler's solution is especially destructive to the silver backing.

c. Use.—(1) In using any visual colorimeter, avoidance of eye strain is important; the retina soon tires when color-matching is done. Rest the eyes frequently by looking up from the eyepiece. Suspend a large sheet of filter paper in front of and in line with the eyes as they are raised from the instrument; this rests the eyes and improves the color-matching ability.

(2) In making a reading, bring the fields to an approximate match rather quickly, then look up for a few seconds and finally complete the matching.

(3) The use of various color filters, transmitting only narrow bands of wave lengths, has made the matching of some colors much easier. Some of the newer methods in blood chemistry specify the filters to be used in making readings.

SECTION II

SPECIMENS FOR ANALYSIS

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97. Oxalated blood.—*a. Apparatus and reagents.*—(1) Syringe and needles, sterile, 18- or 20-gage, 1½ inches long.

(2) Rubber tubing tourniquet.

(3) Tincture iodine, 3½ percent.

(4) Alcohol, ethyl, 60 to 70 percent.

(5) Large tubes, small flasks, or bottles containing oxalate.

(6) Lithium oxalate, 1.5 percent solution, or potassium oxalate, 2 percent solution. In preventing blood coagulation, oxalate is better than citrate. Lithium oxalate is better than either potassium or sodium oxalate. A convenient method of preparing oxalated tubes to receive blood is to pipette 1 cc of 1.5 percent lithium oxalate (or 2 percent po-

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tassium oxalate) into the tube, then dry it in the incubator or in an oven at a temperature *below* 80° C., until the water has evaporated. The oxalate in finely divided form is distributed by this means over the lower part of the tube and goes into solution quickly when the blood is added. Drying at temperatures over 80° C. converts part of the oxalate into carbonate, with consequent production of blood clots when the tube is used.

b. Procedure.—(1) Place the tourniquet around the arm a few inches above the elbow. Have it tight enough to obstruct venous flow but not tight enough to interfere with arterial flow. Select a prominent vein, usually the median basilic, and paint the area over it with the iodine solution. Allow it to dry, then wash off with alcohol and let dry. Holding the vein immobile with the thumb of the left hand, which also supports the elbow, introduce the sharp, sterile needle into the vein with a fairly quick motion. Withdraw the required amount of blood into the syringe, place a sterile piece of gauze or pledget of cotton over the puncture site, and then withdraw the needle, while making pressure over the wound. Have the patient hold the cotton or gauze over the puncture for a few minutes. Transfer the blood from the syringe to the tube, shaking the tube so as to dissolve the oxalate.

(2) Specimens are usually taken in the morning, before breakfast, to obviate changes due to the ingestion of foods. Filtrates should be prepared and analysis made within an hour for changes take place in the blood rapidly, especially at summer temperatures. This is especially true of glucose, a considerable decrease due to naturally occurring glycolytic ferments taking place. If necessary, specimens may be kept in the refrigerator at temperatures near 0° C. if they are first chilled in ice water immediately after being drawn.

98. Separation of serum.—*a. Apparatus.*—The same as for obtaining oxalated blood, except that oxalate is not needed.

b. Method.—(1) Obtain blood by venipuncture and transfer it to an ordinary, chemically clean test tube. Do not use so much pressure on the syringe piston that a froth is formed. Allow the tube to stand for a short time until the clot has formed, chill in ice water, and then place in the refrigerator. When the clot has contracted, gently free the top part from the tube if it sticks, then centrifugalize. Transfer the serum by means of a pipette, or by pouring, to a clean, dry tube. In order to avoid hemolysis which ruins specimens for some determinations, syringe, needles, and tubes must be clean and dry.

(2) If a centrifuge is not available, clear serum may be obtained by placing the tube containing the blood nearly horizontal, thus form-

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ing a long, slanting surface. After clotting is complete, chill thoroughly, then place the blood tube upright in the refrigerator overnight. In the morning, pour off the clear serum from the side of the tube opposite the slant.

99. Preservatives.—Various preservatives have been used for whole oxalated blood. No preservative is satisfactory if gross bacterial contamination has occurred. If the blood is taken with sterile precautions into a sterile tube, it may be preserved with sodium fluoride, 10 mg per cubic centimeter. A mixture of sodium fluoride, 10 mg, and thymol, 1 mg, per cubic centimeter is perhaps better. The use of fluoride by inhibiting the reaction of urease, interferes with urease methods for determining urea.

SECTION III

CHEMICAL EXAMINATION

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100. Preparation of protein-free blood filtrate (Folin and Wu).—*a. Reagents.*—(1) *Sodium tungstate*, 10 percent aqueous solution.

(2) *Sulfuric acid*, 0.66 N. To 66 cc of 1 N sulfuric acid add 33 cc of distilled water.

(3) *Sulfuric acid*, 10 percent.

(4) *Benzoic acid*, 0.25 percent aqueous solution.

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The protein precipitation described below need not be made in a volumetric vessel; the procedure is itself volumetric, and is, therefore, applicable to any measured quantity of blood. Ten cubic centimeters of blood give ample filtrate for a complete analysis, though more may be used if desired; if no determinations must be repeated, or if all the constituents are not to be determined, less blood is sufficient. The fact that the protein precipitation is done volumetrically makes for several advantages; it not only allows use of all of a small sample of blood, but it gives a filtrate, which regardless of the initial quantity of blood used, is itself 10 percent blood. Thus, no matter what amount of blood be taken at first, 10 cc of the filtrate correspond to 1 cc of blood, 5 cc of filtrate to 0.5 cc of blood, and so on. This latter fact considerably simplifies the calculations.

Where it is desired to send a specimen to a distant laboratory for analysis, whole blood cannot be shipped as it would have decomposed before arriving at its destination. Instead, the blood is drawn in the usual manner, and a protein-free blood filtrate prepared as described below, except that 7 volumes of 0.25 percent benzoic acid are substituted for the 7 volumes of distilled water customarily used. This filtrate will keep for a considerable period and may be used for the following determinations: sugar, nonprotein nitrogen, urea nitrogen, creatinine, uric acid, and chlorides.

b. Procedure.—(1) Transfer a measured amount (which is 1 volume) of oxalated blood to a flask having a capacity of 15 or 20 times that of the volume taken. Dilute the blood with 7 times its volume, that is, with 7 volumes of distilled water and mix. If the blood is to be shipped to a distant laboratory for analysis the blood is diluted with 7 volumes of 0.25 percent benzoic acid instead of the 7 volumes of distilled water. Allow to stand until laking is complete. With a pipette add 1 volume of a 10 percent solution of sodium tungstate and mix. With another pipette add, with shaking, 1 volume of 0.66 N sulfuric acid. Close the flask with a rubber stopper and give a few vigorous shakes. If the conditions are right, hardly a single air bubble will form as a result of the shaking. (If benzoic acid is used, considerable frothing occurs.)

(2) When blood is properly coagulated, the color of the coagulum gradually changes from pink to dark brown. If this change does not occur, even after standing 15 or 20 minutes, the coagulation is incomplete, due usually to too much oxalate or citrate. In such an emergency, the sample may sometimes be saved by the cautious addition of 10 percent sulfuric acid; add the acid drop by drop, shaking vigorously after each addition and allowing the mixture to stand for a few minutes before adding more, until coagulation is complete.

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(3) Pour the mixture on a filter large enough to hold the entire contents of the flask, and cover with a watch glass. If the filtration is begun by pouring the first few cubic centimeters of the mixture down the double portion of the filter paper, the filtrate is almost invariably as clear as water from the first drop; if the first portion is not clear, it may have to be returned to the filter.

(4) The only probable sources of error in the above procedure lie in the improper tungstate or sulfuric acid solutions. The sodium tungstate may contain chlorides or too much carbonate. While the sodium tungstate may be purified by recrystallization from alcohol it is preferable and more economical to purchase a tungstate of proven purity. Most of the larger manufacturers of chemicals now prepare a special sodium tungstate for use in the preparation of blood filtrate, which will yield the desired result.

(5) The amount of sulfuric acid used in the precipitation is intended to set free the whole of the tungstic acid with about 10 percent excess to neutralize the carbonate usually present in commercial tungstates. A greater excess of acid must not be used, as a large part of the uric acid will be lost by such procedure; the volumetric sulfuric acid solutions must, therefore, be accurately made and used.

101. Nonprotein nitrogen (Folin and Wu).—*a. Reagents.*—(1) *Sulfuric-phosphoric acid digestion mixture.*—Mix 300 cc of phosphoric acid, sirupy (about 85 percent H_3PO_4) with 100 cc of concentrated sulfuric acid. Transfer to a tall cylinder, cover well to exclude ammonia, and set aside for sedimentation of calcium sulfate. This sedimentation is very slow, but in the course of a week or so the top part is clear, and 50 to 100 cc can be removed by means of a pipette. If this cannot be done, rapid centrifugalization will yield a perfectly clear solution. To 100 cc of the clear acid mixture add 10 cc of a 6 percent copper sulfate solution and 100 cc of distilled water.

(2) *Standard nitrogen solution.*—A concentrated stock solution is prepared by dissolving 4.716 gm of pure, dry ammonium sulfate in 1 liter of 0.2 N sulfuric acid. This solution contains 1 mg of nitrogen per cubic centimeter and the solution for use in the color comparison is made by diluting 10 cc of this solution up to 100 cc with 0.2 N sulfuric acid. In this last dilution 1 cc contains 0.1 mg nitrogen.

(3) *Nessler's solution.*—See paragraph 78*b*(1)(*c*).

b. Procedure.—(1) Introduce 5 cc of the protein-free blood filtrate corresponding to 0.5 cc of blood, into a dry 200- by 25-mm test tube graduated at 35 cc. These tubes must be made of pyrex glass. Add 1 cc of the sulfuric-phosphoric acid digestion mixture and boil vigorously over a micro-burner until the characteristic dense acid fumes begin to fill the test tube, which is usually in from 3 to 7 minutes. If

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the test tube is held in a slightly inclined position, and the heating begun by applying the flame of the micro-burner at the side of the tube and just below the top of the contained mixture, no bumping will occur; as the mixture begins to boil, the flame can be applied lower down, and finally, under the bottom of the tube. Unless this method of heating is followed, bumping is likely to be troublesome, and may even result in the loss of a part or all of the preparation.

(2) When the sulfuric acid fumes are unmistakable, cut down the flame so that the contents of the tube are just visibly boiling, and close the mouth of the test tube with a small watch glass or funnel.

(3) Continue the heating very gently for 2 minutes from the time the fumes begin to be unmistakable, even if the solution has become clear and colorless at the end of 20 to 40 seconds. If the oxidation is not visibly finished at the end of 2 minutes, the heating must be continued until the solution is nearly colorless.

(4) Allow the contents to cool for 70 to 90 seconds, and then add 15 to 25 cc of distilled water; cool further, approximately to room temperature, and add distilled water to the 35-cc mark. Occasionally there is formed a heavy white precipitate, probably silicates; this may settle out, or can be readily centrifugalized or filtered out, *after* Nesslerization, and just before reading against the standard.

(5) When the unknown has been prepared, a standard for comparison is made as follows: Place 3 cc of the standard nitrogen solution, containing 0.3 mg of nitrogen, in a 100-cc volumetric flask; add 2 cc of the sulfuric-phosphoric acid digestion mixture referred to above, and then about 50 cc of distilled water.

(6) Then add to the unknown 15 cc and to the standard 30 cc, respectively, of the Nessler's solution; fill the standard to the mark with distilled water; mix each thoroughly by inverting several times and compare in the colorimeter. It is essential that the unknown and the standard should be Nesslerized at approximately the same time.

c. Calculation.—The reading of the standard, usually 20 mm, multiplied by 30, and divided by the reading of the unknown, gives the nonprotein nitrogen in milligrams per 100 cc of blood.

102. Urea nitrogen (urea).—*a. Reagents.*—The same as those used in method, given in paragraph 78*b*, except that permutit is not required.

b. Procedure.—(1) Place 5 cc of the blood filtrate in a test tube graduated at 25 cc; to a similar tube add 1 cc of the standard nitrogen solution containing 0.1 mg nitrogen and dilute to 5 cc with distilled water. To both standard and unknown add 2 drops of the

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acetate buffer mixture and a piece of urease paper. Stopper and set aside for 30 minutes at room temperature, during which time the tubes are frequently shaken to set free the urease from the paper. At the end of this period add 1 cc of gum acacia solution to each tube and dilute to about 20 cc with distilled water. Then to each tube add 2 cc of Nessler's solution, dilute to the 25-cc mark, and compare in the colorimeter.

(2) The protection against precipitation afforded by the gum acacia is of limited duration and therefore the reading in the colorimeter must be made at once upon the addition of the Nessler's solution to the unknown. No precipitation or change will occur in the standard, consequently a series of unknowns may be read against the same standard, provided that each unknown is Nesslerized and diluted to the 25-cc mark individually just before matching.

c. Calculation.—The reading of the standard, usually 10 mm, multiplied by 20 and divided by the reading of the unknown, gives the urea nitrogen in milligrams per 100 cc of blood. If it is desired to convert this figure to that for urea, multiply by 2.143.

103. Blood urea clearance (Moller, McIntosh, and Van Slyke).—By blood urea clearance is meant the efficiency with which the kidney removes urea from the blood for excretion in the urine. The necessary data are the concentrations of urea in blood and urine and the volume of urine excreted in a measured period of time.

a. Reagents.—Those used in method given in paragraph 78*b*.

b. Procedure.—(1) The subject is not submitted to any previous routine except that vigorous exercise is avoided and the previous meal should be a moderate one, preferably without tea or coffee, which may increase the blood urea clearance. The most desirable period of the day when excretion is least liable to fluctuations is the time between breakfast and lunch. It is well to give a glass of water before the beginning of the test. The subject remains quiet while the urine is collected during two successive periods of about 1 hour each. The length of period, over or under 1 hour, is not essential so long as it is exactly measured and the *urine output per minute* is calculated. One of the chief sources of error may be the possibility of incomplete emptying of the bladder, either at the beginning or end of the period. The collection of two urine specimens affords a check on this factor. A few minutes before the end of the first hour a blood sample is drawn, oxalate being used to prevent coagulation. Its urea content is used for calculation of the clearance during both periods. This usage is permissible inasmuch as under the conditions of this test the blood urea does not change to any extent during 1 hour.

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(2) A protein-free blood filtrate is prepared in the usual manner. Of the urine collected during each hour, place about 10 cc in a small Erlenmeyer flask with 2 gm of permutit and rotate 5 minutes to absorb ammonia. Filter through a dry filter. Place in a 100-cc cylinder a volume of permutit urine (1, 2, or more cc) such that when later diluted acording to the dilution table (table VII) the resultant volume will be between 50 and 100 cc. The urine is then diluted to the volume called for in table VII. A portion of the diluted urine, 2 to 5 cc, is pipetted into a volumetric flask and diluted to 10 times its volume in order to parallel the dilution of the blood. The urea is now determined on a 5-cc portion of the blood filtrate and a similar amount of urine, by the method given. Instead, however, of matching the blood and urine against a standard nitrogen solution the urine is matched directly against the blood.

c. Calculation.—(1) If the colors of the two filtrates are such that the higher scale reading does not exceed twice the lower, the clearance is calculated directly from the colorimeter scale readings. Percent of average normal clearance equals $\frac{100\ (B)}{(U)}$ where B = reading of the blood filtrate and U = reading of the urine.

(2) If the subject is a child or an adult who deviates markedly in size from normal, the volume to which the urine must be diluted as indicated in table VII is corrected by multiplying by a factor obtained from the following formula:

$$\frac{\text{Volume} \times 1.73}{\text{Square meters of body surface}}$$

(3) The determination of the urea is then repeated, diluting the blood filtrate 2, 3, 5, or 10 times as indicated by the preliminary reading, in order to bring the (B)/(U) ratio in the neighborhood of unity. Then, percent of average normal clearance= $\frac{100\ (B)}{D\ (U)}$. D equals the number of times the blood filtrate has been diluted before final Nesslerization in cases where a low clearance necessitates such dilution. If the subject is known to have a low clearance, the time required for the preliminary reading can usually be saved by dilution of the blood filtrate to the probable extent necessary before the initial reading.

(4) The number of times the urine must be diluted for comparison with the blood is found by reference to the following table:

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TABLE VII.—*Dilution table*

Volume per hour in cc	Number of times to be diluted	Volume per hour in cc	Number of times to be diluted	Volume per hour in cc	Number of times to be diluted
20	94	58	55	165	27. 1
21	92	60	54	170	26. 3
22	89	62	53	175	25. 5
23	87	64	52	180	24. 8
24	84. 8	66	51. 5	185	24. 1
25	83	68	50. 8	190	23. 6
26	82	70	50	195	23
27	80. 5	72	49. 5	200	22. 4
28	79	74	48. 8	210	21. 5
29	77. 5	76	48	220	20. 5
30	76	78	47. 3	230	19. 5
31	75	80	46. 7	240	18. 8
32	74	82	46. 2	250	18
33	73	84	45. 5	260	17. 2
34	71. 8	86	45. 1	270	16. 7
35	70. 8	88	44. 7	280	16
36	70	90	44. 1	290	15. 5
37	69	92	43. 8	300	15
38	68	94	43. 2	310	14. 5
39	67. 2	96	42. 6	320	14. 1
40	66. 2	98	42. 1	330	13. 7
41	65. 5	100	41. 7	340	13. 2
42	64. 8	105	40. 8	350	12. 8
43	64	110	40	360	12. 5
44	63. 3	115	39	370	12. 2
45	62. 5	120	37. 2	380	11. 9
46	61. 8	125	35. 9	390	11. 5
47	61	130	34. 5	400	11. 2
48	60. 2	135	33	410	11
49	59. 6	140	32	420	10. 8
50	59	145	31. 1	430	10. 6
52	58	150	30	440	10. 3
54	57	155	29	450	10
56	56	160	28		

NOTE.—If the clearance is less than half average normal, the blood filtrate, when Nesslerized, will be more than twice as deep in color as the urine filtrate. In such a case, the preliminary (B)/(U) is made in the above manner.

104. Creatinine (Folin and Wu).—*a. Reagents.*—(1) *Picric acid, saturated solution.*—(*a*) Most of the U. S. P. picric acid furnished produces considerable color with alkali, which is very undesirable. Certain chemically pure supplies of picric acid may be used without purifying. The grade usually supplied may be purified

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by various methods, of which the simplest is by recrystallization from acetic acid.

(*b*) Dissolve 100 gm of picric acid, previously dried at 80° to 90° C., in 150 cc of glacial acetic acid in an Erlenmeyer flask with the aid of heat. Continue the heating on an electric hot plate until the solution boils. Filter hot through a fluted filter paper in a dry funnel which has been heated previously. Collect the filtrate in a dry beaker and cover with a watch glass. Let stand overnight at room temperature. If crystallization does not occur, seed with a small crystal of pure picric acid. After crystallization is complete, filter through a Büchner funnel, by suction, using a hardened filter paper. Wash in the funnel with about 35 cc of cold glacial acetic acid. Suck as free from acid as possible and dry at 80° to 90° C., with occasional stirring, until free from the odor of acetic acid. Conduct all operations in a good current of air. Weigh the purified, dried picric acid and add 10 percent by weight of distilled water. Picric acid containing this amount of water is perfectly safe, while dry picric acid is very explosive. Since a saturated solution in water is used, the added water makes no difference.

(2) *Sodium hydroxide*, 10 percent solution.

(3) *Standard creatinine solution.*—A stock solution is first prepared by dissolving 1.6106 gm of creatinine zinc chloride in 1 liter of 0.1 N hydrochloric acid. The working standard is prepared by diluting 3 cc of the stock standard to 500 cc with 0.01 N hydrochloric acid. Transfer to a bottle and add 4 or 5 drops of toluene or xylene. Five cubic centimeters of this solution contains 0.03 mg of creatinine.

b. Procedure.—(1) Place 10 cc of the protein-free blood filtrate, corresponding to 1 cc of blood, in a small flask or in a test tube. Place 5 cc of the standard creatinine solution, containing 0.03 mg of creatinine, in another small flask, and dilute to 20 cc with distilled water. Place 25 cc of a saturated solution of picric acid in another small flask and add 5 cc of a 10 percent solution of sodium hydroxide, mixing thoroughly. Then add 5 cc of the alkaline picrate solution, freshly prepared as above, to the blood filtrate, and 10 cc to the diluted creatinine solution. Let stand 8 to 10 minutes and compare in the colorimeter. The readings should be completed within 15 minutes from the time the alkaline picrate solution was added to the filtrate and standard.

(2) The creatinine standard solution is so made that 5 cc contain 0.03 mg of creatinine, and this amount plus 15 cc of water, represents the standard needed for the vast majority of human bloods, for it covers the range from 1 to 2 mg per 100 cc of blood. In the case of bloods representing retention of creatinine take 10 cc of the stand-

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ard plus 10 cc of water, which covers the range from 2 to 4 mg of creatinine per 100 cc of blood; or 15 cc of the standard plus 5 cc of water, by which 4 to 6 mg per 100 cc of blood can be estimated. By taking the full 20-cc volume from the standard solution, at least 8 mg can be estimated; but when working with such bloods, it is better to substitute 5 cc of the blood filtrate plus 5 cc of water for the usual 10 cc of filtrate.

c. Calculation.—(1) The reading of the standard, usually 20 mm, multiplied by 1.5, 3, 4.5, or 6 depending upon whether 5, 10, 15, or 20 cc, respectively, of the standard solution were used, and divided by the reading of the unknown, gives the creatinine in milligrams per 100 cc of blood.

(2) When the amount of the blood filtrate available for the creatinine determination is too small to permit a repetition, it is advantageous to start with more than one standard. If, however, a high creatinine should be encountered unexpectedly without several standards ready, the determination can be saved by diluting the unknown with an appropriate amount of the alkaline picrate solution, using for such dilution a picrate solution first diluted with 2 volumes of water, so as to preserve equality between the standard and the unknown in regard to the concentration of picric acid and sodium hydroxide.

105. Uric acid (Benedict).—*a. Reagents.*—(1) *Standard solution of uric acid.*—(*a*) A stock is first prepared. Transfer exactly 1 gm of uric acid to a small funnel on a liter volumetric flask. Transfer from 0.45 to 0.5 gm of lithium carbonate to a 300-cc beaker, add 150 cc of distilled water and heat to 60° C., shaking or stirring until all the carbonate has dissolved. With the hot carbonate solution rinse the uric acid into its flask and shake. The uric acid dissolves practically at once. As soon as a clear solution is obtained, cool under running water, with shaking, and add distilled water to a volume of 500 cc. Add 25 cc of formaldehyde, and after shaking to insure thorough mixing, acidify by the addition of 3 cc of glacial acetic acid. Shake to remove most of the carbon dioxide, dilute to volume, and mix. Fill up to the neck a series of small bottles, 100 to 150 cc, cork very tightly, label with the date, and keep in a cool, dark place. Stored in this manner the stock solution keeps indefinitely.

(*b*) The working standard is prepared in the following manner: Transfer with an Ostwald pipette 1 cc of the stock solution above, containing 1 mg of uric acid, to a 250-cc volumetric flask. Half fill the flask with distilled water and add 10 cc of the 0.66 N sulfuric acid used in the blood precipitation. Mix and add 1 cc of 40 percent

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formaldehyde. Mix, dilute to volume, and remix. This standard contains 0.02 mg of uric acid per 5 cc and should be prepared fresh every 2 weeks.

(2) *Sodium cyanide.*—Five percent solution containing 2 cc of concentrated ammonia per liter. This should be prepared fresh once a month and when not in use should be kept in the refrigerator.

(3) *Arsenic-phosphoric-tungstic acid.*—Place 100 gm of pure sodium tungstate in a liter flask and dissolve in about 500 cc of distilled water. Add 50 gm of pure arsenic pentoxide (As_2O_5), 25 cc of 85 percent phosphoric acid, and 20 cc of concentrated hydrochloric acid. Boil the mixture for 20 minutes, cool, and dilute to 1 liter with distilled water. This reagent will keep for 1 year at room temperature.

b. Procedure.—(1) Place 5 cc of the protein-free blood filtrate, corresponding to 0.5 cc of blood, in a test tube, and add 5 cc of distilled water; place 5 cc of the uric acid standard containing 0.02 mg of uric acid in a second test tube, and add 5 cc of distilled water; then to each tube add 4 cc of the 5 percent sodium cyanide solution containing ammonia.

(2) The test tubes need not be graduated but should be of uniform size and diameter. Before proceeding further, one should make sure that the water bath is boiling, and that a vessel of cold water is also at hand.

(3) Then to the tube containing the standard add 1 cc of the arsenic-phosphoric-tungstic acid reagent, mix the contents of the tube by inverting once, and quickly immerse in the boiling water. Immediately add 1 cc of the same reagent to the unknown and after mixing by one inversion, quickly immerse this tube also in the boiling water.

(4) The tubes must be placed in the boiling water *immediately* after the addition of the arsenic-phosphoric-tungstic acid reagent; delay here is fatal to the determination, as it makes for the development of turbidity. The time elapsing between the immersion of the two tubes must not exceed 1 minute; a longer interval results in disproportionate color development; and this latter fact makes it better when there are a number of filtrates to be examined, to limit the number run against one standard preparation to three or, at most, four.

(5) Let the tubes remain in the boiling water for exactly 3 minutes from the time of immersion of the last tube; longer heating tends to fade the color; then remove and place in a large beaker of cold water; let stand in the cold water for 3 minutes and compare in the colorimeter.

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(6) Benedict advises making the color comparisons within 5 minutes after removing from the cold water, stating that long standing before reading may lead to development of turbidity. Perhaps more important is the immediate immersion of the tubes in the boiling water bath after adding the reagent.

c. Calculation.—Using the weaker standard, as in the above technic, the reading of the standard, usually 20 mm, multiplied by 4, and divided by the reading of the unknown, gives the uric acid in milligrams per 100 cc of blood.

106. Sugar (Folin and Wu).—*a. In blood.*—(1) *Reagents.*—(*a*) *Stock sugar solution.*—One percent anhydrous dextrose in 0.25 percent benzoic acid. This solution, when prepared with 0.25 percent benzoic acid, will not decompose. Two working standards are prepared from this stock standard. The weaker solution contains 0.1 mg of sugar per cubic centimeter and is prepared by diluting 5 cc of the stock solution to 500 cc with 0.25 percent benzoic acid. The stronger standard is prepared by diluting 5 cc of the stock solution to 250 cc with 0.25 percent benzoic acid solution.

(*b*) *Alkaline copper solution.*—Dissolve 40 gm of pure anhydrous sodium carbonate in about 400 cc of distilled water in a liter flask. Add 7.5 gm of tartaric acid, and when the latter has dissolved, add 4.5 gm of crystallized copper sulfate. Mix and make up to the liter mark with distilled water.

(*c*) *Molybdate-phosphate solution.*—Place 35 gm of molybdic acid and 5 gm of sodium tungstate in a liter beaker. Add 200 cc of 10 percent sodium hydroxide solution and 200 cc of distilled water. Boil vigorously for 20 to 40 minutes. Cool, dilute to about 350 cc with distilled water, and add 125 cc of concentrated (85 percent) phosphoric acid. Dilute to 500 cc with distilled water.

(2) *Procedure.*—(*a*) Place 2 cc of the protein-free blood filtrate, corresponding to 0.2 cc of blood, in a special blood-sugar test tube; these tubes are graduated at 25 cc and are constricted toward the bottom so as to form a bulb which will contain 4 cc, this amount of fluid rising to just within the constricted portion of the tube. In two other similar tubes place 2 cc of the standard sugar solutions containing, respectively, 0.2 and 0.4 mg of dextrose. To each of the three tubes add 2 cc of the alkaline copper solution.

(*b*) The surfaces of the mixtures must now have reached the constricted parts of the tubes and must not lie above these parts. Tubes of either too large or too small capacity should be discarded; the surface of the mixture should lie within the constriction.

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(*c*) Transfer the tubes to a boiling water bath, and heat for 6 minutes; then transfer to a cold water bath and allow to cool, without shaking, for 2 or 3 minutes.

(*d*) Add to each tube 2 cc of the molybdate-phosphate solution. The cuprous oxide dissolves rather slowly if the amount present is large; but the whole, up to the quantity given by 0.8 mg of dextrose, dissolves usually within 2 minutes.

(*e*) When the cuprous oxide is dissolved, dilute the resulting blue solutions to the 25-cc mark with a 1+4 dilution of the molybdate-phosphate reagent, and mix each tube thoroughly by inverting several times, using care to insure complete mixing, as the greater part of the color is developed in the bulb of the tube. Compare in the colorimeter, using the standard which more nearly approximates the unknown.

(3) *Calculation.*—When the weaker standard is used, that is, the one containing 0.2 mg of dextrose, the reading of the standard, usually 20 mm, multiplied by 100 and divided by the reading of the unknown, gives the sugar in milligrams per 100 cc of blood. When the stronger standard is used, substitute 200 for the 100 in the preceding.

b. In cerebrospinal fluid (*Lyttle and Hearn*).—(1) *Reagents.*—Solutions required are the same as those used in the Folin-Wu method for the precipitation of blood proteins and determination of blood sugar.

(2) *Procedure.*—Four volumes of spinal fluid are added to 14 volumes of distilled water, and to this mixture 1 volume of 10 percent sodium tungstate is added, followed by 1 volume of 0.66 N sulfuric acid. Shake and allow to stand for 10 minutes, then filter. The sugar determination is carried out on 2 cc of this filtrate in exactly the same manner as in determining blood sugar.

(3) *Calculation.*—Where the lower standard has been used, the reading of the standard, usually 20 mm, multiplied by 50 and divided by the reading of the unknown equals milligrams of sugar per 100 cc of spinal fluid. When the higher standard is used, substitute 100 for the 50 above.

107. Glucose tolerance test.—Following the ingestion of a definite amount of glucose, blood sugar is determined at intervals. Urine specimens taken at the same time, are tested for glucose and, if positive, the amount present is determined.

a. Reagents.—Those for blood sugar and urine sugar determination and, in addition, glucose. The dosage used at present is 100 gm regardless of body weight, except in children and in persons differing markedly from normal in stature and general muscular build. It is

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given in 50 percent solution to the fasting patient. Lemon juice makes the sugar solution more palatable.

b. Procedure.—Obtain blood and urine specimens on the fasting patient. Give the glucose solution and note the time. Half an hour, 1 hour, 2 hours, and 3 hours after the ingestion of the glucose, take blood and urine specimens. Determine the blood sugar on each specimen and test all urines for glucose. Determine the urinary glucose in any positive specimens.

c. Result.—Record the blood and urine glucose for the fasting, half-hour, 1-hour, 2-hour, and 3-hour specimens, and also the amount of glucose given.

108. Chlorides (Whitehorn).—*a. Reagents.*—(1) *Standard silver nitrate* (M/35.46 solution).—Dissolve 4.791 gm of C. P. silver nitrate in several hundred cubic centimeters of distilled water in a liter volumetric flask. Make up to 1 liter with distilled water, mix thoroughly and preserve in a brown bottle. One cc of this solution corresponds to 1 mg of chlorine.

(2) *Standard potassium or ammonium thiocyanate* (M/35.46 solution).—Dissolve 3 gm of potassium thiocyanate (KCNS) or 2.5 gm of the ammonium salt (NH_4CNS) in a liter of distilled water. Titrate against 10 cc of the standard silver nitrate solution using about 0.3 gm of powdered ferric ammonium sulfate as an indicator; adjust the solution as may be indicated by the titration, so that it will exactly correspond in strength to the silver nitrate solution. The end point in this titration should be the same as that used in the determination of urine chlorides.

(3) *Powdered ferric ammonium sulfate* ($FeNH_4(SO_4)_2 \cdot 12H_2O$).

(4) *Concentrated nitric acid.*—Specific gravity 1.42; halogen-free.

b. Procedure.—Pipette 10 cc of the protein-free blood filtrate, corresponding to 1 cc of blood, into a porcelain dish. Add 5 cc of the standard silver nitrate solution and stir thoroughly; add about 5 cc of the concentrated nitric acid, mix, and let stand for 5 minutes to permit flocking out of the silver chloride. It is to be noted that the silver nitrate and nitric acid are not added to the filtrate simultaneously; to do so may result in the mechanical enclosure of silver nitrate solution within the curds, and a consequent error in the positive direction. Then with a spatula add an abundant amount, about 0.3 gm, of the ferric ammonium sulfate, and titrate the excess of silver nitrate with the standard thiocyanate solution until the definite salmon-red, not yellow, color of the ferric thiocyanate persists in spite of stirring, for at least 10 seconds.

c. Calculation.—(1) Each cubic centimeter of the thiocyanate solution corresponds to 1 cc of the silver nitrate solution; therefore, the

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amount of thiocyanate used represents the excess of silver nitrate remaining after the reaction with chloride was complete. Since 5 cc of silver nitrate were originally used, the difference between 5 and the amount of thiocyanate used for the titration, that is, 5 minus the titer, gives the amount of silver nitrate solution which was used up in the chloride precipitation. Since this solution was so made that 1 cc of it corresponds to 1 mg of chlorine, this figure (5 minus the titer) gives the *chlorine* in milligrams in the filtrate used, representing 1 cc of blood.

(2) It is preferable to report chlorides as *sodium chloride*, rather than as chlorine; and the chlorine value above is converted to NaCl by dividing by 0.606. This latter figure must be multiplied by 100 to give the value per 100 cc of blood.

(3) The actual calculation can be shortened to the following: 5 minus the titer, in cubic centimeters, multiplied by 165, will give the chlorides, as NaCl in milligrams per 100 cc of blood.

109. Cholesterol (Leiboff).—*a. Reagents.*—(1) *Standard solution of cholesterol in chloroform.*—A stock solution is first prepared by dissolving 0.160 gm of pure cholesterol in 100 cc of redistilled chloroform. The working standard is made by diluting 5 cc of the stock standard to 100 cc with chloroform. Five cc equal 0.4 mg of cholesterol. Preserve in dark glass bottles, preferably in the refrigerator.

(2) *Acetic anhydride.*—If not clear and colorless it may be purified by redistillation.

(3) *Concentrated sulfuric acid.*

(4) *Asbestos cloth, medium weave.*—As purchased, this cloth is quite dirty. Cut into strips and extract in a Soxhlet apparatus for an hour or two with chloroform. Dry thoroughly and cut into 2-cm squares.

b. Procedure.—Place a square of asbestos cloth in the special Leiboff extraction tube. From a pipette drop 0.25 cc of oxalated blood onto it, slowly. Dry in a vacuum desiccator over calcium chloride for an hour. Introduce 5 cc of chloroform into the extraction tube, connect to a reflux condenser, immerse in a beaker of water heated over a hot plate, and allow to extract for 1 hour. Detach the tube, remove the cloth, allow to cool to room temperature, and add chloroform to the 5-cc mark. In another tube place 5 cc of the standard cholesterol solution. To both standard and unknown add 2 cc of acetic anhydride and 0.1 cc of concentrated sulfuric acid. Mix and place in a beaker of water at a temperature of 20° to 25° C., in a dark cabinet and allow to remain 30 minutes for the color

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to develop. At the end of this period compare in the colorimeter with the standard set at 15 mm.

c. Calculation.—(1) Twenty-four hundred divided by the reading of the unknown equals milligram of cholesterol per 100 cc of blood.

Caution: The color developed by the action of the acetic anhydride and sulfuric acid on cholesterol in chloroform solution changes rapidly on removal from the water bath and exposure to light, and since the change in the standard is not at the same rate as the unknown, it is essential that the matching be made as rapidly as possible, within 2 minutes from the time the solutions are removed from the dark cabinet. If a series of unknowns are to be read, and this cannot be done in 2 minutes, it is necessary to prepare another standard.

(2) This reaction is also seriously affected by moisture. It is, therefore, essential that all pipettes, tubes, colorimeter cups, and plungers be absolutely dry throughout the procedure.

110. Inorganic phosphorus (Fiske and Subbarow).—*a. Reagents.*—(1) *Sulfuric acid* (10 N).—Four hundred and fifty cc of concentrated sulfuric acid added to 1,300 cc of water.

(2) *Molybdic acid solution.*—Dissolve 25 gm of ammonium molybdate in 200 cc of water. Rinse into a liter volumetric flask containing 300 cc of 10 N sulfuric acid. Dilute to the mark with water and mix.

(3) *Trichloracetic acid*, 10 percent solution.

(4) *Standard phosphate solution.*—A stock standard is prepared by dissolving 0.3509 gm of pure monopotassium phosphate (KH_2PO_4) in 1 liter of distilled water. Add 10 cc of chloroform as a preservative and keep in the refrigerator. To prepare the working standard transfer 10 cc of the stock standard to a 100-cc volumetric flask, add 80 cc of 10 percent trichloracetic acid, and dilute to the mark with distilled water. Five cc equal 0.04 mg of phosphorus.

(5) *Amino-naphthol-sulfonic acid reagent.*—Dissolve 30 gm of sodium bisulfite ($NaHSO_3$) and 1 gm of crystalline sodium sulfite ($Na_2SO_3 \cdot 7H_2O$), or 0.5 gm of anhydrous sodium sulfite, in 200 cc of distilled water. Add 0.5 gm of purified 1, 2, 4-amino-naphthol-sulfonic acid and stir thoroughly. Preserve in a dark glass bottle. This reagent should be prepared freshly once a month. The sediment that forms will settle to the bottom and need not be filtered out if care is taken not to stir it up when pipetting.

b. Procedure.—Either serum or plasma is used in the determination, and there must be no hemolysis present. Transfer 8 cc of trichloracetic acid to a small Erlenmeyer flask. While the flask is

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being gently rotated, run in 2 cc of the serum or plasma from an accurate pipette. Close the mouth of the flask with a rubber stopper and shake vigorously a few times. Filter through an ashless filter such as Whatman No. 42. Transfer 5 cc of the filtrate to a tube graduated at 10 cc. Into a similar tube measure 5 cc of the standard phosphate solution. To both tubes add 1 cc of the molybdic acid reagent and 0.4 cc of the sulfonic acid reagent. Dilute to the mark with distilled water, mix thoroughly, allow to stand 10 minutes, and compare in the colorimeter.

c. Calculation.—The reading of the standard, usually 20 mm, multiplied by 4 and divided by the reading of the unknown equals milligrams of inorganic phosphorus per 100 cc of blood.

111. Phosphatase (Bodansky method, modified).—*a. Reagents.*—Those used for phosphorus determination above and in addition:

(1) *Sodium beta-glycerophosphate solution.*—Make by dissolving 1.5 gm in 50 cc of water.

(2) *Barbital solution.*—Dissolve 1.030 gm of sodium barbital in 50 cc of water.

NOTE.—These solutions must be prepared the same day they are to be used.

(3) *Trichloracetic acid*, 50 percent solution.

b. Procedure.—(1) *Standard.*—Place 1 cc of the stock standard phosphate solution in a small flask or test tube; add 4 cc of barbital solution and 2 cc of distilled water.

(2) *Control.*—In a similar flask or tube place 2 cc of clear, unhemolyzed serum, 4 cc of barbital solution, and 1 cc of water.

(3) *Unknown.*—Similarly, place 1 cc of serum, 2 cc of barbital solution, and 5 cc of water in a flask or tube.

(4) Place standard, control, and unknown in a beaker or glass filled with water at 37.5° C. to a depth which will cover the mixture in the tubes. Then add to the "unknown" 1 cc of the glycerophosphate solution and place the beaker in an incubator at 37.5° C. If available, a water bath set at the same temperature may be used, the glycerophosphate being added to the unknown just as it is placed in the bath. Incubate for exactly 2 hours. At the end of this period, add to each tube 1 cc of the 50 percent trichloracetic acid solution. Then to the "standard" and "control," add 2 cc of the glycerophosphate solution. Filter each mixture through Whatman No. 42 paper, collecting the filtrate in clean tubes. Carry each filtrate through the phosphorus determination as given in the preceding section, using 5 cc of each respective filtrate, 1 cc of molybdic acid solution, and 0.4 cc of the sulfonic acid reagent. Dilute to 10 cc with water, mix, and after

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standing for 10 minutes, compare the unknown and the control against the standard.

c. Calculation.—(1) The reading of the standard, usually 20 mm, multiplied by 4 and divided by the reading of the control gives the milligrams of inorganic phosphorus per 100 cc of serum (same as in par. 110).

(2) The reading of the standard times 8, divided by reading of the unknown, gives the milligrams of inorganic phosphorus originally present plus that converted from the organic state by the action of the enzyme per 100 cc of mixture.

(3) The difference between the values for unknown and control gives the phosphatase units, one unit being that amount which will liberate 1 milligram of inorganic phosphorus per 100 cc of serum from the organic state in 2 hours at 37.5° C. Since normal values differ according to the method used, it is essential in reporting to record the normal values of the method used. Upper limits of normal according to the method given here are: for adults, 10 units; for children, 20 units.

112. Calcium (Roe and Kahn).—*a. Reagents.*—In addition to the solutions required for phosphorus determinations, the following are required:

(1) *Standard calcium solution.*—Prepare a stock solution by dissolving 0.4991 gm of pure calcium carbonate in about 50 cc of 10 percent trichloracetic acid in a 1,000-cc volumetric flask. Iceland spar is preferred, if available. Shake well, and when evolution of CO_2 has ceased, dilute to the mark with 10 percent trichloracetic acid. To prepare the working standard transfer 10 cc of the stock solution to a 100-cc volumetric flask, add 70 cc of 10 percent trichloracetic acid, and dilute to the mark with distilled water. Five cc equal 0.1 mg of calcium.

(2) *Alkaline alcohol wash reagent.*—In a 100-cc cylinder place 58 cc of 95 percent ethyl alcohol, add 10 cc of amyl alcohol, and make up to 100 cc with distilled water. Add 2 drops of 1 percent phenolphthalein and then 5 percent sodium hydroxide, a drop at a time, with repeated shaking until a distinct pink is obtained.

(3) *Sodium hydroxide*, 25 percent solution.

(4) *Trisodium phosphate*, Na_3PO_4, 5 percent solution.

b. Procedure.—(1) Two cc of serum are precipitated with 8 cc of 10 percent trichloracetic acid in the same manner as in the phosphorus determination. Where phosphorus and calcium are to be determined on the same specimen, sufficient filtrate for both determinations may be obtained by precipitating 3 cc of serum with 12 cc of trichloracetic acid.

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(2) To a graduated centrifuge tube, transfer 5 cc of the filtrate and to another similar tube transfer 5 cc of the standard calcium solution. The tips of the centrifuge tubes used must be sufficiently narrow so that the diameter at the 0.1 cc mark will not exceed 7 mm but must not be too finely drawn out. They must be absolutely clean and when not in use should be kept immersed in the dichromate sulfuric acid cleaning solution. No reliance should be placed on the graduation marks, as they have been found to be inaccurate in many tubes and the 10 cc mark should be checked. If found inaccurate, a new mark should be made. To both tubes add 1 cc of 25 percent NaOH, mix by twirling, and allow to stand for 5 minutes. Then add 1 cc of the trisodium phosphate solution and allow to stand for 1 hour to complete precipitation of the calcium phosphate.

(3) Centrifugalize for 2 minutes. Decant the supernatant fluid with one smooth movement that will not disturb the precipitate. With the mouth still inverted, the tube is placed upon a clean filter paper and allowed to drain several minutes, after which time any remaining fluid which may have collected on standing is removed from within the mouth of the tube by touching it with a slip of filter paper or by a clean piece of gauze. Add from a pipette about 3 cc of alkaline alcohol wash reagent in such a manner as to break up the mat of $Ca_3(PO_4)_2$ in the bottom of the tube. This is done by using a bulb pipette with a fine tip and blowing forcefully, directing the stream upon the calcium phosphate precipitate. If the calcium phosphate mat is not broken up completely by this procedure, it must be fragmented thoroughly with a clean glass stirring rod. The walls of the tube are now washed down with an additional 2 cc of the alkaline alcohol wash reagent. The tubes are centrifugalized again for 2 minutes, then decanted and drained as above.

(4) Redissolve the precipitate in both standard and unknown in 4 cc of 10 percent trichloracetic acid; add to each tube 1 cc of the molybdic acid reagent, the same as is used in the phosphorus determination, and 0.4 cc of the sulfonic acid reagent, also the same as for the phosphorus determination. Dilute to the 10-cc mark with distilled water, mix, allow to stand 10 minutes, then compare in the colorimeter.

c. Calculation.—(1) The reading of the standard multiplied by 10 and divided by the reading of the unknown equals the milligrams of calcium per 100 cc of blood.

Caution: This reaction is a reaction for phosphorus based upon the amount of phosphorus contained in the calcium phosphate precipitate. It has been found impossible to obtain reagents which are absolutely calcium-free or free of other substances which would also give

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color. Therefore, a standard calcium solution is prepared and treated in the same manner throughout as the blood filtrate. Consequently, any error produced in the blood calcium determination as a result of color-producing substances in the reagents used will be exactly balanced by a similar error in the standard used, provided the standard is treated in the same manner as the unknown and with the same reagents. Most filter paper contains traces of calcium. It is therefore necessary to use calcium-free filter paper in filtering the proteins from the blood. A double acid-washed paper, such as Whatman No. 42, has been found to meet these requirements. As a result of the action of the 25 percent NaOH and 5 percent trisodium phosphate on the glass bottles, a precipitate of silicates will form which will give a color. If kept in tall bottles, the precipitate will settle to the bottom, and the clear supernatant fluid can then be pipetted from the top, otherwise it is necessary to filter both solutions just before use.

(2) In previous methods calcium was determined by precipitation of the oxalate from the diluted blood serum without prior removal of the blood serum proteins. It has been found that when serum is so treated, about 5 to 15 percent of the calcium fails to be precipitated, consequently, when the precipitation is carried out on the protein-free trichloracetic filtrate, the results will average about 10 percent higher than those usually published as normal based upon the usual precipitation from the blood serum in the presence of the proteins. The method herein described gives results similar to those obtained with the oxalate precipitation on the protein-free trichloracetic filtrate. The normals are consequently higher than usually published, being 10 to 12 mg per 100 cc of blood instead of 9 to 11 mg.

(3) Should it be necessary to send specimens for calcium and phosphorus determination to a distant laboratory, clear serum may be sent for calcium, but since the action of the enzyme phosphatase continues in the serum, changing organic to inorganic phosphorus, only the trichloracetic acid filtrate is suitable for this purpose. Since both Ca and P are usually determined together, the filtrate is sent in for both.

113. Bile pigment in serum.—*a. Icterus index (Bernheim).*—(1) *Reagents.*—(*a*) *Potassium dichromate*, 1:10,000 solution. Dissolve 0.1 gm of chemically pure potassium dichromate in about 500 cc of distilled water in a liter volumetric flask. Add 4 drops of concentrated sulfuric acid and dilute to the mark with distilled water. Keep in a dark-glass bottle in the dark. This is the standard against which the color of the serum is matched.

(*b*) *Sodium chloride*, 0.9 percent solution.

(2) *Procedure.*—Accurately dilute 1 cc of clear serum with the

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sodium chloride solution until its color matches the standard approximately. This dilution may be 1:2, 1:5, 1:10, or even more. Place the standard in one colorimeter cup and set it at 15 mm. Place the serum, undiluted or diluted as necessary, in the other cup and match against the standard.

(3) *Calculation.*—The reading of the standard, 15 mm, divided by the reading of the serum, times the dilution, if any, gives the icterus index.

NOTE.—Since this test is simply a measurement of the color of the serum, it is evident that even the slightest trace of hemolysis will vitiate the result and it is of utmost importance that hemolysis be avoided. The needle and syringe used should be entirely dry. The blood is allowed to clot in a dry centrifuge tube, protected from the light, and then centrifugalized so as to obtain a clear serum.

Carrots in the diet impart a yellow color to the blood serum which gives a high index. No carrots should be eaten the day preceding the test, and the blood should be drawn before breakfast to avoid chyle.

b. Determination of bilirubin (Gibson and Goodrich modification of van den Bergh method).—This quantitative method supersedes the older direct, indirect, and quantitative van den Bergh reactions. It has been found that the three types of direct van den Bergh reaction, thought due to differences in the chemical or physical character of the bilirubin in the blood, are in reality due to its concentration. The present method compares the color of diazotized serum or plasma with a standard solution of diazotized bilirubin.

(1) *Reagents.*—(*a*) *Sulfanilic acid solution.*—Dissolve 1 gm of sulfanilic acid in 15 cc of concentrated hydrochloric acid and dilute to 1 liter with distilled water.

(*b*) *Sodium nitrite*, 0.5 percent solution, freshly prepared.

The finished reagent, hereafter called diazo reagent, is prepared by mixing 25 cc of solution (*a*) and 0.75 cc of solution (*b*) immediately before use.

(*c*) *Ammonium sulfate*, saturated aqueous solution.

(*d*) *Ethyl alcohol*, 95 percent.

(*e*) *Hydrochloric acid*, concentrated.

(*f*) *Bilirubin standard.*—Dissolve 10 mg of pure bilirubin in 10 to 20 cc of water with the aid of a few drops of 10 percent sodium hydroxide and make up to 100 cc with water. Add 50 cc of freshly prepared diazo reagent, 100 cc of saturated ammonium sulfate, and 400 cc of alcohol. When the red color has developed add 100 cc of concentrated hydrochloric acid and let stand at least 3 hours before using. If kept in the refrigerator this standard will keep 3 months. Smaller proportionate amounts may be used in preparing this standard.

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(2) *Procedure.*—Serum or plasma from oxalated blood may be used but there must be no hemolysis and the test should be done as soon as possible after the blood is drawn. To 2 cc of serum or plasma in a 15-cc centrifuge tube add 1 cc of freshly prepared diazo reagent and mix. The appearance of a red color at this point indicates a "direct" reaction. Add 2 cc of saturated ammonium sulfate solution, 8 cc of alcohol, and mix. When the red color has developed, add 2 cc of concentrated hydrochloric acid, mix, and let stand several minutes. Centrifugalize (or filter), pour the clear supernatant into a colorimeter cup and compare with the bilirubin standard.

(3) *Calculation.*—The reading of the standard, times 10, divided by the reading of the unknown equal milligrams bilirubin per 100 cc of serum or plasma. One "unit" of bilirubin is 1 part in 200,000 or 0.5 mg per 100 cc.

NOTE.—The normal icterus index is from 4 to 6. Latent jaundice (hyperbilirubinemia without apparent jaundice) gives readings between 6 and 15. With an icterus index of 15 and over, visible jaundice occurs.

By the quantitative test, normal serum contains from 0.2 to 0.5 units of bilirubin (0.1 to 0.25 mg per 100 cc), and the serum of cases of latent jaundice contains from 0.5 to 2 units (0.25 to 1.0 mg of bilirubin per 100 cc).

114. Blood proteins (Andersch and Gibson modification of method of Wu and Ling).—*a. Determination of serum proteins.*—Ordinarily it is the serum proteins which are determined, that is, albumin and globulin. The method given below determines total protein and albumin directly, the globulin being found by difference. When it becomes necessary to determine fibrinogen, oxalated plasma must be used. The fibrinogen is precipitated as fibrin and separated from the plasma solution. It is then determined directly as given under *b* below.

(1) *Reagents.*—(*a*) *Trichloracetic acid*, 20 percent solution.

(*b*) *Sodium hydroxide*, 10 percent.

(*c*) *Sodium chloride*, 0.9 percent.

(*d*) *Standard tyrosine solution.*—Dissolve 200 mg of pure dry tyrosine in 1 liter of 0.1 N HCl.

(*e*) *Saturated sodium carbonate solution.*

(*f*) *Saturated ammonium sulfate solution*, 56 gm per 100 cc.

(*g*) *Phenol reagent of Folin and Ciocalteu.*—Transfer 100 gm of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$) and 25 gm of sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) together with 700 cc of water to a 1,500-cc Florence flask. Add 50 cc of 85 percent phosphoric acid and 100 cc of concentrated hydrochloric acid. Connect with a reflux condenser by means of a cork or rubber stopper wrapped in tinfoil and boil gently for 10 hours. At the end of the boiling period add 150 gm of lithium

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sulfate, 50 cc of water, and a few drops of bromine. Boil the mixture without the condenser for about 15 minutes to remove the excess bromine. Cool, dilute to 1 liter, and filter. The finished reagent should have no greenish tint, as this means the presence of blue reduction products which will lessen the range of true proportionality.

(2) *Procedure for total serum proteins.*—The serum is diluted 1 to 10 with 0.9 percent NaCl. One cubic centimeter of this dilution is transferred to a 15-cc centrifuge tube and 4 cc of water added, followed by 1 cc of the 20 percent trichloracetic acid. The precipitate is centrifugalized out and the supernatant fluid discarded. The precipitate is dissolved in 0.5 cc of 10 percent NaOH and heated in a boiling water bath for 30 minutes. Two cubic centimeters of the standard tyrosine solution are measured into a centrifuge tube similar to the one containing the unknown. Both tubes should be accurately graduated at the 10-cc mark. Dilute both standard and unknown to the 5-cc mark with distilled water and add 1 cc of the phenol reagent and 3 cc of saturated sodium carbonate solution. Make up both to the 10-cc mark with distilled water, mix, and compare in the colorimeter after standing for 30 minutes.

(3) *Procedure for albumin.*—To 1 cc of serum add 4 cc of water and 5 cc of saturated ammonium sulfate solution and place in an incubator at 37° C. for 15 minutes, then filter through a fine filter paper such as Whatman No. 42; if the filtrate is not clear, return to the paper. To 2 cc of the filtrate in a centrifuge tube accurately graduated at 10 cc, add 3 cc of water and 1 cc of the 20 percent tricloracetic acid. Centrifugalize, pour off the supernatant fluid, dissolve the precipitate in 0.5 cc of 10 percent NaOH, heat in the boiling water bath for 30 minutes, and then develop the color and compare against a standard in the same manner as described above for total protein.

(4) *Procedure for globulin.*—The percent of globulin is obtained by subtracting the albumin from the total protein.

(5) *Calculations.*—(*a*) *For total protein.*—The reading of the standard, usually 10 mm, multiplied by 5.2 and divided by the reading of the unknown equals the percent of total protein.

(*b*) *For albumin.*—The reading of the standard multiplied by 2.58 and divided by the reading of the unknown equals the percent of albumin.

(*c*) *For globulin.*—Percent of total protein minus percent of albumin equals percent of globulin.

b. Determination of fibrinogen.—(1) *Reagents.*—The same as in *a* above.

(2) *Procedure.*—To 1 cc of plasma from oxalated blood in a 15-cc

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centrifuge tube, add 2 cc of water and 1 cc of saturated ammonium sulfate solution. Mix, let stand for a few minutes, and then centrifugalize to throw down the precipitated fibrinogen (fibrin). Pour off the supernatant fluid completely. Dissolve the fibrin precipitate in 0.5 cc of 10 percent sodium hydroxide and heat in the boiling water bath for 30 minutes. For the standard, place 2 cc of the tyrosine solution in another centrifuge tube. Dilute both standard and unknown to the 5-cc mark, add to each 1 cc of the phenol reagent, and then 3 cc of saturated sodium carbonate solution. Make both up to the 10-cc mark with water, mix, and let stand for 30 minutes. Compare in the colorimeter, with the standard set at 10 mm.

(3) *Calculation.*—The reading of the standard, times 0.52, divided by the reading of the unknown equals percent of fibrinogen.

NOTE.—When fibrinogen, albumin, and globulin are desired on the same case, secure enough oxalated blood to give at least 2 cc of clear plasma. Determine fibrinogen as above on a 1-cc portion. Treat another 1-cc portion exactly as serum is treated in *a* above. In this case the total protein equals albumin plus globulin plus fibrinogen. Having the figures for total protein, albumin, and fibrinogen, the globulin is found by difference.

115. Alkali reserve, direct method, carbon dioxide capacity of blood plasma (Van Slyke and Cullen).—The plasma from oxalated blood is shaken in a separatory funnel filled with an air mixture whose carbon dioxide tension approximates that of normal arterial blood, by which treatment it combines with as much carbon dioxide as it is able to hold under normal tension. A known quantity of the saturated plasma is then acidified within a suitable pipette, and its carbon dioxide is liberated by the production of a partial vacuum. The liberated carbon dioxide is then placed under atmospheric pressure, its volume accurately measured, and the volume corresponding to 100 cc of plasma calculated.

a. Reagents and apparatus.—(1) *Apparatus.*—(*a*) The apparatus used is illustrated in figure 11. It is made of strong glass in order to withstand the weight of the mercury without danger of breaking, and is held in a strong clamp, the jaws of which are lined with rubber. In order to prevent accidental slipping of the apparatus from the clamp, an iron rod of 6- or 8-mm diameter should be so arranged as to project under the cock *f* between *c* and *d*. Three hooks or rings at the levels 1, 2, and 3 serve to hold the leveling bulb at different stages of the analysis. The bulb is connected with the bottom of the apparatus by a heavy-walled rubber tube.

(*b*) It is necessary that both stopcocks be properly greased and absolutely airtight; and it is also essential that they (especially *f*) should be held in place so that they cannot be forced out by pressure of the mercury. Rubber bands may be used for this purpose, but it

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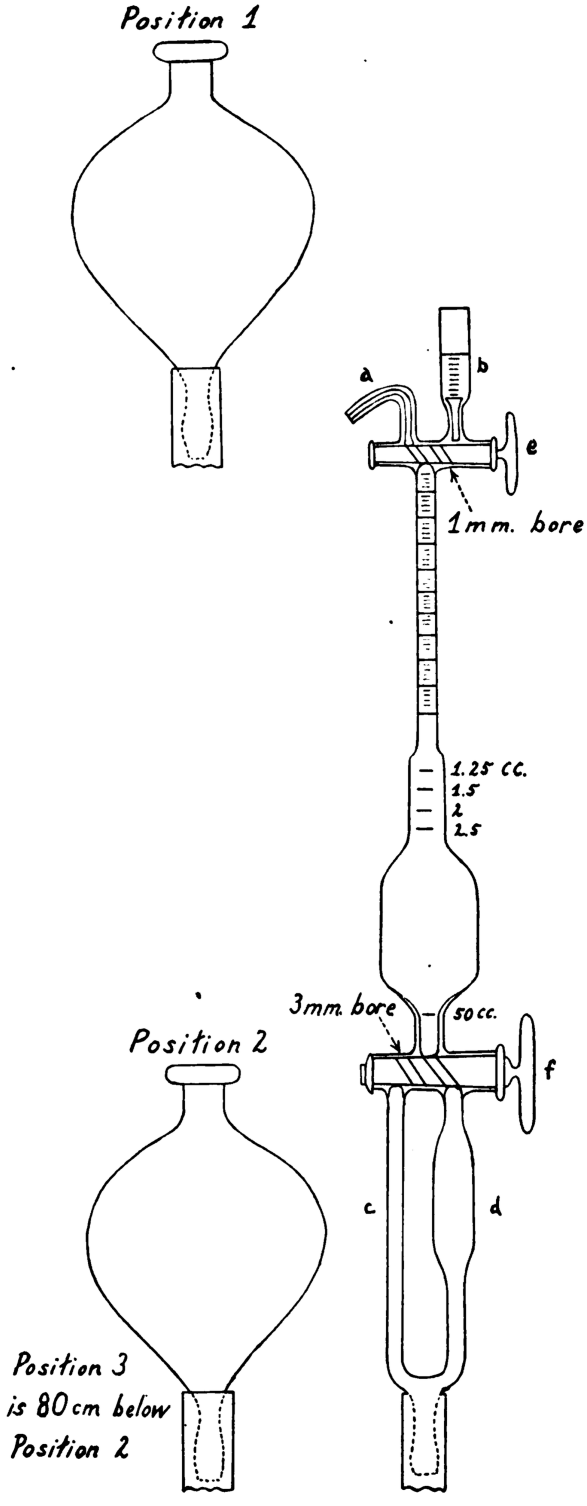


FIGURE 11.—Van Slyke CO_2 apparatus.

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has been found that elastic cords of fine wire spirals, applied in the same manner as the rubber bands, are stronger and more durable.

(*c*) Before the apparatus has been used, or whenever air has been admitted to it, as in renewing mercury or, perhaps, through leaks, the rubber tubing and even the glass walls may contain considerable amounts of gases; these must be evacuated before using it. To test the apparatus for tightness and freedom from gases, raise the leveling bulb and completely fill the apparatus with mercury. The bulb is then lowered to position 3, so that a Torricellian vacuum is obtained, the mercury falling to about the middle of *d*; the bulb is then raised again. If the apparatus is tight and gas-free, the mercury will refill it completely and will strike the upper cock with a sharp click. If there is any gas in the apparatus, it will act as a cushion; the click will not be heard and a bubble will remain above the mercury. In this case expel the collected gas through cock *e*, and repeat the procedure until all the gas has been evacuated. After the apparatus has been freed from gases, it can be used repeatedly and indefinitely without further trouble from this source, if no air is admitted and there is no leak. It is always desirable, however, before making the first determination of a series, to test the apparatus as described above.

(*d*) After a determination is finished, the leveling bulb is lowered without opening the upper cock, and most of the mercury is withdrawn from the pipette through *c*. The water solution from *d* is readmitted, and, with leveling bulb raised to position 1, the water solution together with a little mercury is forced out of the apparatus through *a*. The apparatus may then be used immediately for another determination; the slight amount of the old solution which remains on the inner surface of the pipette is negligible; if it is to be set aside it is well to rinse it several times with water, and then to leave it completely filled with mercury. If the apparatus is used only occasionally, it is well to stopper lightly both the cup *b* and the mercury bulb.

(2) *Reagents.*—(*a*) *Sulfuric acid*, 5 percent.

(*b*) *Ammonium hydroxide* (carbonate-free).—Dilute 1 cc of 28 percent ammonium hydroxide to 100 cc with distilled water. Ordinary ammonia water can be made carbonate-free by adding a small amount of saturated barium hydroxide solution. Filter off the barium carbonate formed and precipitate the excess of barium with a little ammonium sulfate.

(*c*) *Caprylic alcohol.*

b. Procedure.—(1) *Drawing blood.*—(*a*) Without using a tourniquet, draw about 6 to 7 cc of venous blood into a centrifuge tube containing a very little powdered potassium oxalate. Use 20 mg for 10 cc of blood, and not more; less should be used if less blood is drawn. The

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estimation may be made with as little as 2 or 3 cc of blood, using only 0.5 cc of plasma, but the larger quantity of blood, allowing 1 cc of plasma to be used, is preferable. The addition to the oxalate in the tube, before the blood is drawn, of sufficient paraffin oil to cover the quantity of blood taken is also recommended but if the determination is proceeded with promptly, and if only the very minimum of shaking or stirring sufficient to mix the blood and oxalate is done, the paraffin oil can be omitted except at higher altitudes.

(*b*) The tube and contents are then centrifugalized to obtain the separated plasma and the determination continued.

(*c*) If it is desired to keep the plasma for the estimation of the carbon dioxide at a later time, it should be transferred to a paraffined tube, covered with paraffin oil, stoppered lightly, and kept cold. Under these conditions, if sterile, it may be kept for over a week without alteration of its carbon dioxide capacity.

(2) *Saturation of plasma with carbon dioxide.*—(*a*) After centrifugalization, about 3 cc of the plasma are transferred to a 300-cc

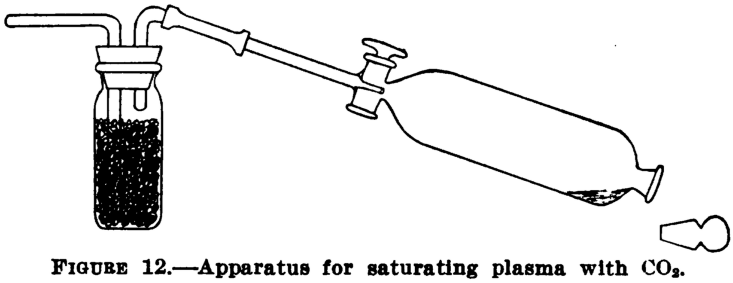


FIGURE 12.—Apparatus for saturating plasma with CO_2.

separatory funnel; the air within the funnel is displaced either by alveolar air from the lungs of the operator or 5.5 percent carbon dioxide mixture from a tank. In either case the gas mixture must be passed over moist glass beads before it enters the funnel; this may be done conveniently by the use of some such apparatus as is shown in figure 12.

(*b*) When alveolar air is used, the operator, without inspiring more deeply than normal, expires as quickly and as completely as possible through the glass beads and separatory funnel. The stopper of the funnel should be inserted just before the expiration is finished, so that no air will be drawn back into the funnel; the funnel cock is then closed. In order to saturate the plasma, the separatory funnel is slowly turned for 2 minutes, distributing the plasma in a thin layer as completely as possible over the interior surface of the funnel. After saturation is complete, the stoppered funnel is placed upright and allowed to stand until the fluid has drained from the walls into the bottom.

(3) *Determination of carbon dioxide.*—(*a*) Before beginning a determination, it is necessary that the entire apparatus (fig. 11), includ-

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ing the chamber *d*, the communicating tube *c*, and the pipette proper, be filled with mercury to the top of both capillary tubes above the cock *e*; this is done by raising the mercury bulb with the cock open, and closing the cock when the mercury reaches the proper level. Then, using a cotton-covered applicator, wash out the cup *b* with the 1 percent carbonate-free ammonia solution and remove any excess of the ammonia with a dry applicator. This alkaline treatment of the cup is essential to prevent any premature liberation of the carbon dioxide from the plasma, especially since the last solution added in any preceding determination was the 5 percent sulfuric acid.

(*b*) Then, the tight and gas-free apparatus being filled with mercury and the cup *b* washed with ammonia as described above, 1 cc of plasma, accurately pipetted, is placed in the cup, the tip of the pipette remaining below the surface of the plasma as it is added. With the mercury bulb at position 2 and the cock *f* in the position shown in figure 11, the plasma is admitted from the cup into chamber, leaving just enough above the cock *e* to fill the capillary so that no air will be introduced when the next solution is added. The cup is then washed with two portions of about 0.5 cc of water, each portion being admitted to the pipette in turn. A small drop of caprylic alcohol is then permitted to flow entirely into the capillary above *e*, and finally, 0.5 cc of 5 percent sulfuric acid is run in.

(*c*) It is not necessary that exactly 1 cc of wash water and 0.5 cc of acid shall be taken but the total volume of the water solution introduced must extend exactly to the 2.5-cc mark on the apparatus; this can readily be accomplished by placing slightly more than sufficient acid solution in the cup, and then drawing the solution exactly down to the mark.

(*d*) After the acid has been added, the cock *e* is closed and a drop of mercury is placed in the cup and allowed to run down the capillary as far as the cock in order to seal the latter. Whatever excess of sulfuric acid remains is then removed by a cotton-covered applicator.

(*e*) If the amount of plasma available is small, a little more than 0.5 cc is saturated with carbon dioxide in a 50-cc funnel, and exactly 0.5 cc used for the determination. In this case the quantities of water and acid used to wash the plasma into the apparatus are halved, so that the total volume of water solution introduced is only 1.25 cc and the observed volume of gas is multiplied by 2 before proceeding with the calculation.

(*f*) The mercury bulb is now lowered and hung at position 3 and the mercury in the pipette allowed to run down to the 50-cc mark, producing a Torricellian vacuum in the apparatus. When the mercury meniscus has fallen to the 50-cc mark, the lower cock *f* is

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closed and the pipette removed from the stand. Equilibrium of the carbon dioxide between the 2.5 cc of water solution and the 47.5 cc of free space is obtained by turning the pipette upside down 15 or more times, thus thoroughly agitating the contents. The pipette is then replaced in the clamp.

(*g*) By opening the cock *f*, the water solution is now allowed to flow from the pipette completely into the chamber *d* without, however, allowing any of the gas to follow it. The leveling bulb is then raised in the left hand, while with the right the cock *f* is turned so as to connect the pipette with *c*. The mercury flowing in from *c* fills the body of the pipette and as much of the calibrated stem at the top as is not occupied by the gas extracted from the solution. A few hundredths of a cubic centimeter of water, which could not be completely drained into *d*, floats on top of the mercury in the pipette; the error caused by the reabsorption of carbon dioxide into this small volume of water is negligible if the reading is made at once. The mercury bulb is held at such a level that the gas in the pipette is under atmospheric pressure, that is, level with the top of the mercury column in the pipette stem, and the volume of gas read on the scale.

(*h*) In order to have the column in the pipette exactly balanced by that outside, the surface of the mercury in the leveling bulb should be raised until it is level with the mercury meniscus in the pipette and then, for entire accuracy, raised above the latter meniscus by a distance equal to one-thirteenth of the height of the column of water above the mercury in the pipette. As the water column should never be more than 10 mm high, the correction that has to be estimated is less than 1 mm of mercury, not enough to influence results appreciably.

c. Calculation.—In the above procedure, not only is the volume of carbon dioxide set free from bicarbonate by acidification read, but also included are the air and carbon dioxide physically in solution in the plasma and water. It is only the carbon dioxide bound as bicarbonate which measures the alkali reserve, hence the values for the dissolved gases must be subtracted. It is also necessary to reduce the volume of CO_2 as observed to standard temperature of 0° C. and pressure of 760 mm of mercury. This has all been done and table VIII by Van Slyke and Cullen comprises all the necessary corrections. To use the table multiply the observed volume of CO_2 by the observed barometric pressure divided by 760. Opposite this figure in the left-hand column of the table, find the corrected value under the proper temperature column. This gives the cubic centimeters of carbon dioxide, reduced to 0° C., and 760 mm of mercury

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pressure, bound as bicarbonate in 100 cc of plasma; that is, the combining power of the plasma in "volumes percent."

TABLE VIII.—*Table for calculation of carbon dioxide combining power of plasma (Van Slyke and Cullen)*

Observed volume gas $\times \frac{B}{760}$	Cc of CO_2 reduced to 0° C., 760 mm, bound as bicarbonate by 100 cc of plasma				Observed volume gas $\times \frac{B}{760}$	Cc of CO_2 reduced to 0° C., 760 mm, bound as bicarbonate by 100 cc of plasma			
	15°	20°	25°	30°		15°	20°	25°	30°
0.20	9.1	9.9	10.7	11.8	0.60	47.7	48.1	48.5	48.6
1	10.1	10.9	11.7	12.6	1	48.7	49.0	49.4	49.5
2	11.0	11.8	12.6	13.5	2	49.7	50.0	50.4	50.4
3	12.0	12.8	13.6	14.3	3	50.7	51.0	51.3	51.4
4	13.0	13.7	14.5	15.2	4	51.6	51.9	52.2	52.3
5	13.9	14.7	15.5	16.1	5	52.6	52.8	53.2	53.2
6	14.9	15.7	16.4	17.0	6	53.6	53.8	54.1	54.1
7	15.9	16.6	17.4	18.0	7	54.5	54.8	55.1	55.1
8	16.8	17.6	18.3	18.9	8	55.5	55.7	56.0	56.0
9	17.8	18.5	19.2	19.8	9	56.5	56.7	57.0	56.9
0.30	18.8	19.5	20.2	20.8	0.70	57.4	57.6	57.9	57.9
1	19.7	20.4	21.1	21.7	1	58.4	58.6	58.9	58.8
2	20.7	21.4	22.1	22.6	2	59.4	59.5	59.8	59.7
3	21.7	22.3	23.0	23.5	3	60.3	60.5	60.7	60.6
4	22.6	23.3	24.0	24.5	4	61.3	61.4	61.7	61.6
5	23.6	24.2	24.9	25.4	5	62.3	62.4	62.6	62.5
6	24.6	25.2	25.8	26.3	6	63.2	63.3	63.6	63.4
7	25.5	26.2	26.8	27.3	7	64.2	64.3	64.5	64.3
8	26.5	27.1	27.7	28.2	8	65.2	65.3	65.5	65.3
9	27.5	28.1	28.7	29.1	9	66.1	66.2	66.4	66.2
0.40	28.4	29.0	29.6	30.0	0.80	67.1	67.2	67.3	67.1
1	29.4	30.0	30.5	31.0	1	68.1	68.1	68.3	68.0
2	30.3	30.9	31.5	31.9	2	69.0	69.1	69.2	69.0
3	31.3	31.9	32.4	32.8	3	70.0	70.0	70.2	69.9
4	32.3	32.8	33.4	33.8	4	71.0	71.0	71.1	70.8
5	33.2	33.8	34.3	34.7	5	71.9	72.0	72.1	71.8
6	34.2	34.7	35.3	35.6	6	72.9	72.9	73.0	72.7
7	35.2	35.7	36.2	36.5	7	73.9	73.9	74.0	73.6
8	36.1	36.6	37.2	37.4	8	74.8	74.8	74.9	74.5
9	37.1	37.6	38.1	38.4	9	75.8	75.8	75.8	75.4
0.50	38.1	38.5	39.0	39.3	0.90	76.8	76.7	76.8	76.4
1	39.1	39.5	40.0	40.3	1	77.8	77.7	77.7	77.3
2	40.0	40.4	40.9	41.2	2	78.7	78.6	78.7	78.2
3	41.0	41.4	41.9	42.1	3	79.7	79.6	79.6	79.2
4	42.0	42.4	42.8	43.0	4	80.7	80.5	80.6	80.1
5	42.9	43.3	43.8	43.9	5	81.6	81.5	81.5	81.0
6	43.9	44.3	44.7	44.9	6	82.6	82.5	82.4	82.0
7	44.9	45.3	45.7	45.8	7	83.6	83.4	83.4	82.9
8	45.8	46.2	46.6	46.7	8	84.5	84.4	84.3	83.8
9	46.8	47.1	47.5	47.6	9	85.5	85.3	85.2	84.8
0.60	47.7	48.1	48.5	48.6	1.00	86.5	86.2	86.2	85.7

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116. Carbon monoxide.—*a. Carbon monoxide in blood* (*Sayers, Yant, and Jones*).—This method depends upon the fact that carbon monoxide, CO, combines with the hemoglobin of the blood, displacing the oxygen and changing oxyhemoglobin, HbO, to carbon monoxide hemoglobin, HbCO. The blood under examination is treated with absolutely fresh pyrotannic acid solution, which in the presence of CO develops a specific color. This color is compared with a set of standards representing various degrees of saturation with CO from 0 percent to 100 percent in steps of 10.

(1) *Reagents.*—(*a*) *Pyrogallol*, 2 percent aqueous solution.

(*b*) *Tannic acid*, 2 percent aqueous solution.

Equal volumes of (*a*) and (*b*) are mixed just prior to use.

(*c*) *Preparation of standards.*—Five cubic centimeters or more of human or animal blood are drawn and kept from clotting by the addition of 50 mg of potassium citrate per 10 cc of blood. This blood is divided into equal parts, one of which is immediately diluted 1 to 10 with distilled water forming solution No. 1, which is all oxyhemoglobin; the other is saturated with CO by bubbling ordinary illuminating gas through it. Where illuminating gas is not available, CO may be generated by heating sulfuric acid with oxalic acid, passing the gas produced through a solution of sodium hydroxide to remove the carbon dioxide, before passing it through the blood. The saturated blood is now diluted 1 to 10 with distilled water, forming solution No. 2, all carbon monoxide hemoglobin.

From solution No. 1 and solution No. 2, mixtures are made in small test tubes, approximately 8 mm in diameter, which total 1 cc but vary from 0 percent to 100 percent HbCO in steps of 10. For example: To the first tube 1 cc of the oxyhemoglobin, solution No. 1 only, is added; to the second tube 0.9 cc of solution No. 1 and 0.1 cc of solution No. 2; to the third tube 0.8 cc of solution No. 1 and 0.2 cc of solution No. 2, and so on in each of the succeeding tubes, solution No. 1 diminishing by 0.1 cc in each tube, and solution No. 2 increasing by 0.1 cc in each tube until the last tube which contains 1 cc of solution No. 2 only and will represent 100 percent HbCO. Thus, the percentage of HbCO increases from 0 percent in the first tube, by steps of 10 percent in each succeeding tube, to 100 percent in the last tube.

To each standard thus prepared add 1 cc of the mixture of equal parts of strictly fresh solutions of 2 percent pyrogallic acid and 2 percent tannic acid, after which the tube is inverted twice to insure thorough mixing. The tube should be sealed immediately by pouring a little melted paraffin on top of the contents while immersed in cold water to prevent overheating. When the walls of the tube be-

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come dry the remainder of the tube should be filled with ordinary sealing wax. Care should be taken to exclude all air. These standards develop their full color in about 30 minutes, and if kept in a cool, dark place, will keep for 2 weeks or more.

(2) *Procedure.*—Collect 0.1 cc of blood from the tip of the finger and dilute with 0.9 cc of distilled water in a test tube of the same size used in the preparation of the standards. Add 1 cc of the strictly fresh pyrotannic acid mixture used in the preparation of the standards. This mixture must be made fresh every day. Invert the tube several times. Place in a rack and allow to stand for 15 minutes, at the end of which period comparison is made with the standards by interposing between them until the standard is found which most nearly matches. If CO is indicated the tube should be allowed to stand for 15 minutes longer and another reading made.

(3) *Calculation.*—The percentage of HbCO is estimated from the value of the standard most closely matched.

b. Carbon monoxide in air (Sayers, Yant, and Jones).—(1) *Reagents.*—Same as in *a* above.

(2) *Procedure.*—Fresh human or animal blood is obtained and diluted 1 to 10 with distilled water. Two cubic centimeters of this solution are then introduced into a sample bottle containing the air to be analyzed, the manipulation being such as to allow as little of the air as possible to escape. The sample bottle is a 250-cc bottle or flask in which the air to be analyzed is collected by inserting the glass tube on the end of a scrubber containing soda lime and pumping air through the scrubber and bottle by means of an aspirating bulb. At the last squeeze of the bulb, the glass tube is quickly removed and a rubber stopper inserted in the bottle.

The bottle containing the blood is equilibrated by being rotated constantly for 15 to 20 minutes, as much as possible of the surface of the bottle being covered with the blood solution. When this equilibration has been finished, 1 cc of the equilibrated blood solution is placed in a test tube, 1 cc of fresh pyrotannic acid mixture added, and the comparison of colors and determination of HbCO made according to the procedure for blood.

(3) *Calculation.*— $\frac{\text{HbCO}}{100-\text{HbCO}} \times \frac{2093}{300} =$ parts of CO per 10,000 parts of air.

Example: A blood solution equilibrated with a gas sample was found to contain 50 percent HbCO by comparison with the standards. Substituting this value in the above equation gives the following:

$\frac{50}{100-50} \times \frac{2093}{300} = 7$ parts CO per 10,000 parts of air.

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The equilibration of the blood with the air sample should be done at a temperature not varying more than 3° C. plus or minus from 20° C. (5° plus or minus from 68° F.). Within this range the temperature correction is so small as to be negligible.

117. Ethyl alcohol in blood, urine, or spinal fluid.—*a. General.*—(1) The method used is a modification of that originally devised by the French toxicologist, Nicloux. It consists of the oxidation of the ethyl alcohol by means of potassium dichromate and sulfuric acid with the coincident reduction of the dichromate to chromium sulfate to a degree corresponding to the amount of alcohol oxidized. The chromium salt is green in color, and with small quantities of alcohol the intensity of the green color measures exactly the amount of alcohol oxidized.

(2) Various methods for preparing the standards have been used in the past, all of them having certain objectionable features. A combination of methods has been worked out obviating some of the earlier difficulties, using a stronger reagent than that given in various laboratory manuals and journals. For that reason, the Anstie's reagent as given here, called "Anstie's reagent, modified, stronger" must not be confused with that used in other methods. This newer reagent is about 11 percent stronger than the older modified reagent. The new reagent may be converted into the older one by diluting 9 parts of the new reagent with 1 part of water.

b. Reagents.—(1) *Anstie's reagent, modified, stronger.*—Dissolve 3.70 gm of chemically pure reagent quality potassium dichromate in 150 cc of distilled water. Add slowly, with constant stirring, 280 cc of concentrated sulfuric acid, chemically pure. Dilute to 500 cc with distilled water.

(2) *Standard alcohol solution.*—Place about 50 cc of distilled water in a 100-cc volumetric flask. To it add 2.53 cc of absolute ethyl alcohol by means of an accurately graduated pipette, keeping the pipette tip near the surface of the water to prevent loss of alcohol by evaporation. Make up to 100 cc with distilled water.

(3) *Scott-Wilson reagent.*—(*a*) Mercuric cyanide, 5 gm dissolved in 300 cc of water.

(*b*) Sodium hydroxide, 90 gm dissolved in 300 cc of water.

(*c*) Silver nitrate, 1.45 gm dissolved in 200 cc of water.

Add (*b*), thoroughly cooled to (*a*); mix thoroughly, then add (*c*) to the mixture with constant stirring. This solution will keep for 6 months. If it becomes cloudy, or a precipitate forms, filter it. *Never pipette this solution—it is very poisonous.*

(4) *Color comparison standards.*—(*a*) Arrange 9 test tubes of uniform diameter and color (color comparison tubes are better) in a test

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tube rack and place 9 cc of Anstie's reagent in each tube. Then add to each tube the standard alcohol solution and distilled water in the amounts shown in table IX.

TABLE IX.—*Alcohol standards*

Tube number	Alcohol solution (cc)	Distilled water (cc)	Corresponds to alcohol in the specimen (mg per cc)	Tube number	Alcohol solution (cc)	Distilled water (cc)	Corresponds to alcohol in the specimen (mg per cc)
1	None	1.0	Negative	6	0.5	0.5	2.5
2	0.1	.9	0.5	7	.6	.4	3.0
3	.2	.8	1.0	8	.7	.3	3.5
4	.3	.7	1.5	9	.8	.2	4.0
5	.4	.6	2.0				

(*b*) The contents of each tube must then be thoroughly mixed. This may be accomplished by drawing the contents of each tube up into a 10-cc pipette and allowing it to run back into the tube several times.

(*c*) These standards may be kept for several weeks if tightly stoppered and kept in a vertical position in a test tube rack. The solutions in the standards must not come into contact with the stoppers, as both cork and rubber stoppers contain reducing substances which may cause a change in color of the standards.

(*d*) Each standard should be labeled with the number of milligrams of alcohol to which it corresponds (e. g., tube No. 1 should be labeled "negative," tube No. 5 should be labeled "2.0 mg" etc.).

(*e*) It is useless to try to make standards for readings greater than 4.0 mg of alcohol per cubic centimeter, as the Anstie's reagent is apparently completely changed by that amount of alcohol, and no difference can be detected between the 4.0, the 4.5, and the 5.0 mg per cubic centimeter standards no matter by which method they are prepared. Should specimens be encountered which have 4.0 mg of alcohol per cubic centimeter, or more, a second determination should be made using half the quantity of the specimen (2 cc instead of 4 cc) and the result multiplied by two to give the final reading.

c. Procedure—urine, blood, or spinal fluid.—(1) Arrange two 25- by 210-mm tubes with two-holed rubber stoppers and inlet and outlet tubes. The inlet tubes should extend nearly to the bottom of the tube and the outlet tube just below the stopper. Using well-washed rubber tubing, connect the inlets and outlets in such a manner

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that a current of air may be aspirated through the specimen tube over into the tube containing Anstie's reagent.

(2) In the specimen tube place 4 cc of specimen, 2 to 4 cc of Scott-Wilson reagent, and sufficient water to make 10 cc. Halfway between the upper level of the fluid contents and the bottom of the stopper place a wad of glass wool.

(3) In the second tube place 10 cc of the Anstie's reagent.

(4) Stopper the tubes, and adjust the suction so that a reasonable current of air is aspirated through the tubes. Immerse the tubes in a water bath previously brought to boiling. Continue the boiling and aspiration for 12 to 15 minutes.

(5) Cool the dichromate solution and compare with the standards which read directly in milligrams per cubic centimenter.

d. Result.—Report in milligrams per cubic centimeter as read from the standard matched, each milligram per cubic centimeter corresponding to tenths of percent.

118. Sulfonamides in blood or urine (method of Marshall and Litchfield, modified).—The methods for sulfanilamide, sulfapyridine, sulfathiazole, and sulfaguanidine are the same except for the preparation of standards and the calculations. The procedure is based on the diazotization of the drug and subsequent coupling to form a colored compound which is compared colorimetrically with a standard treated in the same manner.

a. Sulfanilamide.—(1) *Reagents.*—(*a*) *Sodium nitrite*, 0.1 percent aqueous solution, freshly prepared each day.

(*b*) *Sodium phosphate, monobasic, and ammonium sulfamate solution.*—Dissolve 13.8 gm of monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$) and 0.5 gm of ammonium sulfamate in water and make up to 100 cc. This solution is one molal in sodium phosphate.

(*c*) *Dimethyl-a-naphthylamine*, 0.4 percent solution in 95 percent ethyl alcohol. Kept in a dark bottle in the refrigerator, this reagent will keep at least 6 months.

(*d*) *Trichloracetic acid*, 15 percent aqueous solution.

(*e*) *Saponin*, 0.05 percent aqueous solution.

(*f*) *Hydrochloric acid*, 2 N.

(*g*) *Stock sulfanilamide solution.*—Dissolve 100 mg of pure sulfanilamide crystals in water and make up to 1 liter. Do not use tablets.

(*h*) *Working standard.*—Dilute 10 cc of stock solution to 100 cc with water. Each cubic centimeter of the working standard contains 0.01 mg sulfanilamide.

(2) *Procedure for blood.*—Lake 1 volume of oxalated blood with

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15 volumes of water. Let stand 15 minutes. If the saponin solution is substituted for the distilled water, laking will be complete in 1 to 2 minutes. To the laked blood add 4 volumes of trichloracetic acid and after standing 5 minutes, filter.

(*a*) *Free sulfanilamide.*—To 10 cc of filtrate add 1 cc of the sodium nitrite solution and mix. After 3 minutes, add 1 cc of the sodium phosphate-ammonium sulfamate solution, mix, and let stand 2 minutes. Add 5 cc of the dimethyl-a-naphthylamine solution and after standing 10 minutes compare in the colorimeter with a standard prepared at the same time and treated in the same manner. For blood, two standards will cover the range of concentrations ordinarily encountered. The weaker standard consists of 2 cc of the working standard solution (total 0.02 mg sulfanilamide), 2 cc of trichloracetic acid solution, and 6 cc of water. In the stronger standard use 5 cc of working standard (0.05 mg sulfanilamide), 2 cc of trichloracetic acid, and 3 cc of water.

(*b*) *Total sulfanilamide.*—Treat 10 cc of blood filtrate prepared as above with 1 cc of 2 N hydrochloric acid in a boiling water bath for 1 hour. Cool and adjust the volume to 10 cc. Proceed as for free sulfanilamide except that a 2 molal sodium phosphate, 0.5 percent ammonium sulfamate solution (27.6 gm $N.H_2PO_4 \cdot H_2O$ to 100 cc) is used.

(*c*) *Acetylsulfanilamide.*—The difference between the total and the free gives the amount of conjugated acetylsulfanilamide.

(3) *Procedure for urine.*—Urine is diluted so as to contain 0.5 to 2.0 mg percent of sulfanilamide. A dilution of 1:100 usually is satisfactory. Treat 10 cc of diluted urine with 1 cc of the trichloracetic solution and then proceed in exactly the same manner as for blood. Two standards cover the usual range of concentration in the urine. The weaker consists of 5 cc of the working standard, 1 cc of trichloracetic acid, and 5 cc of water (sulfanilamide content, 0.05 mg). The stronger standard contains 10 cc of working standard and 1 cc of trichloracetic acid (0.1 mg sulfanilamide).

(4) *Calculations.*—In general the following formula applies:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \frac{\text{mg sulfanilamide}}{\text{in standard used}} \times \frac{\text{dilution of unknown}}{\text{cc of filtrate or dilution taken}} \times 100 = \frac{\text{mg per}}{100\text{ cc}}$$

With the procedure given above, the formula may be reduced to:

For blood: $\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 4$ (if weaker standard is used, 10 if stronger is used) $= \frac{\text{mg per 100}}{\text{cc}}$

For urine: $\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times 50$ (if weaker standard is used, 100 if stronger is used) $= \frac{\text{mg per 100}}{\text{cc}}$

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b. Sulfapyridine.—(1) *Reagents.*—Exactly the same as for sulfanilamide, *a* above.

(2) *Procedures for blood and urine.*—Exactly as for sulfanilamide and using the same sulfanilamide working standard.

(3) *Calculations.*—Since sulfapyridine has a lower color-producing value than the sulfanilamide in the standard used (0.8 compared with 1.0), the results must be multiplied by the factor 1.25 to give the true values.

c. Sulfathiazole.—The reagents, procedures, and calculations are exactly the same as those used in the sulfanilamide determinations except for the standard.

Since the color developed by sulfathiazole differs considerably in shade from that of sulfanilamide, the stock standard must be made by dissolving 100 mg of pure sulfathiazole crystals in water and making it up to 1 liter.

d. Sulfaguanidine.—The reagents, procedures, and calculations are exactly the same as for sulfanilamide except for the standard used. The stock standard should be prepared from pure sulfaguanidine crystals.

TABLE X.—*Normal values of blood*

(All amounts are in milligrams per 100 cc of whole blood unless otherwise stated. These values are for bloods taken in the morning after a fast of at least 10 hours.)

	Normal	Remarks
Nonprotein nitrogen	25 to 35	During digestion there is a rise of about 4 mg per 100 cc. Anything below 30 mg is to be considered normal, but values up to 35 mg are to be found without any evidence of kidney retention.
Urea nitrogen	10 to 15	During digestion of a full meal containing meat, a rise of 2 or 3 or more mg occurs. In the usual run of clinical cases, values as high as 20 mg may be encountered.
Creatinine	1 to 2	In a selected series of normals the upper limit may be as low as 1.7 mg; 2 mg is the more common upper limit of normal.
Uric acid	2 to 4	The figures given are based on Benedict's method which gives somewhat higher figures than does that of Folin and Wu. Values as high as 4.5 mg are frequently found in bloods, all the other values of which are well within normal range.

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TABLE X.—*Normal values of blood*—Continued

	Normal	Remarks
Sugar	80 to 120	During the absorptive period after food there is a marked increase, dependent on the carbohydrate content of the food. The extent of this rise after a standard carbohydrate meal is the basis of the "sugar tolerance test."
Chlorides	450 to 500	Figures for plasma are somewhat higher than those for whole blood: 575 to 625 mg per 100 cc.
Cholesterol	140 to 190	Blood gives an average figure of 210 mg per 100 cc and others regard the normal as lying even higher. It is probable, however, that 150 mg is a fairly representative normal standard.
Calcium	10 to 12	These values are for the serum alone and represent the total calcium present in the serum after clotting and separation of the clot.
Phosphorus	3 to 4.5	These values are for the inorganic phosphorus of the serum after separation from the clot.
Alkali reserve (CO_2 combining power of the blood plasma).	53 to 77 volumes percent.	Austin and Jonas state that the minimum normal should be regarded as 60 instead of 53 volumes percent. Certainly anything below 55 volumes percent may be regarded as pathological.
Bilirubin	0.1 to 0.25	One van den Bergh unit is equivalent to 1 part of bilirubin in 200,000 parts of serum, or 0.5 mg per 100 cc. Normal serum therefore contains 0.2 to 0.5 van den Bergh units.
Icterus index	4 to 6	An icterus index below the normal limit of 4 has so far been found only in cases of secondary anemia. An icterus index of 15 is necessary for jaundice to be evident clinically. Hence, an index between 6 and 15 is termed "latent jaundice."
Total serum proteins	6.5 to 8.0 percent.	Low in nephritis with edema.
Serum albumin	4.5 to 6.5 percent.	Low in nephrosis.

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TABLE X.—*Normal values of blood*—Continued

	Normal	Remarks
Serum globulin	1.5 to 2.5 percent.	In lipoid nephrosis the globulin is usually normal and the reduction is in serum albumin giving an inverse ratio.
Fibrinogen (plasma)	0.3 to 0.6 percent.	
Albumin-globulin ratio.	2 to 2.3	
pH of blood	7.35 ± 0.05	In extreme cases variations of 0.2 to 0.5 may occur

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CHAPTER 7

GENERAL BACTERIOLOGICAL METHODS

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SECTION I

GENERAL CHARACTERISTICS OF BACTERIA

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119. General.—*a.* Bacteria are minute, living, single-celled organisms of the vegetable kingdom which reproduce under suitable conditions with exceeding rapidity by transverse fission and grow without the aid of chlorophyl. They have no nucleus, but contain nuclear material diffused throughout the cell. They are characterized and differentiated by their size and shape (morphology), their manner of movement (motility), their staining peculiarities, conditions of growth, food requirements, results of growth, and their ability to produce disease.

b. They are so small as to be visible only by means of a high magnification of the compound microscope. A dust particle, a drop of saliva, a finger tip may harbor hundreds of assorted bacteria. A drop of pus may contain thousands of a single type.

c. They are single-celled, not having the complex structure of the higher forms of life with specialized tissues and organs of many individual cells. Bacteria are so simple in their apparent structure

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that the single cells carry on all of the life processes. Each cell is an individual, which can absorb nutriment, digest it, excrete the waste products, form new chemical compounds, move about to a certain extent (see flagella, par. 120*c*), protect itself (see capsule, spore, par. 120*c*), and under favorable conditions reproduce itself in a very simple way, by simple fission.

d. The classification of orders, families, genera, and species of bacteria is based upon morphological characteristics, and physiological, biochemical, immunological, and pathogenic properties.

e. Under favorable conditions micro-organisms retain most of their characteristics for a long time, but some of their characteristics, particularly their virulence, and even their form and colony appearance may become modified by their environment. Thus a pathogenic micro-organism propagated for many years in a test tube may lose its virulence, although other characteristics remain the same. Most of a micro-organism's process peculiarities may thus become modified or lost in the course of time, creating difficulties in accurately identifying and classifying the micro-organisms.

120. Morphology.—This refers to the physical characteristics (size, shape, and structure).

a. Size.—The size, being microscopic, is expressed in microns as the unit of measurement. It varies with genus and species from the very large forms of 40 to 50 microns in length to the very minute forms, hardly visible, even with the highest magnification of a compound microscope, 0.2 micron in diameter, even to species which are so small as to be invisible and therefore called "ultra-microscopic." The latter are so small that they pass through the pores of the finest earthen filters and are therefore called "filtrable." Although there is this great variation in sizes, there is usually a strict uniformity of size of all individuals of the same species.

b. Shape.—(1) The shape, also fairly uniform for each species, varies from round forms to long rod forms, with all intermediate shapes. The round forms are called "cocci" (singular coccus), the rods "bacilli" (bacillus), and the curved rod forms "spirilla" (spirillum). Of these, the bacilli are the most numerous, the cocci moderately so, and the spirilla least numerous among the bacterial species. There are still other shapes (spirochetes, yeasts, molds) among the higher forms of bacteria. Nothing is known about the shape of the ultra-microscopic forms, known as "filtrable viruses" (virus). Usually all individuals of a species are of the same shape; occasionally they show considerable variation in shape, being then spoken of as "pleomorphic."

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(2) The individuals, each a distinct living entity, may occur singly or grouped into pairs, cubes, chains, clusters, or packets. Certain descriptive terms are applied to express this grouping of cells: a coccus occurring alone is a "micrococcus," cocci in pairs are "diplococci," in groups of four "tetrads," in packets (cubes) of 8 or more "sarcinae," in chains "streptococci," and in clusters like a bunch of grapes "staphylococci;" bacilli occurring in chains may be called "streptobacilli," but these descriptive terms are less applied to bacilli and spirilla than to cocci.

c. Structure.—When examined, living, under the microscope, bacteria appear as colorless, refractile cells, sometimes with highly refractile areas or spores. Many bacteria possess a capsule, a gelatinous envelope around the cell demonstrable by special staining methods. The "vegetative stage" of bacteria is that stage of their life when they grow and multiply; most bacteria have only this one stage or form of life; a few bacterial species may assume another form to resist destruction, the "bacterial spore." Spores (condensed cell contents) are round or oval bodies appearing as bright, refractile, or specially staining bodies within the bacteria; even the body of the bacterial cell may disintegrate, leaving the spore apart. "Metachromatic granules" are granular fragments of the bacterial cell which stain deeper or differently than the balance of the cell; their presence is a peculiarity of some kinds of bacteria. "Flagella" are fine, hairlike appendages which give motility to some bacteria, particularly to bacilli and spirilla, never to cocci; flagella are not readily stained and will not be seen by ordinary staining methods.

121. Motility.—"Brownian movement" is the back-and-forth movement which all particles partake of in a liquid medium, the movement being magnified to the same extent as is the micro-organism so that when seen by the high magnification it may appear to be a considerable commotion. All micro-organisms, whether living or dead, as well as inert particles, when observed in a fluid, will have this type of movement. "Active motility" is the capacity for individual movement possessed by some living micro-organisms, by which they move around in the field more or less freely, dart across the microscopic field, and change their positions; this movement is accomplished by means of flagella; it occurs only in the case of living, active micro-organisms and is lost upon their death. Both types of movement can be observed only in fluid specimens; movement cannot be seen in specimens which have been fixed to a slide and stained.

122. Staining.—Most bacteria can be stained easily with simple stains (solutions of analine dyes); other bacteria can be stained with

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difficulty or not at all with simple stains and require a special staining technic. Simple stains are used in studying the morphology of bacteria; differential stains give us information of value in classifying bacteria, such as ease of staining and decolorization, color taken by the bacterium, and the bringing out of special morphological features (capsules, spores, flagella, granules, etc.). "Gram positive" bacteria retain the primary stain (blue) when stained by Gram's method; "Gram negative" bacteria take the color of the counterstain (red), having lost the primary stain during decolorization. An "acidfast" bacterium can be stained only with difficulty but when once stained retains the dye even when washed with acid-alcohol. Staining characteristics are constant for each species.

123. Growth and reproduction.—Under favorable conditions bacteria grow rapidly to a certain size, fairly constant for each species, and then divide by fission into two equal halves. The average time required for this process, under favorable conditions, is 20 minutes. A mass of individuals reproduced from a single organism is called a "colony." Spore formation is not reproduction but the formation of a resistant form of the organism. When a spore is placed into an environment favorable for growth, it germinates into the vegetative form which then proceeds to reproduction and colony growth. Several other modes of bacterial reproduction have been described.

124. Condition of growth.—Most pathogenic bacteria grow best at a temperature of 37° C. but will grow at temperatures 1° or 2° higher or several degrees lower. Many saprophytic bacteria will grow at 37° C. but most prefer and some require a lower temperature (20° C.). Some bacteria (thermophilic bacteria) grow at high temperatures (40° to 80° C.). Most bacteria will grow on simple culture media; some require special nutrient material such as blood, while others cannot be successfully cultivated. The results of the growth of bacteria in the presence of dyes, carbohydrates, and other chemicals vary for genera and species.

125. Morphology of colonies.—After incubation of a single bacterium on suitable media, the growth has developed in 24 hours or more to become visible to the eye and may be particularly studied by a low-power magnification. Colonies of bacteria differ in form, texture, size, shape, color, and the degree of adherence to the medium. The characteristics of colonies are fairly constant and are valuable aids in the differentiation of bacteria. Many conditions affect the morphology of colonies: the nature of the nutriment, degree of moisture of the medium, and the age of the culture. Other physical and chemical factors influence the form, texture, and color of colonies.

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a. Smooth and rough types.—A single species is capable of giving rise to two distinct types of colonies. The smooth or S-type of colonies has a dome shape with more or less smoothly curved edge and even texture. The rough or R-type of colony has more sharply angular edge, roughened surface, and granular consistency. There are types intermediate between the rough and smooth colonies. One type of colony may arise from the other. Properties of morphology, virulence, biochemical activities and antigenic composition are correlated with the S- and R-type of colonies.

b. Secondary or daughter colonies.—After relatively prolonged incubation, secondary growth may appear at the edge or on the surface of colonies, formed by the multiplication of cells somewhat different from the cells which formed the original colony.

126. Ability to produce disease.—Most bacteria grow on dead matter as saprophytes, or on or in living animals or plants as commensals; other bacteria, when inoculated into a susceptible host, give rise to typical pathological conditions. Mice, rats, guinea pigs, rabbits, monkeys, and many other animals are used. The results of inoculation are constant for each species. The species of animal attacked, the type disease produced including tissues attacked and type lesions, and the result of inoculation into animals previously immunized against known bacteria are of differential value.

127. Other micro-organisms.—Bacteria have been defined above and will receive major mention hereafter. There will also be considered, without specific definition or classification, a number of other forms of microscopic life closely related to bacteria: higher bacteria, yeasts, molds, rickettsia, and filtrable viruses.

SECTION II

PREPARATION OF GLASSWARE

Paragraph
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128. Cleaning.—*a. New glassware.*—Boil in water to which has been added sufficient white soap or washing soda (type supplied for dishwashing machines) to provide a good foam. Cool water to 45° to 50° C. and wash thoroughly inside and outside, using a washrag and the proper type brush. Rinse thoroughly in running tap water, preferably hot. Rerinse in distilled water and invert on drain board or place in hot air oven at 160° C. to dry.

b. Used glassware.—Sterilize test tubes, flasks, etc., that have contained cultures of pathogenic bacteria in autoclave or by placing in

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a 3 to 5 percent solution of cresol for several hours, and empty contents into sink (broth cultures) or garbage can (agar cultures). Glassware smeared with petrolatum, paraffin, or wax pencil should be given a preliminary cleansing with xyol. Wash as described for new glassware but use greater care to cleanse inside of articles.

c. Dichromate-sulfuric acid cleaning solution.—Cloudy glassware, which cannot be cleansed by soap and water, and used pipettes and slides, after preliminary washing, should be soaked in a cleaning solution in a large glass or earthenware container for 2 to 24 hours. For formula see paragraph 43.

d. Cleaning special glassware.—(1) *Pipettes.*—Immediately after use, place in tall jars containing 2 to 5 percent cresol solution. To wash inside of pipette and force out cotton plug, attach rubber tube to cold water faucet and insert the tip end of pipette into the hose; turn on water and force it through pipette until clean; rinse in distilled water and dry. Inspect pipettes, pick out any that are not perfectly clean, and soak overnight in acid cleaning solution (par. 43).

(2) *Used glass slides and cover slips.*—After use, place in jar of 5 percent cresol solution, as a cleansing disinfectant. Wash in hot, running water; rub individual slides between thumb and forefinger with soap scouring powder and rinse in tap water. Either soak overnight in acid cleaning solution (par. 43) and rinse in tap water followed by distilled water and alcohol, or rinse in distilled water and soak in alcohol containing 2 percent acetic acid; dry and polish with soft cloth.

(3) *Syringes.*—Wash in tap water immediately after use for withdrawing blood, or fill with cresol solution for several hours after contamination with pathogenic bacteria. Wash, rinse, and dry.

129. Plugging, wrapping, and storage.—*a. Pipettes.*—Plug base of each loosely with cotton. Wrap individually with paper and sterilize with dry heat.

b. Syringes.—Dismantle syringe and wrap in heavy unbleached muslin. Smaller syringes may be sterilized in large cotton-plugged test tubes.

c. Test tubes.—Plug with cotton.

d. Flasks.—Plug with cotton wrapped in cheesecloth.

e. Centrifuge tubes.—Plug firmly with cotton contained in piece of cheesecloth large enough to leave apron about 1 inch wide around mouth of tube.

f. Petri dishes.—Place in round or square metal boxes for sterilization, or wrap in paper.

g. Storage.—Sterilize, label, and store in cabinet protected from dust.

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SECTION III

STERILIZATION

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130. Dry heat.—A temperature of 160° to 190° C. in a gas or electric oven for at least 1 hour is sufficient for the sterilization of small articles. A prolonged exposure to temperatures over 175° C. will char or burn cotton and paper. The following rules apply for routine use:

a. Sterilize glassware, wrapped in cloth or paper or plugged with cotton, by heating for minimum of 2 hours at 160° to 175° C.

b. Sterilize glassware, closed with glass stopper or packed in metal containers, by heating for minimum of 2 hours at 160° to 190° C.

c. Glassware, closely packed or in large container, must be heated for longer period of time to insure penetration of heat to, and sterilization of, central portion.

131. Steam under pressure (autoclave).—The standard laboratory autoclave is an unjacketed, horizontal type, set at 15 to 17 pounds pressure. It is used for the sterilization of linen, cotton goods, rubber, glassware, and culture media which are not injured by high temperatures, and for killing old cultures. Routinely, sterilize for 15 minutes at 15 pounds pressure. Large packages or media in bulk will require from 30 minutes to 1 hour. Example: 600 cc of media in 1,000-cc flask require 30 minutes, the additional time attaining penetration of the bulky material to the sterilizing temperature.

a. Place material in autoclave, leave door open, open escape valve, and turn on steam.

b. Close door when steam starts to flow from autoclave.

c. Leave escape valve open until steam escapes rapidly, then close, leaving crack wide enough for trickle of steam to escape.

d. Allow pressure to rise to 15 pounds and sterilize for desired length of time.

132. Steam not under pressure.—The Arnold sterilizer is used for materials, such as nutrient gelatin, milk media, and media containing carbohydrates, that may be damaged by overheating.

a. Place sterilizer over good gas burner and bring water in pan to a boil.

b. Remove top, or open door, and place media in inner compartment, then close.

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c. Heat for 20 to 30 minutes.

d. Leave media at room temperature and repeat the procedure on the 2 succeeding days.

133. Boiling in water.—Syringes, used for injections or drawing blood, and dissecting instruments are sometimes sterilized by boiling in water for 30 minutes.

134. Chemicals.—*a.* A 2 to 5 percent solution of compound cresol may be used to sterilize discarded cultures, used glassware, or rubber gloves, and for disinfecting laboratory floors and table tops.

b. Bacterial antigens and vaccines may be sterilized with 0.2 percent formalin or 0.5 percent phenol.

c. Antisera may be sterilized by addition of 0.5 percent phenol and normal serum for preparation of Loeffler's medium by addition of 2 percent chloroform.

135. Filtration.—Berkefeld, Mandler, Seitz, or other filters are employed for removing bacteria from liquids that may be damaged or destroyed by heat, such as antisera, dextrose solution, and ascitic fluid. The process is described in section VI, chapter 10.

SECTION IV

PREPARATION OF STAINS AND SOLUTIONS

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136. General.—Bacteria are stained by the *basic* aniline dyes. The *acid* aniline dyes, including eosin and acid fuchsin, are not suitable for bacterial staining. The basic aniline dyes mentioned below, and others if needed, may be conveniently kept as stock solutions of the powder in alcohol to saturation, from which are prepared various simple and compound staining solutions.

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137. Stock solutions.—Stock solutions of dyes consist of these dyes in saturated alcoholic solution. They are prepared by placing the measured amount for solution, or slightly more, into 95 percent ethyl alcohol at room temperature and, after shaking for complete solution, filtering through paper to remove surplus dye and debris. Label "_____, stock solution."

Dye:	*Solubility in 100 cc of 95 percent alcohol*
Crystal violet	13.87 gm
Fuchsin (basic)	8.16 gm
Methylene blue	1.48 gm
Safranin	3.41 gm

Example: To prepare stock solution of safranin, place slightly in excess of 3.41 gm of the dye in 100 cc of 95 percent alcohol, shake to solution over period of 2 or 3 days, filter through paper, and label.

138. Simple stain solutions.—*a. General formula.*

Stock dye solution	10 cc
Distilled water	90 cc

b. Uses.—Simple stains or as elements of compound stains.

c. Application.—Apply stain to a fixed slide for 2 to 5 minutes, wash with water, and blot dry.

139. Loeffler's alkaline methylene blue.—*a. Formula.*

Potassium hydroxide, 10 percent solution	0.07 cc
Distilled water	70 cc
Mix and add:	
Methylene blue, stock solution	30 cc

b. Preparation.—The KOH is first added to H_2O to make 1:10,000 dilution, then dye added.

c. Uses.—General bacterial stain.

140. Carbol-fuchsin, dilute.—*a. Formula.*

Carbol-fuchsin (formula elsewhere)	10 cc
Distilled water	90 cc

b. Uses.—General bacterial stain.

141. Bismarck brown.—This is not kept as a stock alcoholic solution.

Bismarck brown powder	0.5 gm
Boiling water	100 cc

Cool and filter.

142. Gram's method.—This is the most important of all bacteriological stains. It includes the application in turn, to a fixed

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slide, of a violet stain, Gram's iodine, a decolorizing agent, and a contrast counterstain.

a. Reagents.—(1) *Primary stain.*—Crystal violet-ammonium oxalate solution.

Crystal violet, stock solution	5 cc
Alcohol, 95 percent	5 cc
Mix and add:	
Ammonium oxalate, 1 percent aqueous solution	40 cc

(2) *Gram's iodine.*

Iodine			1 gm
Potassium iodide			2 gm
Distilled water	240 cc	or distilled water	300 cc
Sodium bicarbonate, 5 percent aqueous solution	60 cc		

(3) *Decolorizer.*—Ninety-five percent ethyl alcohol, or acetone, or alcohol-acetone (50–50).

(4) *Counterstain.*—Any simple contrast stain: safranin, bismarck brown, or dilute carbol-fuchsin.

b. Technic of Gram-staining.—(1) Prepare thin, even slide spreads, air-dry, and pass through flame for fixation.

(2) Crystal violet stain is applied for 1 minute, then excess stain is poured off.

(3) Gram's iodine is then applied for 1 minute. Wash in water.

(4) Decolorizer is applied in several washes until no further traces of the stain can be washed out of the preparation (½ to 2 minutes). Wash in water.

(5) Apply counterstain (e. g., safranin) for ½ minute. Wash in water. Blot and air-dry.

c. Results.—(1) Gram-positive organisms are stained violet.

(2) Gram-negative organisms are stained pink (brown or red).

(3) Gram-ambophile organisms give a variable result.

143. General rules of Gram behavior of organisms.—*a.* Cocci are Gram-positive except gonococcus, meningococcus, and catarrhalis group.

b. Bacilli are Gram-negative except the diphtheria, the acidfast group, and most spore bearers.

c. Spirilla and spirochetes are Gram-negative.

d. Older cultures of Gram-positive organisms tend to become Gram-ambophile or negative.

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144. Neisser's method for polar body staining.—*a. Formulas.*
(1) *Polar body stain (Neisser No. 1).*

Methylene blue, stock solution	10 cc
Acetic acid, 5 percent solution, freshly prepared	50 cc

(2) *Counterstain (Neisser No. 2).*—Bismarck brown, formula paragraph 141, or use the safranin as prepared for Gram's counterstain.

b. Technic of stain.—(1) Prepare even, thin spreads on slides, air-dry, and fix by heat.

(2) Apply polar body stain for 1 to 3 minutes, then wash.

(3) Apply counterstain for 1 minute. Wash and dry.

c. Result.—Polar bodies will be stained blue; bacillary bodies take the counterstain.

d. Uses.—Differential stain of the diphtheria bacillus, the plague bacillus, and others having metachromatic granules.

145. Acidfast stain method (Ziehl-Neelsen's carbol-fuchsin).—*a. Formulas.*—Include a primary stain, a decolorizer, and a counterstain.

(1) *Carbol-fuchsin.*

Basic fuchsin, stock solution	10 cc
Phenol, 5 percent solution	90 cc

(2) *Acid alcohol.*

Acid, hydrochloric	3 cc
Alcohol, ethyl, 95 percent	97 cc

(3) *Counterstain.*—Loeffler's methylene blue.

b. Technic.—(1) Prepare spreads of suspect material on slides, air-dry, and fix by heat.

(2) Apply carbol-fuchsin and heat gently until steam appears over the surface. Allow to steam for 5 minutes. Wash in water.

(3) Decolorize with acid alcohol by renewal washer to a faint pink. Wash in water.

(4) Counterstain with methylene blue for ½ minute, wash in water, and blot dry.

c. Results.—(1) Acidfast organisms are stained red.

(2) Non-acidfast organisms are stained blue.

d. Application.—The detection of tubercle bacilli, leprosy bacilli, and a few other acidfast organisms.

146. Hiss' method for capsules.—*a. Formulas.*—(1) *Staining solution.*

Crystal violet, stock solution	10 cc
Distilled water	90 cc

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Gram's crystal violet or Ziehl-Neelsen's carbol-fuchsin may be substituted.

(2) *Mordant.*—Copper sulfate, 20 percent aqueous solution.

b. Technic.—(1) (*a*) *Exudate.*—Spread evenly on clean slide.

(*b*) *Cultured organism.*—Mix with equal parts of animal serum and spread.

(2) Air-dry, but do not heat-fix.

(3) Apply staining solution for 1 minute, heated to steaming.

(4) Wash off the stain with copper sulfate. Do not wash with water.

(5) Blot dry or examine wet under a cover slip.

c. Results.—Capsule, if present, appears as a faint blue halo about a dark purple cell body.

147. Spore stain (Dorner's method).—*a. Formulas.*—Carbol-fuchsin and methylene blue as in acidfast stain.

b. Technic.—(1) Prepare slide spread and stain with carbol-fuchsin as for Ziehl-Neelsen method.

(2) Wash in hot tap water.

(3) Rinse rapidly with 95 percent alcohol.

(4) Apply Loeffler's methylene blue for 2 to 5 minutes. Wash, then blot dry.

c. Results.—Spores are red and cell body blue.

148. Nigrosine method (for spirochetes).—*a. Formulas.*

Nigrosine	10	gm
Distilled water	90	cc

Boil in flask for 30 minutes, then add as preservative:

Formalin (40 percent)	0.5	cc

Filter twice through double filter paper. Store in small sealed test tubes.

b. Technic.—A loopful of fresh exudate or culture fluid is mixed on a slide with a loopful of nigrosine solution, then spread over the slide and dried.

c. Result.—Spirochetes are not stained but are demonstrated negatively as unstained light areas on a smoky background.

149. Van Gieson's stain (for Negri bodies).—*a. Formulas.*

(1) *Fixative.*

Methanol (neutral)	100	cc
Picric acid	.1	gm

} Freshly prepared.

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(2) *Stain.*

Basic fuchsin, stock solution	.5 to 1 cc	Made fresh just before use. Fuchsin varied to desired result.
Methylene blue, stock solution	10 cc	
Distilled water	30 cc	

b. Technic.—(1) Make impression or smear spreads of gray matter of hippocampus or cerebellum of brain of suspected rabid animal.

(2) Fix with a momentary flood of methanol. Wash at once.

(3) Stain is applied for 5 minutes, heated gently to steaming. Wash, then blot dry.

c. Result.—Negri bodies are magenta with blue granules. Nerve cells are blue. Erythrocytes are salmon or bronze color.

150. Wright's and Giemsa's stains.—See references to hematology and parasitology, chapters 2, 12, 13, and 14.

151. Miscellaneous solutions.—*a. Sodium chloride solution* (saline or physiological salt solution).

Sodium chloride	8.5 gm
Distilled water	1,000 cc

b. Buffer solution.

Sodium dihydrogen phosphate (NaH_2PO_4)	28.81 gm
Disodium hydrogen phosphate (Na_2HPO_4)	125 gm
Distilled water	to 1,000 cc

c. Sodium chloride solution, buffered.

Buffer solution (above)	20 cc
Sodium chloride	8.5 gm
Distilled water	to 1,000 cc

d. Sodium citrate-sodium chloride solutions (anticoagulant).

	1 percent	*2 percent*	*10 percent*	
Sodium citrate CP	10 gm	20 gm	100 gm	Filter after solution.
Sodium chloride CP	8.5 gm	8.5 gm	8.5 gm	
Distilled water	1,000 cc	1,000 cc	1,000 cc	

May be sterilized in autoclave at 15 pounds for 20 minutes.

When used as anticoagulant of blood, a final sodium citrate concentration of over 0.25 percent is required; therefore, use to each 10 cc of blood 3.3 cc of the 1 percent, 1.4 cc of the 2 percent or 0.26 cc of the 10 percent solution.

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e. Potassium oxalate solution (anticoagulant).

Potassium oxalate	2 gm	One cc of this to 10 cc of blood prevents coagulation.
Sodium chloride	6 gm	
Distilled water	100 cc	

f. Sodium carbonate solution.—Two gm per liter of water. May be used for boiling instruments, as it prevents corrosion.

g. Disinfectant solution (desk jar use).

	2 percent	*5 percent*
Liquor cresolis compound USP	20	50 cc
Tap water to make	1,000	1,000 cc

h. Preparation of percentage solutions by dilution.—(1) Measure number of cubic centimeters of more concentrated solution corresponding to the percentage desired for the new solution.

(2) Add distilled water to total volume corresponding to the percentage numeral of the original solution. For example, to prepare a 70 percent solution from a 95 percent solution, measure 70 cc of the latter and add 25 cc of distilled water, giving therefore 95 cc of a 70 percent solution.

SECTION V

DETERMINATION OF BIOLOGICAL ACTIVITY

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152. Acid and alkali formation by bacteria.—A definite amount of an indicator solution is incorporated with the basic media and the pH of the resultant growth determined by presence or absence of a color change.

Indicator	Amount used per 1,000 cc of medium	Color change	pH range
Phenol red (0.02 percent aqueous solution).	50 cc	yellow→red	6.8 to 8.4
Bromthymol blue (0.04 percent aqueous solution).	50 cc	yellow→blue	6.0 to 7.6
Bromcresol purple (1.6 percent alcoholic solution).	1 cc	yellow→purple	5.2 to 6.8

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153. Gas formation.—Culture is made in liquid media containing a small inverted tube and indicator as above. Observe daily for the collection of gas in this inverted tube. Record, approximately, the portion of vial filled with gas as percent gas present.

154. Test for indol.—*a. Reagent.*

1. Para dimethyl-amino benzaldehyde	5 gm
(2) Amyl alcohol	75 cc
(3) Hydrochloric acid	25 cc

Dissolve (1) in (2) and then add the acid. The completed reagent should have a yellow or light-brown color.

b. Procedure.—(1) Inoculate tube of tryptone broth. Incubate at 37° C. for 24 hours.

(2) Add 0.2 to 0.3 cc of the amyl alcohol indol reagent and shake gently.

(3) Allow reagent to rise to surface of medium and observe result. Dark red color is a positive indol test; original color of reagent is a negative test.

155. Nitrate reduction (Ilosvay's method).—Nitrate broth culture, after 5 days incubation at 37° C. Add 1 cc of solution *A* and 1 cc of solution *B*. Positive reaction, red, purple, or maroon; negative reaction, no color change.

Solution A

A-naphthylamine	1 gm	Dissolve, filter, then add:
Water, distilled	22 cc	
Acetic acid, dilute (sp. gr. 1.04)		180 cc

Solution B

Sulfanilic acid	0.5 gm
Acetic acid, dilute	150 cc

156. Ammonia production.—Peptone water culture, grown for 5 days at 37° C. Add: Nessler's reagent 0.5 cc. Note color change. Positive, brown; negative, faint yellow.

157. Hydrogen sulfide production.—Lead acetate agar—stab culture, incubated at 37° C. Observe daily for 5 days. Positive reaction, brown or black color; negative reaction, no color change.

158. Reductase test.—Broth culture—24 hours at 37° C. Add 1 drop methylene blue (1 percent aqueous solution). Incubate at 37° C. Note color. Positive (strong reduction), complete decolorization; weak positive, green color; negative, no color change.

159. Methyl red test (M. R.).—Peptone medium (Clark and Lubs' medium) culture, grown for 4 days at 37° C. Add 5 drops methyl red indicator solution (0.04 percent in 60 percent alcohol). Positive reaction, red color; negative reaction, yellow color.

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160. Voges-Proskauer test (V. P.).—To a 5-cc portion, removed asceptically from the peptone medium inoculated for the methyl red test, or to a separately inoculated tube of the same medium, after 24 to 48 hours' incubation, add an equal volume of 10 percent potassium hydroxide solution. Place in incubator at 37° or 45° C for 6 hours or overnight; observe periodically. A positive test is indicated by an eosin pink color.

161. Citrate utilization test.—Inoculate tube of sodium citrate water or of Simmon's citrate agar for 4 days at 37° C. Growth indicates positive; no growth, negative.

162. Tartrate utilization.—Make deep stab inoculation of tube of Jordan's tartrate agar. Positive reaction, acid butt; negative reaction, no change in color.

SECTION VI

PREPARATION OF CULTURE MEDIA

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163. General.—Bacteria may be cultivated upon a great many different kinds of artificial culture media, all of which have the common characteristics of supplying food for the bacteria, having a naturally or artificially adjusted reaction (hydrogen ion concentration or pH) and being of such a nature that they can be readily sterilized. They must meet certain requirements as to surface tension and osmotic pressure and they must be stable.

164. Adjustment of reaction.—The hydrogen ion concentration of media can be determined and adjusted by the electrometric or colorimetric methods. The colorimetric method is the one most frequently used.

a. Equipment, colorimetric method.—For the titration and adjustment of media, a set of color standards are used. Color standard sets may be prepared with several different indicators, such as phenol red with a color range from pH 6.8 to 8.4 and brom-thymol blue with a range from pH 6.0 to 7.6. These sets are prepared at the Army Medical School for distribution to Army laboratories. The set of phenol red indicator standards, which are generally used, consists of nine tubes whose pH range is from 6.8 (yellow) to 8.4 (red); each tube increasing by 0.2.

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b. Technic.—(1) Select the standard tube of the desired pH (most culture media are adjusted to pH 7.2 to 7.6) and place it in the right front hole of a "comparator block."

(2) Place immediately behind it a tube of medium to which no indicator is added.

(3) Place a tube containing 10 cc of medium to which has been added 0.5 cc of a 0.02 percent phenol red solution in the left front hole.

(4) Place immediately behind it a tube of distilled water.

(5) Hold the comparator block toward the daylight and determine whether the medium plus indicator solution has the same color as the pH standard plus medium without indicator.

(6) If the medium is acid, as most freshly prepared media are, add measured quantities of N/10 NaOH until the color of the medium matches the standard tube. (If the medium is very alkaline, add measured quantities of N/10 HCl until the medium matches the standard tube.)

(7) From the volume of N/10 NaOH (or HCl) used to adjust 10 cc of medium to the desired pH, the amount of N/1 NaOH (or HCl) required for 1,000 cc may be estimated by multiplying the number of cubic centimeters of N/10 solution used by 10.

(8) After the final addition of NaOH (or HCl), recheck the pH and readjust if necessary.

NOTE.—As some change in reaction may take place during sterilization, it is desirable to make suitable corrections for this. Broth media with an initial pH of 7.0 changes very little, but if the initial pH is over 7.0 it may become about 0.2 more acid.

165. Clarification.—Media may be clarified by filtration through cotton, gauze, filter paper, or by use of a Berkefeld filter.

166. Distribution.—The media should be placed in sterile glass flasks or tubes before sterilization. This can be done by placing the fluid medium in a large container or funnel from which it is delivered through a rubber tube controlled by a pinchcock.

167. Storage.—After sterilization, label media and store in refrigerator or cold room. Cotton-stoppered flasks and tubes should be protected with a cap of paper or lead foil.

168. Meat extract broth.

Beef extract	3 gm
Peptone	10 gm
Sodium chloride	5 gm
Distilled water	1,000 cc

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Add the weighed ingredients to distilled water and heat slowly to 65° C., stirring until dissolved. Adjust loss of volume with distilled water, clarify by filtration through paper, and titrate to pH 7.2 to 7.6. Autoclave 15 minutes at 15 pounds.

169. Meat extract agar.

Meat extract broth	1,000 cc
Agar	20 to 30 gm

Add agar to broth and autoclave 15 minutes at 15 pounds. Filter through cotton, adjust pH to 7.2 to 7.6, and resterilize.

170. Meat infusion broth.

Beef or veal round, free from fat, ground	500 gm
Distilled water	1,000 cc

Mix and infuse in ice box 18 to 24 hours. Remove and heat over low flame 1 hour. Squeeze through gauze until 1,000 cc are obtained.

Sodium chloride	5 gm
Peptone	10 gm

Add to infusion and dissolve over flame. Titrate to pH 8.0. Filter through paper. Cook in autoclave 45 minutes at 15 pounds. Refilter through paper while hot and make up to volume with distilled water. Autoclave one 10-cc portion 15 minutes at 15 pounds, titrate for acid drift, adjust pH of lot, tube, or flask and autoclave 15 minutes at 15 pounds.

171. Meat infusion agar.

Meat infusion broth	1,000 cc
Agar	20 gm

Add agar and proceed as in preparation of meat extract agar. Adjust to pH 7.4.

172. Nutrient gelatin.

Meat extract broth	1,000 cc
Sheet gelatin (purified)	120 gm

Place the broth in double boiler and add the gelatin. Dissolve gelatin by heating and adjust reaction to pH 7.4. Make up to original volume with distilled water. Add one egg, mixed with small amount of water, to clarify; reheat slowly until egg is firmly coagulated. Filter through cotton. Tube in 10-cc portions and sterilize in Arnold sterilizer for 20 minutes on 3 successive days. After each heating, cool rapidly to prevent lowering of melting point.

173. Nutrient broth (for water analysis).

Beef extract	3 gm
Peptone (Bacto)	5 gm
Distilled water	1,000 cc

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Prepare as for meat extract broth in basic media but adjust pH between 6.4 and 7.0.

174. Nutrient agar (for water analysis).

Meat extract broth (as above)	1,000 cc
Agar	15 gm

Add agar to broth and prepare as for meat extract agar in basic media but adjust pH between 6.4 and 7.0.

175. Lactose broth (for water analysis).—Prepare in same manner as for meat extract broth but with addition of 0.5 percent lactose and 1 cc of 1.6 percent alcoholic solution of bromcresol purple per liter. Adjust to pH 6.4 to 7.0, preferably pH 6.9.

176. Levine's eosin methylene blue agar (for water analysis).

Peptone	10 gm
Dipotassium phosphate (K_2HPO_4)	2 gm
Agar	15 gm
Distilled water	1,000 cc

Add ingredients, dissolve by boiling, and make up volume lost. No adjustment of reaction is necessary. Place 100-cc amounts in flasks and autoclave 15 minutes at 15 pounds. Just prior to use, melt above and to each 100 cc add:

Lactose, 20 percent, sterile solution	5 cc
Eosin, yellowish, 2 percent aqueous solution	2 cc
Methylene blue, 0.5 percent aqueous solution	2 cc

Mix thoroughly and pour plates. Allow to harden and incubate for sterility.

NOTE.—It is permissible to add all of the ingredients to the stock agar at the time of preparation. Place in flasks and sterilize; plates may be prepared from this stock directly.

177. Brilliant green lactose bile broth (for water analysis).

Peptone (Bacto)	10 gm
Lactose	10 gm
Distilled water	500 cc

Dissolve and add—

Fresh ox bile	200 cc

or

20 gm dehydrated ox bile, dissolved in 200 cc distilled water.

Then add—

Distilled water q. s. ad	975 cc

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Adjust pH to 7.4 and add—

Brilliant green, 0.1 percent aqueous solution	13.3 cc
Distilled water, q. s. ad	1,000 cc

Filter through cotton and autoclave for 15 minutes at 15 pounds. Final pH should be not less than pH 7.1 and not more than 7.4.

178. Tryptone-glucose-extract-milk agar (for milk analysis).

Agar	15 gm
Beef extract	3 gm
Tryptone	5 gm
Glucose	1 gm
Distilled water	1,000 cc

Dissolve by boiling over a free flame, make up volume lost, and adjust reaction to pH 7.0. Add—

Skim milk	10 cc

Dispense measured amounts (100 or 200 cc) in flasks, or place 10 to 12 cc in test tubes. Autoclave 15 minutes at 15 pounds.

179. McLeod's gonococcus medium.—*a. Stock medium.*

Peptone	10 gm
Sodium phosphate, dibasic (Na_2HPO_4)	2 gm
Distilled water	1,000 cc

Dissolve by heating to 60° C. Then add—

Ground lean meat	1 lb

Heat to 60° C. for 45 minutes, then steam in autoclave for 30 minutes. Filter, adjust to pH 7.4, and add—

Agar	12 gm

Dissolve with scant boiling, bottle in 100-cc amounts, autoclave 15 minutes at 15 pounds, and store.

b. Completed medium.

Stock medium	100 cc
Blood, human, citrated	10 cc

Add blood to melted stock medium while still very hot. Mix thoroughly and pour plates. Use same day as prepared.

NOTE.—Concentrated amino-acids and peptone inhibit growth of gonococcus; the heating of blood checks this inhibition, hence the use of "chocolate" or heated blood agar. The inhibiting effect of peptone on delicately growing micro-organisms is due to some oxidized substance produced in heat sterilization of the medium, hence the McLeod method reduces these oxidized bodies by having the peptone in contact with the meat during extraction.

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180. Semisolid media.—*a. General.*—General media containing 0.1 to 0.3 percent agar are known as semisolid. Many different types of semisolid media may be prepared, differing in agar content, in the basic liquid medium used (infusion broth, peptone broth, etc.) and in special ingredients that may be added (serum, ascitic fluid, carbohydrates, indicators, etc.). These media are used especially in the study of anaerobic bacteria and in the cultivation and fermentation studies of gonococci and meningococci.

b. Basic formula.

Infusion broth (beef, veal, etc.) or other type liquid media	1,000 cc
Agar	1 gm

Add agar to broth and dissolve by boiling. Adjust reaction to pH 7.6. Tube in 10-cc quantities and serilize in autoclave at 15 pounds for 15 minutes. Before use, melt agar and drive off dissolved oxygen by boiling for 10 minutes; cool to 45° to 50° C. and inoculate.

181. Calcium carbonate broth (for pneumococci).

Meat infusion broth	1,000 cc
Glucose	10 gm

Dissolve by heating. Adjust pH to 7.6. Place clean marble chips in bottom of test tube and add broth. Sterilize for 15 minutes by the fractional method in Arnold sterilizer.

182. Potato-glycerin-blood agar (for H. pertussis).

Sliced potato	100 gm
Glycerin, 4 percent aqueous	200 cc

Mix and steam in autoclave. Remove 50 cc of the resulting glycerin extract of potato.

Glycerin extract	50 cc
Sodium chloride 0.6 percent	150 cc
Agar	5 gm

Add ingredients, melt in autoclave, and place 2 to 3 cc in test tube and sterilize.

Sterile defibrinated rabbit blood or human blood	2 to 3 cc

Add in equal volume to sterile tubes of above media, mix, and slant. pH is approximately 6 and no adjustment is necessary.

183. Robertson's medium (for anaerobes).

Beef heart, fresh, with all fat, fascia, and blood vessels removed, ground	500 gm
Peptone	10 gm
Distilled water	1,000 cc

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Mix ingredients and bring to a boil. Adjust pH to 8.0 and allow to simmer 1½ hours; readjust pH. Separate broth from meat and place in flasks. Autoclave 15 minutes at 15 pounds. Place meat on clean filter paper and dry in oven at 56° C. for 48 hours. Place the desired quantity of dried heart in a test tube and add 10 cc of above broth. Autoclave, cool broth, and titrate. Adjust reaction for acid drift and adjust so that media will have a final pH of 7.4 to 7.6. Resterilize.

184. Dieudonne's alkaline blood agar (for Vibrio comma).

Blood, beef, defibrinated	150 cc
Potassium hydroxide (normal)	150 cc

Mix and steam in Arnold sterilizer 30 minutes. Then melt—

Nutrient agar (pH 6.8)	700 cc

To 7 parts of agar add 3 parts of above alkali-blood mixture. Pour plates and allow to harden uncovered but protected with paper. Place strips of filter paper between dish and cover to absorb moisture and ammonia. Incubate 15 hours at 37° C. before using.

185. Petroff's medium (for M. tuberculosis).—This medium is composed of meat juice, eggs, and a minute amount of gentian violet or brilliant green.

a. In a cool place infuse 500 gm of beef or veal in 500 cc of a 15 percent solution of glycerol in water; after 24 hours place in sterile press and collect the extract in a sterile container.

b. Immerse washed eggs in 70 percent alcohol for 10 minutes. Pick out with sterile tongs, flame, and break in a sterile container. Add 1 part meat juice to 2 parts eggs by volume (1 egg equals approximately 25 cc).

c. Add 1 percent alcoholic solution of gentian violet or brilliant green to make a final proportion of 1 to 10,000. Thoroughly mix ingredients, tube, slant, inspissate, and sterilize in Arnold sterilizer by discontinuous method.

186. Cystine blood agar (for P. tularensis).

Beef or veal infusion	1,000 cc
Agar	15 gm
Proteose-peptone (Difco)	10 gm
Sodium chloride	5 gm

Dissolve by heating; adjust reaction to pH 7.8; autoclave 15 minutes at 15 pounds, and filter. Place in flasks and resterilize. Final

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pH of medium should be between 7.3 and 7.4. Before use, add to the above—

Cystine (preferable) or cystine hydrochloride	1 gm
Glucose	10 gm

Dissolve by heating in Arnold sterilizer and then sterilize for 30 minutes. Cool to 50° C. and add—

Rabbit or horse blood or serum, sterile	50 cc

Tube aseptically, slant, and incubate for sterility.

187. Hiss' serum-water medium (for fermentation tests).

Clear, sterile serum	1 part
Distilled water	3 parts

Mix and heat in Arnold sterilizer for 15 minutes. Add 1 percent of the desired carbohydrates, dissolved in a little hot water. To each 1,000 cc of medium, add 1 cc (or more, if required) of a 1.6 percent alcoholic solution of bromcresol purple. Tube and sterilize for 20 minutes in Arnold sterilizer on 3 successive days. Incubate for sterility.

188. Potato medium.—Select large white potatoes, peel, and scrub thoroughly under running water. Cut cylinders from the potato with cork borer. Cut these obliquely into wedge-shaped pieces and place in running water overnight to reduce acidity. Place in tubes and add 2 cc of distilled water; autoclave for 15 minutes at 15 pounds.

189. Glycerol agar.

Meat infusion agar	1,000 cc
Glycerol (pure)	30 cc

Mix by heating and adjust pH to 7.2. Tube, autoclave for 15 minutes at 15 pounds, and slant.

190. Loeffler's medium (for C. diphtheriae).—Collect beef blood in sterile containers. Allow to clot without removing. Loosen clot with sterile rod and store in refrigerator. Pipette off clear serum. To 3 parts serum add 1 part meat infusion broth, pH 7.6, containing 1 percent glucose. Mix by stirring, tube, and inspissate on a slant, gently raising temperature to about 85° C. Hold at this temperature until the serum is firmly coagulated. Sterilize by fractional method (for 20 minutes on 3 successive days) in the Arnold sterilizer. After sterilization, paraffinize cotton plugs and incubate tubes for sterility.

191. Tellurite medium (for C. diphtheriae).—Melt meat extract agar or 0.2 percent dextrose agar, 10 cc in tube or larger measured quantity in flask, and cool to 50° C. For each 10 cc of medium,

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add 1 cc of citrated or defibrinated rabbit blood and 1 cc of a sterile 2 percent solution of potassium tellurite. Mix and pour into Petri dishes.

NOTE.—An excellent tellurite medium may also be prepared by adding 5 cc of Bacto-tellurite blood solution to 100 cc of Bacto-dextrose proteose No. 3 agar, heating to 80° C., cooling to 50° C., and pouring plates.

192. Sabouraud's media (for fungi).—*a. Conservation.*

Peptone	30 gm
Agar	20 gm
Tap water	1,000 cc

Dissolve, tube, and autoclave 30 minutes at 8 pounds.

b. Differential.

Maltose, crude	40 gm
Peptone	10 gm
Agar	20 gm
Tap water	1,000 cc

Mix all ingredients except maltose, bring to a boil, and then add maltose; filter if necessary. Autoclave 30 minutes at 8 pounds. No adjustment of reaction is necessary.

193. Corn meal agar.

Water, distilled	1,500 cc
Corn meal	62.5 gm

Heat at 60° C. for 1 hour. Filter through paper and adjust volume to 1,500 cc. Add—

Agar	19 gm

Heat in Arnold sterilizer for 1¼ hours. Filter through cotton, tube, and sterilize in autoclave at 15 pounds for 15 minutes. Adjustment of reaction is not necessary.

194. Chocolate blood agar.—Add 5 percent of sterile defibrinated blood to melted meat infusion agar at 50° to 55° C., mix carefully to avoid bubbles, and slowly raise the temperature to 75° C. Pour into plates or tube and slant. Incubate to determine sterility.

195. Enriched agar media.—*a. Blood agar.*—Add 5 to 10 percent of sterile defibrinated or whole blood (human, rabbit, or horse) to infusion agar (preferred) or extract agar that has been melted and cooled to 45° C. Pour plates or prepare slants and incubate for sterility.

b. Serum agar.—To 1,000 cc of melted infusion agar at 45° to 50° C., add 100 cc of sterile normal horse serum. Place in sterile tubes and slant, or pour into plates. Incubate to determine sterility. Sterile ascitic or hydrocele fluid may be used instead of the serum.

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196. Glucose agar.—To 1,000 cc of melted extract agar or infusion agar, add 10 gm of glucose and heat slowly until dissolved. Titrate and adjust the reaction to original pH of the agar. Sterilize by the fractional method.

197. Glucose brain broth (for streptococci and anaerobes).

Fresh calf brain	5 to 10 gm
1 percent glucose veal infusion broth pH 7.8	35 cc

a. Prepare flask of veal infusion broth (par. 170), adjust reaction to pH 7.8, and add glucose (1 gm per 100 cc of medium).

b. Wash several pieces of calf brain, 1 cc in size, in running water and place into the bottom of a large tube (200 by 25 mm); add the infusion broth. Autoclave at 15 pounds for 20 minutes and cool.

c. Remove 10 cc of the supernatant fluid and check the reaction; if the reaction is pH 7.4 to 7.6, it is satisfactory; but if there has been a greater acid drift, adjust to pH 7.6. Estimate from the titration of the 10-cc portion the amount of NaOH required to adjust reaction for bulk of the broth.

d. Then fill the desired number of tubes with similar quantities of the brain tissue and broth (pH adjusted, if necessary). Sterilize in the autoclave at 15 pounds for 20 minutes. Incubate at 37° C. to determine sterility.

198. Liver infusion agar (for Brucella).—*a. Basic formula.*

Agar	20 gm
Distilled water	500 cc
Beef liver infusion	500 cc
Peptone (Difco)	5 gm
Sodium chloride	5 gm

Place all ingredients in a suitable container, cover, and place in flowing steam for 1 hour. Remove and cool to 60° C. Adjust the pH at this time to 7.0. Place in flowing steam again for ½ hour. Decant and place in sterile flasks or tubes and sterilize 30 minutes at 15 pounds. The pH will drop to 6.6 during this sterilization and no adjustment is necessary as the organisms grow best at this pH.

Two separate media are prepared, using one of the following dyes:

(1) Basic fuchsin 1/100,000 (1.0 cc of 0.1 percent aqueous solution to 100 cc of medium).

(2) Thionin 1/200,000 (1.0 cc of 0.1 percent aqueous solution to 200 cc of medium).

b. Preparation of beef liver infusion for above.—Fresh beef liver, free from fat, is ground. To 500 gm of ground liver add 500 cc of tap water and place in flowing steam for 20 minutes. Remove lid and stir with glass rod in order to mix thoroughly. Continue heating

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for 1½ hours. Remove and strain through wire screen or four layers of gauze. Autoclave 15 minutes at 15 pounds.

199. Liver infusion broth (for culturing blood for Brucella).—Same as liver infusion agar (par. 198), except omit agar and do not add dyes.

200. Russell's double sugar.

Meat extract agar, melted	1,000 cc
Lactose (sterile 25 percent solution)	40 cc
Glucose (sterile 25 percent solution)	4 cc

Mix melted agar and two sugars and adjust pH to 7.2. Add 50 cc of 0.02 percent aqueous phenol red. Filter if necessary, tube, and sterilize in autoclave 25 minutes at 8 pounds.

201. Eosin methylene blue agar (for typhoid-dysentery isolation).—Prepare as for Levine's eosin methylene blue agar in water analysis (par. 176) but reduce dye content one-half by using 1 cc of 2 percent eosin and 1 cc of 0.5 percent methylene blue per 100 cc of medium.

202. Bismuth sulfite agar (Wilson and Blair) (for typhoid group).—Use Bacto or other dehydrated product. Follow directions on bottle for preparation and sterilization.

203. Desoxycholate agar and desoxycholate-citrate agar (Liefson) (for typhoid-dysentery group).—To prepare these media, use a dehydrated product (such as those prepared by the Baltimore Biological Laboratory, Baltimore, Md.) and follow directions on bottles for preparation, sterilization, and use.

204. Selinite-F enrichment medium (for typhoid group isolation from feces and urine).

Sodium acid selinite (anhydrous)	4 gm
Peptone	5 gm
Sodium phosphate (anhydrous)	10 gm
Distilled water	1,000 cc

Determine experimentally the portion of the monobasic and dibasic sodium phosphate which together with the peptone and sodium acid selinite will give a pH of 7.0. Dissolve the weighed ingredients in the distilled water by gentle heat, tube in 10-cc amounts, and sterilize in Arnold sterilizer for not over 30 minutes.

205. Bile medium (for typhoid group).

Ox bile (or equal volume of 10 percent solution of Bacto-oxgall)	900 cc
Glycerol	100 cc
Peptone	20 gm

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Dissolve over water bath, tube or place in flasks, and autoclave 15 minutes at 15 pounds.

206. Simmons' citrate agar.

Magnesium sulfate	0.20 gm
Sodium chloride	5.0 gm
Ammonium acid phosphate ($NH_4H_2PO_4$)	1.0 gm
Sodium citrate ($5\frac{1}{2}H_2O$)	2.77 gm
Distilled water	1,000 cc
Agar	20 gm

Dissolve chemicals in the distilled water; add agar and heat to dissolve. Adjust reaction to pH 7.2 and add—

Bromthymol blue, 1.5 percent alcoholic solution	10 cc

Filter through cotton, tube, and autoclave 15 minutes at 15 pounds. Slant with deep butt.

NOTE.—Check reaction of medium with known cultures of *E. coli*, *A. aerogenes*, *S. schottmuelleri*, and *E. typhosa* before using routinely.

207. Jordan's tartrate agar.

Agar	20 gm
Peptone (Difco)	10 gm
Sodium potassium tartrate	10 gm
Sodium chloride	5 gm
Distilled water	1,000 cc

Dissolve ingredients by heating. Adjust pH to 7.4 and add—

Phenol red, 0.2 percent alcoholic solution	12 cc

Tube in 10-cc quantities and autoclave 15 minutes at 15 pounds.

NOTE.—Check reaction of medium with known cultures of *S. typhimurium*, *S. enteritidis*, *S. paratyphi*, and *S. schottmuelleri* before using routinely.

208. Lead acetate agar (for H_2S production).

Bacto-tryptone	20 gm
Agar	15 gm
Distilled water	1,000 cc

Prepare basic medium, adjust to pH 6.8 to 7.0, and sterilize in autoclave. Prepare for use by adding to each 100 cc of melted medium 0.4 cc of a sterile 25 percent solution of dextrose and 10 cc of a sterile 0.5 percent solution of basic lead acetate. Mix and dispense in tubes aseptically, with slant and deep butt. Incubate for sterility. Check reaction of medium with known strains of *S. paratyphi* and *S. schottmuelleri*.

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209. Tryptone broth (for indol test).

Distilled water	1,000 cc
Tryptone (Bacto)	10 gm

Dissolve by heating and stirring, place 5-cc amounts in test tubes, and autoclave 15 minutes at 15 pounds.

210. Clark and Lubs' medium (for Voges-Proskauer and methyl red tests).

Proteose-peptone (Difco)	5 gm
Dextrose	5 gm
Dipotassium phosphate (K_2HPO_4)	5 gm
Distilled water	1,000 cc

Dissolve by heating, filter, and make up volume lost. Tube in 10-cc quantities and sterilize by fractional method.

211. Dunham's peptone solution (for "cholera red" reaction and as base for carbohydrate media).

Proteose-peptone (Difco)	10 gm
Sodium chloride	5 gm
Distilled water	1,000 cc

Dissolve by heating, adjust pH to 7.6, filter, tube in 10-cc amounts, and autoclave 15 minutes at 15 pounds.

212. Nitrate broth (for nitrate reduction test).

Peptone (Bacto)	10 gm
Potassium nitrate (KNO_3) nitrite-free	1 gm
Water, distilled, ammonia-free	1,000 cc

Dissolve by heating, adjust reaction to pH 7.4 to 7.6, filter, tube in 10-cc amounts, and sterilize in autoclave at 15 pounds for 15 minutes.

213. Carbohydrate broth (for fermentation tests).—To 1,000 cc meat extract broth (par. 173), add 1 percent of the desired fermentable substance, dissolved in a little hot water. Then add 1 cc of 1.6 percent alcoholic solution of bromcresol purple per liter. Place 5-cc portions in fermentation tubes and autoclave 10 minutes at 7 pounds or by fractional method in Arnold sterilizer.

214. Bromcresol purple milk.—Remove the cream from sweet milk or purchase fresh, skimmed milk. Heat the comparatively fat-free milk in the Arnold sterilizer for 20 minutes. Siphon off the central four-fifths for use (discard the cream layer on top and the bottom portion containing sediment). To each 1,000 cc of fat-free milk, add 1 cc (or more, if required to give distinct blue color) of a 1.6 percent alcoholic solution of bromcresol purple. Tube in 10-cc amounts and sterilize in Arnold sterilizer by the fractional method.

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Other indicators may be substituted for the bromcresol purple, such as azolitmin (10 cc of a neutralized 5 percent aqueous solution) to make litmus milk.

NOTE.—For the many media not described in this section refer to "A Compilation of Culture Media" by Levine and Schoenlein, published by Williams and Wilkins Company, Baltimore, Md., or to any standard textbook on bacteriology.

Media in a dehydrated powder form are obtainable and may be substituted for most of the media described in this section. They are accurately prepared by reliable commercial manufacturers, supplied in powder form, and include a wide range of complex media. Being dry, they may be preserved without deterioration in stoppered bottles and kept at room temperature.

SECTION VII

CARE OF LABORATORY ANIMALS

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Rabbits	218
Guinea pigs	219
Mice	220
Albino rats	221
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215. Types of animals.—Five types of laboratory animals are the rabbit, guinea pig, mouse, albino rat, and monkey.

216. Reception quarantine.—All animals received from an outside source should be isolated for 10 days to 3 weeks in previously disinfected quarters, and found to be free from disease before mixing with regular stock.

217. Housing.—Animal quarters should be kept clean, dry, and completely free from vermin. The optimum temperature for most animals is 65° to 70° F. with adequate ventilation. The standard large (10½-inch) and small (8-inch) animal jars are suitable for mice and rats; the large jar also can be used for a small guinea pig. The standard galvanized-iron animal cage (14 by 14 by 16 inches) will hold one rabbit or several guinea pigs. For use in breeding rabbits or guinea pigs, larger cages or pens, preferably with outside runways, should be built. The bottom of the jar or tray in cage should contain an absorbent bed material, such as wood shavings; hay or straw may be used in large breeding cages. Clean quarters and renew bedding twice per week.

218. Rabbits.—*a.* The diet recommended consists of commercial "Rabbit Pellets" supplemented once or twice per week with feeding of greenstuff, such as carrots, lettuce, or celery tops. A diet con-

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sisting of equal parts of oats, wheat, and barley, plus 10 percent of legume, soybean, or linseed meal is suitable. Alfalfa or timothy hay will serve both for food and bedding. Always keep plenty of water and a small piece of rock salt in the cage.

b. Diseases.—(1) "Coccidiosis," an intense and fatal enteritis, is the most serious disease. Observe new rabbits for this several days before adding to stock.

(2) "Ear mange" is caused by a mite; can be cured by local application of a parasiticide.

(3) "Snuffles" is a cold-like disease caused by a filterable virus. Isolate infected rabbits until 3 weeks after recovery.

c. Breeding.—Keep one male (buck) for each 8 to 10 females (does). Females are ready for mating at age of 10 months and may be bred every 3 months thereafter (4 litters per year). Keep record of date bred; gestation period 31 days; 2 or 3 days before expected arrival of litter place small breeding box and ample supply of bedding in cage. Wean young after 8 weeks and separate sexes.

219. Guinea pigs.—*a. Feeding.*—Same as for rabbits, except they must have supplementary feeding of greenstuffs at least twice per week to supply vitamin C.

b. Diseases.—(1) Salmonella infections, chiefly *Salmonella typhimurium* and *S. enteritidis*, are most dangerous of common diseases. Best method of control: Kill all potentially infected animals, sterilize room and cages, and obtain new stock.

(2) Vitamin C deficiency is caused by lack of sufficient greenstuffs in diet. Characterized by coarse hair and mangy appearance. It is transmissible to young through mother. Treatment: improved diet.

(3) *Balantidium coli* type of enteritis.

c. Breeding.—Use colony breeding with four or five females in cage with one male; duration of pregnancy, 63 days. Wean young and separate sexes when 4 or 5 weeks old.

220. Mice.—Several different strains are used, such as, white mice, Swiss mice (also white), and C 57 strain (black).

a. Feeding.—Commercial dog- or fox-chow checkers furnish an ample, balanced diet for growth and breeding; occasionally add piece of carrot or other greenstuff. Must have supply of fresh clean water in cage at all times. Mice will do well on simpler diets, such as the mixed grain diet listed above for rabbits, or dry bread with water or skimmed milk, with addition of cod liver oil once per week.

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b. Diseases.—Salmonella infections (mouse typhoid), caused by same organisms as for guinea pigs, are common and very dangerous. To control: Destroy all infected stock, sterilize room and cages, and obtain fresh stock.

c. Breeding.—Colony breeding, with four or five females to one male; gestation period, 21 days; when well advanced pregnancy is observed, place female in individual jar. After 21 days, isolate young and return mother to breeding jar. Feed young same as adults, but addition of evaporated milk to diet hastens growth.

221. Albino rats.—*a. Feeding.*—Same as for mice.

b. Diseases.—If cages are kept clean and ample diet provided, rats are very resistant to disease.

c. Breeding.—Young females are ready for breeding when 4 months old. Use colony method of breeding with four females and one male in cage; duration of pregnancy, 22 days; not necessary to remove pregnant female from breeding cage. Wean young and separate sexes when 21 days old.

222. Monkeys.—*a. Feeding.*—Monkeys will do very well on dog-chow checkers plus canned tomatoes, with occasional feeding of fruits and nuts (oranges, apples, bananas, peanuts, sunflower seeds, etc.).

b. Diseases.—(1) Pneumonia, usually fatal.

(2) Miliary tuberculosis.

c. Breeding.—Breeding in captivity in small laboratories is not practical.

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CHAPTER 8

METHODS OF STUDYING BACTERIA

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SECTION I

GENERAL

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General	181

223. General.—Micro-organisms may be studied and their characteristics determined by—

a. Direct microscopical examination.

b. Stained film examination.

c. Cultivation on artificial culture media.

d. Serological reactions—determination of certain immune substances, by agglutination, complement fixation, animal protection, and other tests.

e. Pathogenicity—the disease-producing capacity, if any, on man, animals, or plants.

SECTION II

DIRECT METHODS

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224. Hanging drop.—With a small applicator ring the concavity of a concave slide with vaseline. Place a loopful or small drop of bacterial suspension on the center of a clean cover glass. Invert the slide and place its concavity over the cover glass so that the drop of fluid lies in the center. Turn over the slide and its attached cover glass. Press the cover slip firmly in place to prevent evaporation. Place the slide on the microscope stage and, with the low-power

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lens, focus on the edge of the drop. Without changing the focus change to the high-dry objective, locate the drop, and focus on the bacteria. The oil immersion objective is used for still higher magnification. This permits the observer to see the micro-organism in the fresh state, to note some of its characteristics, particularly its motility if present, its size, shape, sometimes spores, capsules, and even to estimate its purity and observe its growth. It cannot be studied as well in this fresh state as when stained.

225. Cover glass.—Place a small loopful of the bacterial suspension on a clean slide; over it place a cover glass and gently press the latter until it no longer floats. Examine as above. This method is simpler than the hanging-drop method and for most purposes equally satisfactory. It has the advantage of permitting observation with darkfield as well as by ordinary illumination.

226. Darkfield.—The illumination principle of this method is comparable to that which causes dust particles to be illuminated in a ray of sunlight. A cover glass preparation (ringed with vaseline)

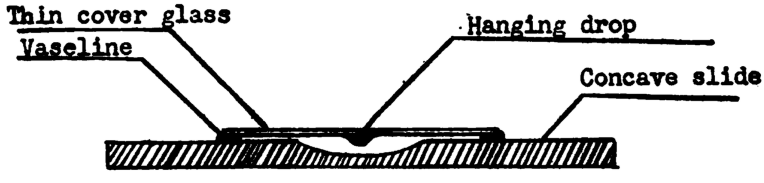


FIGURE 13.—Hanging drop.

of material under examination is prepared, using thin slide and cover glass, and is placed on a specially prepared microscope; a darkfield condenser replaces the ordinary condenser, a "funnel stop" is placed in the oil immersion objective, specially intense light is used, and immersion oil is placed between the slide and condenser as well as on top of cover glass. All highly refractile objects, including bacteria, will be seen as bright objects against a black background. This is particularly used in the study of spirochetes in order to observe their peculiar motility.

227. Brownian movement.—All micro-organisms, living or dead, and also all other microscopic objects, observed by above methods, will have a dancing, trembling motion in the field, which will be magnified just as much as is the organism itself; this is "Brownian movement." It is entirely a passive movement, just as is the bobbing up and down of a bubble on the surface of a pool.

228. Active motility.—This is a movement actively produced by the organism itself, causing it to change its position, often very rapidly, even to the extent of having individuals dart across the

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field, as if they were in a hurry to get away. Not all micro-organisms possess this quality of active motility, a feature of value in identification.

SECTION III

STAINING METHODS

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Special stains 230

229. Simple stains.—These are merely weak solutions of aniline dyes, applied to the slide for a brief period, then washed off. Those most commonly used for the staining of bacteria are methylene blue, fuchsin (red), crystal violet, safranin (pink), and bismarck brown.

230. Special stains.—More than a simple stain is required to make apparent the flagella, capsules, spores, and other special characteristics of a micro-organism. Differential stains utilize more than one dye, sometimes aided by a decolorizing agent and a "mordant" (any agent such as heat or phenol that will fix the dye more firmly); these include Gram's stain, acidfast stains (for tubercle and lepra bacilli), Neisser's stain (for diphtheria bacilli), and Wright's stain (for protozoa).

SECTION IV

CULTURE METHODS

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231. Cultural characteristics.—Under favorable conditions of food, moisture, and temperature, an organism multiplies to become, after many generations, a colony of many like organisms, visible to the naked eye. In fluid media these organisms scatter throughout the liquid, giving it a cloudy appearance, sometimes developing a color, odor, or appearance peculiar to that species. In solid media, such as agar, dispersion of the new growth is impossible; the organisms

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pile up into a mass (a colony) the size, shape, and color of which may characterize the species. The differences in growth on different media, the temperature at which it grows best, and the requirements as to the presence or absence of oxygen are also important characteristics of each kind of micro-organism.

232. Cultural requirements.—Food materials and conditions of life and growth must be suitable before an organism will grow. Disease-producing bacteria will utilize as food material substances resembling the fluids and tissues of the body; beef broth (infusion or extract) is a basic constituent, sodium chloride an essential salt, and agar a hardening substance, of general culture media. The reaction of the medium, or its degree of acidity or alkalinity must be suitably adjusted; most bacteria prefer a neutral or slightly alkaline medium, others prefer one that is acid. Some bacterial species are more fastidious—grow only in media containing some special enrichment substance. There are some bacteria for which the particular artificial media for cultivation have not yet been found.

233. Culture media.—This may be liquid or solid, the latter made so by the addition of gelatin or agar. As the melting point of gelatin is 28° C., it can be used only for cultivations at or below room temperature. The melting point of agar is about 99° C. and its solidifying point about 39° C., so agar may be used for those organisms which grow at body temperature (37° C.) or below. Certain substances may be added to media, not for nutrition but to gain some specific reaction of the organism. The final step in the preparation of culture media consists of killing all the living organisms in it, that is, sterilizing it so that any growth present following subsequent inoculation may be assured to be from the organism present in inoculum. A medium so prepared and sterilized will retain its serviceability until it desiccates, this tendency being reduced by storage in the ice box.

234. Culture planting.—Inoculation or transfer is usually accomplished with a wire needle (platinum) supported in a holder, the wire end being either straight or bent in a loop. This wire must be sterilized before and after use by heating to redness through an open flame. Occasionally a sterile cotton swab or pipette may be used for transfer of inoculum. Every effort must be used to make the transfer without risk of contamination.

235. Transfer from test tube to test tube.—The best technic is to hold both tubes in one hand while making the transfer so that inoculating needle may pass from tube to tube without risk of dust contamination. The steps of the inoculation are as follows:

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a. Sterilize needle (deposit upright in tumbler while preparing tubes).

b. Place both tubes in left hand and remove cotton plugs (plugs to be kept sterile, held between fingers of right hand).

c. Pass mouth of both test tubes through flame.

d. Enter needle into tube No. 1 to obtain inoculum.

e. Enter needle into tube No. 2 to plant bacteria.

(1) Liquid media merely receives a touch of needle loop.

(2) "Stab" agar receives a stab of straight needle.

(3) "Slant" agar receives surface stroke or broad brush of needle.

f. Sterilize needle.

g. Pass mouth of each tube through flame.

h. Replug tubes.

i. Label tubes with identification and date.

236. Pour plate transfer.—*a.* Agar in tube is melted and cooled to about 42° C. in water bath.

b. Inoculation is made as in paragraph 235*e*(1).

c. Inoculated agar is poured into sterile Petri dishes, agitated, and cooled (cover left partially off for a few minutes to permit water vapor to escape).

d. Result is dispersion of bacteria throughout media, surface and deep.

e. For individual colony study, three such tubes are inoculated in series and each poured as above.

237. Plate method.—*a.* Sterilize needle.

b. Touch tip of needle to specimen colony or culture to be transferred.

c. Streak gently the infected needle over agar surface.

(1) Several strokes of needle in one area.

(2) Sterilize needle.

(3) Several strokes of needle at right angles to and crossing (1) above.

(4) Sterilize needle.

(5) Complete strokes on plate area, crossing lines (3) above, avoiding lines (1) above.

d. Result: variable inoculation from heavy in (1) to scant in (3) area.

e. Modify procedure to a single massive streak if inoculum is known to be light.

f. Two to four different plants may be made on a single plate if caution is practiced not to permit the inoculation line to cross the plate sector line. This is frequently used to obtain additional growth of known pure cultures when well-isolated colonies are not required.

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238. Colony picking.—Having developed discrete colonies on either a pour agar or plate culture, a pure culture may be transferred by following steps:

a. Observe colony with hand lens and be assured that it is but one colony, well separated from other colonies.

b. Ring colony with wax pencil on bottom of plate.

c. Sterilize a straight needle.

d. Touch tip of needle to colony, checking by a hand lens, freedom from touching any other colony.

e. Inoculate selected culture mediate with this infected needle.

(1) *Liquid media.*—The needle is merely touched to media.

(2) *Plate media.*—The needle makes the first streak as for plate method (par. 237), the cross streak being made by another loop or bent needle.

f. Result: a culture of known purity, all from an original single micro-organism.

239. Incubation.—Cultures are placed for growth in incubators which maintain constant temperature. Most of the pathogenic bacteria grow well at a temperature of 37° C. Many saprophytic bacteria and fungi grow best at lower temperatures and may be incubated at 20° to 30° C. Cultures in gelatin must be kept below the melting point of that medium (28° C.) either in a "cold" incubator or at room temperature, or the gelatin culture may be incubated at 37° C. and later chilled to determine whether the medium will again solidify. The length of time for incubation required for different species of bacteria varies from 24 hours to several weeks.

240. Atmospheric conditions.—Bacteria differ greatly in their growth under differing amounts of oxygen in the atmosphere.

a. Aerobes are those which grow in the average atmosphere and therefore require no special provisions. Most pathogenic bacteria are in this group.

b. Micro-aerophiles require an atmosphere containing less oxygen than in *a* above.

c. Anaerobes require the absence of oxygen and require special provision for exclusion of oxygen.

(1) Obligate anaerobes strictly require absence of oxygen.

(2) Facultative anaerobes may grow under partial aerobic or anaerobic atmosphere.

241. Deep tube-tissue anaerobic method.—Deep tubes of weak agar containing a carbohydrate with or without piece of fresh, sterile animal tissue, may provide, at various levels, proper atmosphere for all but the strictest of anaerobes. Such tubes need to be boiled just

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before their inoculation to drive off the absorbed oxygen, then quickly cooled, and later may be covered with sterile petrolatum to preserve absence of oxygen. Such cultures are inoculated with a long, platinum needle or capillary pipette. They are used for maintenance of stock cultures rather than the isolation of individual bacteria from contaminated mixtures.

242. Anaerobic jar method.—The jar here described is a greatly modified "Brown Jar," omitting its electric heating unit. It can be made locally; the catalyzer by monthly restoration may be durable for several years.

a. Cultures, plates, or tubes, after inoculation, are placed in the jar, made and assembled as below, and entire jar placed in incubator for the required incubation period.

b. Jar—glass, round, museum type, about 15 inches high, 6 inches in diameter, with a glass lid.

c. Hole is drilled (dental drill will do it) in side of lid; a short length of 6-mm glass tube is sealed into this hole with sealing wax. Rubber tubing passes from this tube to bottom of jar.

d. Catalyzing capsule is suspended from under surface of lid.

To make catalyzing capsule, place 1.4 gm of asbestos wool in porcelain evaporating dish. Add 10 cc of 10 percent platinum chloride (with some HCl to aid solution). Stir this into the asbestos wool with spatula. Dry slowly in incubator or hot-air oven. Carbonize in yellow flame (thoroughly, with frequent stirring of wool). When well coated with soot, heat to glowing by use of hot flame to reduce the mixture. Place this platinized asbestos between two layers of very fine wire screen. Complete capsule by sewing rim of double-layer wire screen with wire. Reactivate capsule monthly by thoroughly carbonizing it in yellow flame, then heating it to a red glow.

e. Indicator in a small test tube is attached to inside wall of jar—alkaline dextrose broth with just enough methylene blue to give slight blue color. Anaerobiasis is indicated by complete decolorization of the methylene blue (after overnight exposure).

f. Molding clay (such as in children's play sets) is placed around rim of jar, readjusted each time jar is used.

g. Culture plates or tubes are placed in bottom of jar.

h. Lid is applied to molding clay, loosely at first, to permit release of oxygen at first as hydrogen first enters, then pressed to airtightness and held in place by clamp after partial replacement has occurred.

i. Connection is made, as in figure 14, to a hydrogen supply.

j. Hydrogen is provided by Kipp generator, storage tank, or improvised generator.

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k. Kipp generator production of hydrogen:

(1) Moss zinc is placed in central bulb.

(2) HCl, diluted with 2 parts of water, or 20 percent H_2SO_4, is poured into upper bulb, passes into bottom chamber, and rises to contact zinc.

(3) Wash bottle is placed in series between generator and jar.

l. Flow continues until no more hydrogen enters jar. Action of catalyzer is evidenced by vapor within jar and the warming of the catalyzer. If catalyzer becomes very hot, the flow of hydrogen is to be reduced. If catalyzer fails to become warm, it requires reactivation.

m. Jar is disconnected from hydrogen source and placed in incubator.

n. Care is required to prevent explosion. Allow most of the oxygen to escape from the jar before clamping down lid. Do not allow the

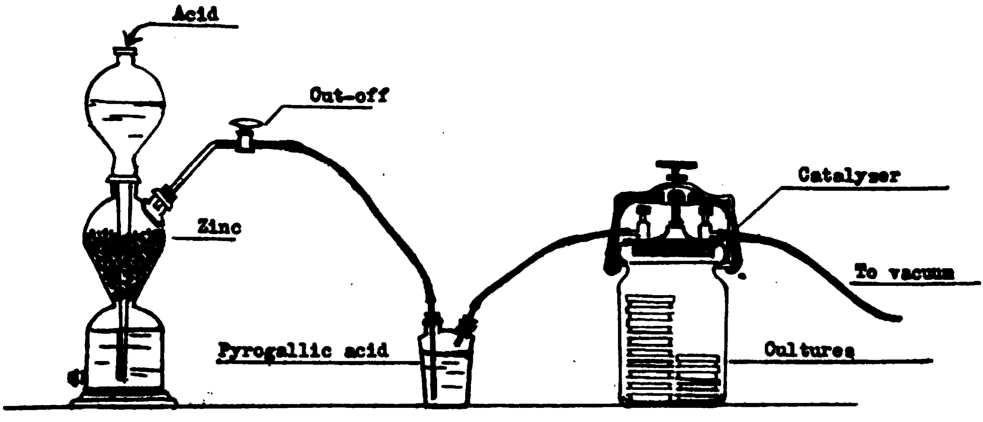


FIGURE 14.—Anaerobic jar showing Kipp generator, wash bottle, and anaerobe jar containing cultures.

capsule to reach a red heat. In opening the jar after incubation, avoid sudden inrush of air.

243. Partial oxygen tension method.—Certain organisms, including some *Brucella* and *Neisseria*, grow best on primary culture in an atmosphere containing 10 percent CO_2. This may be provided as follows:

a. Jar, museum type, with lid, about 5 inches in diameter and 8 inches high.

b. Measure inside volume and permanently label jar.

c. Calculate amounts of reagents required for CO_2 production and label jar.

(1) Na_2CO_3—0.24 gm per liter.

(2) Ten percent H_2SO_4—4 cc per liter.

d. Place culture plates and tubes in jar.

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e. Place the reagents into a small, open container in jar.
f. When reaction begins to subside, place airtight cover on the jar.
g. Place jar in the incubator.

SECTION V

SEROLOGICAL METHODS

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Macroscopic agglutination test (preferred method) 245
Microscopic agglutination test 246
Rapid slide agglutination test (presumptive only) 247

244. Antigen-antibody reaction.—*a. Purposes.*—(1) Identification of bacterial species by using known sera.

(2) Identification of specific antibodies in sera by using known bacterial antigens.

b. Materials used for agglutination tests.—(1) Immune serum containing specific agglutinins or serum of unknown content.

(2) Antigen (suspension of either living or dead bacteria).

(3) Electrolytic solution such as 0.85 percent sodium chloride.

(4) Series of test tubes.

c. Results of agglutination test.—When antigen and serum are mixed in proper proportion and incubated, the organisms become clumped, recognizable by microscopic or naked eye examination. To be of diagnostic significance, agglutination must occur in certain measured dilutions of serum which are sufficiently high to rule out nonspecific reactions.

245. Macroscopic agglutination test (preferred method).—*a. Antigen preparation.*—(1) Antigens are prepared by making an even suspension of young agar slant cultures of the known or unknown organism, so diluted with salt solution that news print can just be read through the final test-tube suspension.

(2) Stock antigens may be kept in heavy, sterile suspensions and diluted to proper strength as required for test.

b. Technic.—(1) Series of small test tubes (10 or less) are placed in a metal rack.

(2) Salt solution, 0.9 cc in first tube, 0.5 cc in other tubes.

(3) Serum 0.1 cc added to the first tube (=1 cc of 1:10 serum). Mix.

(4) Remove 0.5 cc from tube No. 1 and add to tube No. 2; mix (=1 cc of 1:20 serum).

(5) Remove 0.5 cc from tube No. 2 and add to tube No. 3 and so through all tubes except last.

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(6) Discard 0.5 cc from next to last tube after mixing. Last tube is salt solution control.

(7) Series may extend to more tubes if necessary to extend beyond titer of the serum.

(8) Bacterial suspension 0.5 cc is added to each tube. Shake.

(9) Final serum dilutions are 1/20, 1/40, 1/80, 1/160, 1/320, 1/640, 1/1280, 1/2560, 1/5120, 0.

c. Incubation.—(1) *Temperature.*—The optimum is 50° to 55° C. in water bath; 37° C. or even room temperature are admissible for large-clumping, motile bacteria when 50° to 55° is impossible. For small-clumping suspension 37° C. is not desirable.

(2) *Time.*—(*a*) *For motile bacteria.*—Two hours at 50° to 55° C., 8 hours at 37° C., or 24 hours at room temperature.

(*b*) *For nonmotile bacteria.*—Four to twenty-four hours at 50° to 55° C., or 24 hours at 37° C.

(*c*) *For "O" suspensions.*—Twenty-four hours at 50° to 55° C.

d. Reading.—(1) Preliminary reading of agglutination in 2 hours, final in 24 hours.

(2) Control tubes should show no clumping.

(3) Negative (−) tubes show no clumping.

(4) Positive tubes show clumped and sedimented organisms.

++ reaction = completely clumped and sedimented.
+ reaction = half clumped and sedimented.
+− reaction = less than half clumped and sedimented.

(5) Each tube is separately read and recorded.

(6) Titer of the serum is the highest dilution which shows complete agglutination, e. g., "positive in serum dilution 1 : 640."

(7) Notation made whether agglutination is floccular or granular type.

246. Microscopic agglutination test.—*a.* Place 1 drop of serum in a watch crystal or small tube.

b. Add 9 drops of 0.85 percent salt solution. Mix (1:10).

c. Transfer 5 drops to second tube. Add 5 drops of salt solution (1:20).

d. Similarly prepare a third, fourth, or more dilutions.

e. Place loopful of each dilution on separate cover glasses.

f. Add loopful of living broth culture of organism to each of *e* above and mix.

g. Place each cover glass over a ringed concave slide (see hanging-drop method, par. 224).

h. Similarly prepare a control having only salt solution and broth culture.

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i. Observe with the high-dry objective for loss of motility and clumping of the bacteria, compared to the control.

j. Make final reading at the end of 1 hour.

k. Record as positive highest titer giving microscopic clumping.

247. Rapid slide agglutination test (presumptive only).—*a.* Prepare 1: 50 dilution of a known high titer antiserum.

b. Place drop of this on a slide. Another drop of salt solution is placed nearby.

c. Emulsify a loopful of bacteria from an agar slant culture with each drop.

d. Observe under low power of microscope for presence or absence of clumping in the mixture of bacteria and antiserum, and the smoothness of the emulsion (absence of clumps) in the salt solution control.

SECTION VI

ANIMAL EXPERIMENTATION

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248. Purposes.—*a.* General study of pathogenic micro-organisms.

b. Virulence determination of a specific organism for a definite animal species.

c. Lesions produced are a characteristic, identifying some species.

d. Isolation of such organisms as are not readily grown initially on culture media.

e. Immunity studies.

f. Maintenance of such bacteria or viruses as cannot be kept on culture media.

249. Animals used.—*a. Routine use.*—Guinea pig, rabbit, white mouse, and white rat.

b. Occasional use.—Horse, monkey, fowl, canary, and ferret.

250. Methods of inoculation.—*a.* Subcutaneous (under the skin).

b. Intracutaneous (into the skin).

c. Intramuscular (into the muscle).

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d. Intravenous (into a vein).

e. Intraperitoneal (into the abdominal cavity).

f. Intrapleural (into the pleural cavity).

g. Intracerebral (into the brain).

h. Subdural (into membranes of brain or spinal cord).

i. Per os (by mouth).

j. Per rectum (by rectal injection).

k. Other channels of application.

251. Subcutaneous inoculation of guinea pig.—*a.* Choice of site is the abdominal wall or inner thigh.

b. Remove hair from site by pulling it out, small tufts at a time, or by shaving.

Depilatory paste (alternate method, to be done day prior to injection): Barium sulfide and cornstarch, equal parts, with water enough to make a thin paste. Apply to part for 3 to 4 minutes with applicator, then wash off and dry.

c. Sterilize skin site with tincture of iodine, alcohol, or other germicide.

d. Prepare inoculum in glass syringe with fine-gage needle.

e. Plunge needle into skin while area is raised between the fingers, so as to avoid penetrating the abdominal wall into peritoneal cavity.

252. Intraperitoneal inoculation.—*a.* Preparation is as for above.

b. Plunge needle through abdominal wall by two movements:

(1) Through the skin in an oblique direction.

(2) Through the muscle and peritoneum, by a very short and careful vertical stab.

c. Exercise care not to puncture the gut.

253. Intravenous inoculation of rabbit.—*a.* Choice of site, veins along margins of ears.

b. Hair over the site is shaved and animal head held downward for short time.

c. Animal is held by assistant or placed in animal box, head out.

d. Ear is placed in position over a light, base of ear slightly compressed (both devices will make the veins more prominent).

e. Holding tip of ear with left hand, operator inserts needle with right hand, the needle entering vein nearly parallel to its course.

f. Injection is started slowly, until assured that inoculum is entering vein; if it passes along course of vein by perivascular leakage, another puncture of vein is attempted.

254. Mouse inoculation.—*a. Intraperitoneal.*—(1) Mouse is held in one hand by fingers and thumb grasp to back of neck, root of tail

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held between little finger and palm, the mouse body resting in palm.

(2) Needle puncture is made into abdomen by a quick, short jab.

b. Subcutaneous.—(1) Mouse is placed in a jar, tail held out, with jar top holding mouse within.

(2) Injection is made under the skin near the base of the tail.

(3) Caution is to be practiced against injuring mouse by this holding method.

c. Intravenous.—Injection is made with a fine needle into vein of tail.

255. Bleeding of animals.—*a. Rabbit.*—To obtain a few cubic centimeters of blood from ear:

(1) Ear is shaved and immersed in warm water (to expand vessels).

(2) Thrust is made into vein with a broad, cutting needle of Hagedorn type.

(3) Blood is caught in glass tube.

b. Rabbit or guinea pig.—To obtain blood from heart:

(1) Animal is held in place (with or without anaesthesia) resting on its back.

(2) Left anterior chest is shaved and painted with iodine.

(3) Twenty-two gage, 2-inch needle, on a glass syringe is used.

(4) Needle is passed downward into heart, at third intercostal space, close to left sternum. Slight suction is made by syringe plunger until blood appears.

(5) Needle is quickly removed after sufficient blood is withdrawn.

(6) Animal is restored to its cage for several weeks rest before reuse.

c. Rat and mouse.—To obtain blood from tail:

(1) Animal is placed in a small box, with tail out and held.

(2) Cut made at tail end with scissors.

(3) Stripping movement of tail from base will produce a few drops of blood at tail tip.

d. Horses and sheep.—To obtain blood from veins: Large needle is inserted into external jugular vein in neck which runs from a line just behind the angle of the lower jaw to the sternoclavicular junction.

256. Blood handling for various purposes.—*a. Whole blood for culture media.*—The blood may be placed at once into media.

b. Citrated blood for culture media.—The blood is placed at once into a sterile flask containing an anticoagulant, such as enough sodium citrate to have 0.25 percent concentration after blood is added.

c. Defibrinated blood for culture media or serum.—The blood is placed at once into sterile flasks containing glass beads and defibrinated by several minutes of gentle shaking, then left on ice until used or separated.

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d. Serum for serological tests.—The blood is placed into tubes or plates, allowed to clot in the ice box, and the serum removed after clot has contracted. Centrifugalization may be required to remove all of the cells.

257. Post morten examination of small animals.—*a.* Perform autopsy as soon after death as possible, especially if cultures are to be made.

b. Instruments required are—

(1) Scissors, 1 point sharp, 4½-inch for larger animals; or scissors, strabismus, straight and scissors, iris, curved for mice.

(2) Sharp scalpels.

(3) Tissue forceps.

(4) Hemostatic forceps.

(5) Autopsy board.

(6) Thumb tacks.

(7) Instruments, such as bone forceps, bone saw, and curettes for special examinations. Use sterile instruments if cultures are to be made.

c. Sterilize surface of small animals by immersing entire animal, except nose and mouth, in 3 percent cresol solution; sterilize desired portion of larger animals with cresol or iodine after step *d* below.

d. Tack down small animals through each foot to a wooden board; larger animals are tied down with abdomen up by each foot on autopsy pan or board.

e. Snip skin of abdomen in pubic region, insert blunt point of scissors, and make cut along median line to inferior maxillary; cut the skin on each side at a right angle to the ends of the median cut and continue out the legs to expose the inguinal and axillary lymph glands.

f. Using tissue forceps and blunt point of scissors, separate skin from abdomen and retract; examine exposed tissue and glands for abnormality.

g. If sterility is desired, paint abdominal wall with iodine and/or wash with acetone-alcohol, and use set of sterile instruments for further examination.

h. Open abdomen by making median incision, as in *e* above, from genital region to the diaphragm, followed by terminal lateral cuts; lay back abdominal wall as flaps on each side of animal.

i. Examine abdominal organs for gross pathology, prepare smears, and make cultures.

j. To enter thorax, first cut the diaphragm free from the thoracic cavity; then cut ribs on each side, beginning at the floating ribs at

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an angle up to the suprasternal notch; remove this section or lay it back to expose heart and lungs. Study for presence of pathological lesions and prepare smears and cultures.

k. Place organs or tissues to be given histopathological study in a bottle of 10 percent formalin or Zenker's fluid.

l. Remove animal from the board, wrap it firmly in paper, and dispose of it as circumstances permit, preferably by immediate incineration.

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CHAPTER 9

PATHOGENIC BACTERIA

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SECTION I

CLASSIFICATION OF BACTERIA

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258. General.—*a.* Bergey's "Manual of Determinative Bacteriology" provides classification system and criteria for bacteriology. The genera and species names used in the following paragraphs are those found in the fifth edition (1939) of that manual. The outline classification, herein given, omits many groups, including only those species mentioned elsewhere in this text. For brevity and simplicity, no effort is made to keep each genus in its proper tribe or family as shown by Bergey. Bacteria in this key are classified, first, on basis of morphology of organism, second, on Gram-staining qualities, and third, on basis of other special characteristics.

b. The full name of a bacterium consists of the name of the genus with initial letter capitalized, followed by the name of the species in small letters, with the entire name underscored when written, italicized when printed. The name of the genus may be abbreviated.

259. Cocci.—Cells spherical or somewhat elliptical; aerobic.

a. Gram positive.—(1) *Streptococcus forms.*—Cells in short or long chains, never in packets. Genus *Streptococcus.*

(*a*) Pyogenic group; generally (beta) hemolytic; four species and several serological types. Most important species, *S. pyogenes.*

(*b*) Viridans group; not beta hemolytic but may show varying degrees of greening (alpha) of blood. Most important species, *S. salivarius.*

(*c*) Saprophytic group; no hemolysis or only indistinct zone (alpha prime type); usually not pathogenic. Example, *S. faecalis.*

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1 and 2. Long and short bacilli, singly, in pairs, and short chains.
3. Types of flagellated bacilli.
4. Various sizes, shapes, and positions of bacterial spores.
5. Fusiform bacilli.
6. *C. diphtheriae*, banded forms and polar bodies.
7. *Past. pestis*, bipolar staining rods and involution forms.
8. *a, Borrelia. b, Treponema. c, Leptospira.*
9. *D. pneumoniae*, encapsulated.
10. Diplococci.
11. Biscuit-shaped diplococci of *Neisseria.*
12. Tetrads.
13. Staphylococci.
14. Streptococci.
15. *Vibrio comma.*

FIGURE 15.—Morphology of bacteria.

(2) *Diplococcus forms.*—Cells usually in pairs; alpha hemolysis; colonies greenish on blood agar. *Diplococcus pneumoniae.*

(3) *Micrococcus forms.*—Cells in plates, groups, or irregular packets or masses, never in chains; usually nonpathogenic. Genus *Micrococcus.*

(4) *Staphylococcus forms.*—Cells as a rule in irregular groups; usually pathogenic. Genus *Staphylococcus.*

(*a*) Orange pigment. *Staph. aureus.*

(*b*) Lemon-yellow pigment. *Staph. citreus.*

(*c*) White or colorless growth on solid media. *Staph. albus.*

(5) *Tetragena forms.*—Occur in pairs and tetrads. *Gaffkya tetragena.*

(6) *Sarcina forms.*—Division occurs in three planes, producing regular packets. Example: *Sarcina lutea.*

b. Gram-negative.—Cells normally in pairs, with adjacent sides usually flattened. Genus *Neisseria.*

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(1) Grow best on special culture media at 37° C.

(*a*) Acid from dextrose, not from maltose. *N. gonorrhoeae.*

(*b*) Acid from dextrose and maltose. *N. intracellularis.*

(2) Grow well on ordinary culture media at 22° C. Usually not pathogenic. Several species. Examples:

(*a*) Nonchromogenic, *N. catarrhalis.*

(*b*) Chromogenic, *N. flava.*

260. Curved forms.—Cells elongated, more or less spirally curved, Gram-negative. *Vibrio comma.*

261. Nonsporulating bacilli.—Elongated, rod-shaped cells, without endospores. Gram-positive or Gram-negative. Aerobic.

a. Gram-positive.—Long, slender, nonmotile rods, occurring singly, in pairs, and in chains. Genus *Lactobacillus.*

b. Gram-negative.—(1) Small, motile or nonmotile rods; not active in fermentation of carbohydrates; usually parasitic on warm-blooded animals; frequently require body fluids for growth. (Family *Parvobacteriaceae*).

(*a*) Majority grow on ordinary media; show bipolar staining; majority ferment carbohydrates. Tribe *Pasteurelleae*, genus *Pasteurella.*

1. Grow on ordinary media; indol and H_2S produced; no growth in bile; sorbitol fermented. Animal pasteurellas (5 species).

2. Grow on ordinary media; neither indol nor H_2S produced; growth in bile; sorbitol not fermented. *Pasteurella pestis.*

3. No growth on ordinary media. *Pasteurella tularensis.*

(*b*) Majority grow on ordinary media; do not show bipolar staining; none ferment carbohydrates; nonmotile. Three species of genus *Brucella* are:

Species	Growth in media containing	
	Thionin	Basic fuchsin
Br. melitensis	+++	+++
Br. abortus	−−−	+++
Br. suis	+++	−−−

(*c*) On first isolation require some factor or factors contained in blood or plant tissues. Usually do not show bipolar staining. Genus *Hemophilus.*

1. X and V factors required—*H. influenzae.*

2. Neither X nor V factors required—*H. pertussis* and *H. ducreyi.*

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(2) Motile or nonmotile rods widely distributed in nature. Majority of species attack carbohydrates forming acid, or acid and gas. Grow well on artificial media.

(*a*) Do not produce acid in media containing carbohydrates.

1. Rounded colony, no pigment produced. *Alcaligenes faecalis.*

2. Large spreading colony, yellowish-green pigment produced. *Pseudomonas aeruginosa.*

(*b*) Ferment dextrose and sucrose but not lactose with formation of acid and small amount of gas. Produce characteristic spreading, amoeboid colonies. Liquefy gelatin. *Proteus vulgaris.*

(*c*) Ferment dextrose and lactose with formation of acid and gas. So-called coli-aerogenes group.

NOTE.—See table XIII showing differential characteristics of this group in discussion of coli-aerogenes group (sec. II).

(*d*) Ferment dextrose with formation of acid, or acid and gas. A few species of genus *Shigella* ferment lactose with formation of acid, but never visible gas. Tribe *Salmonelleae.*

1. Ferment dextrose with formation of acid and gas. Genus *Salmonella.*

2. Ferment dextrose with the formation of acid but no gas.

(a) Motile, *Eberthella typhosa.*

(b) Nonmotile, Genus *Shigella.*

Salmonella—typhoid-dysentery group

Species	Dextrose	Mannitol	Maltose	Xylose	Dulcitol	Lactose	Saccharose	Inositol	Indol	H_2S production	Citrate utilization	d-tartrate utilization	Motility
Salmonella choleraesuis	AG	AG	AG	AG	(AG)	—	—	—	—	—	+	+	+
Salmonella pullorum	AG	AG	—	(AG)	—	—	—	—	—	+	---	—	—
Salmonella paratyphi	AG	AG	AG	—	(AG)	—	—	—	—	(+)	—	—	+
Salmonella enteritidis	AG	AG	AG	AG	AG	—	—	—	—	+	+	+	+
Salmonella schottmuelleri	AG	AG	AG	(AG)	(AG)	—	—	(AG)	—	+	+	—	+
Salmonella typhimurium	AG	AG	AG	AG	AG	—	—	(AG)	—	+	+	+	+
Eberthella typhosa	A	A	A	(A)	(A)	—	—	—	—	+	—	+	+
Shigella dysenteriae	A	—	—	—	—	—	—	—	—	—	—	---	—
Shigella paradysenteriae	A	A	(A)	—	—	—	(A)	—	+	—	—	---	—
Shigella alkalescens	A	A	A	A	A	—	(A)	—	+	---	—	---	—
Shigella sonnei	A	A	A	—	—	A	A	—	—	---	—	---	—
Shigella madampensis	A	A	A	A	—	A	A	—	+	—	—	---	—

NOTE.—Parentheses in symbols denote variable or delayed reaction.

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262. Sporulating bacilli.—Rods producing endospores, usually Gram-positive. Often decompose protein media actively.

a. Grow aerobically.—Mostly saprophytes. Genus *Bacillus.*

(1) *Pathogenic forms.*—Nonmotile rods with square-cut to concave ends, occurring in long chains; central spores. *Bacillus anthracis.*

(2) *Nonpathogenic forms.*—Usually motile, having central or excentric spores. *B. subtilis* group (145 species).

b. Grow only anaerobically.—Often parasitic. Genus *Clostridium.*

(1) *Nonmotile rods.*—Rods not swollen at sporulation; spores central or excentric. *Cl. perfringens.*

(2) *Motile.*—Rods swollen at sporulation.

(*a*) Spores terminal or subterminal. Spherical or nearly so. *Cl. tetani.*

(*b*) Spores oval, central, or excentric.

1. Pathogenic to man—due to preformed toxin.

(a) *Cl. parabotulinum.*

(b) *Cl. botulinum.*

2. Pathogenic to man—associated with gas gangrene.

(a) *Cl. novyi.*

(b) *Cl. septicum.*

(c) *Cl. bifermentans.*

(d) *Cl. histolyticum.*

(e) *Cl. fallax.*

3. Not pathogenic to man; many species. Examples:

(a) *Cl. sporogenes.*

(b) *Cl. tertium.*

263. Bacilli having branching characteristics.—Show parallelism, slight branching, curving forms, V-shapes, clubbing at ends, and segmental staining. Gram-positive.

a. Not acidfast.—Colonies more flat and moist like other bacteria; rods frequently club-shaped. Genus *Corynebacterium.*

(1) True diphtheria organism. Slender rods, curved or straight, of variable lengths; granular or segmented; generally club-shaped. Metachromatic granules large except in *gravis* type. Moderate growth on ordinary media. *C. diphtheriae.*

(2) The diphtheroid group of bacteria; 20 species.

(*a*) Short, thick, straight rods; stain uniformly; luxuriant growth on ordinary media. *C. pseudodiphthericum.*

(*b*) Medium-sized rods showing solid and barred forms; metachromatic granules small; scanty and slow growth on ordinary media. *C. xerose.*

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Species	Dextrose	Maltose	Dextrin	Glycerol	Galactose	Saccharose	Litmus milk	Production of an exotoxin	Production of hemolysis
C. diphtheriae, type I	+	+	+	−	−	−	−	+	+
C. diphtheriae, type II	+	−	−	−	−	−	−	+	+
C. diphtheriae, types III, IV, V	+	+	+	+	+	−	−	+	+
C. pseudodiphthericum	−	−	−	−	−	−	−	−	−
C. xerose	+	+	−	−	+	+	−	−	−
C. segmentosum	+	+	−	+	−	±	acid	−	−

b. Acidfast.—Colonies more or less wrinkled and dry, more like molds. Slender rods, seldom filaments, which are stained with difficulty, but when once stained are acidfast. Cells sometimes show swollen, clavate or cuneate forms and sometimes even branched forms. Genus *Mycobacterium.*

(1) Saprophytes, or parasites on cold-blooded animals; grow rapidly on most media at room temperature (8 species). Examples:

(*a*) *M. lacticola.*

(*b*) *M. phlei.*

(2) Parasites on warm-blooded animals; grow slowly on all media.

(*a*) Pathogenic for man.

1. M. tuberculosis variety *hominis.*

2. M. tuberculosis variety *bovis.*

(*b*) Not pathogenic for man. *M. avium.*

(3) Pathogenic for man. Will not grow on usual culture media. *M. leprae.*

SECTION II

BACTERIA OF MEDICAL IMPORTANCE

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264. General.—A brief description of the principal pathogenic bacteria and of certain nonpathogenic species commonly encountered in the examination of pathological materials is presented in this section. Herein will be shown their important generic and specific char-

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acteristics and the chief laboratory procedures required for their identification, or for the identification of the infections in man caused by them. Organisms possessing similar morphological and Gram-staining properties or, in case of the enteric group, bacteria frequently found associated in pathological specimens, are presented together without regard to the sequence of presentation in the Bergey classification, with the exception that genera belonging to the same family are discussed together.

265. Genus Staphylococcus.—*a. Habitat.*—Common, potential or actual parasites, occurring on normal skin and body orifices, and in feces, therefore in dust, soils, and as culture contaminants; frequently the cause of suppurative lesions in man.

b. Characteristics.—Moderate size cocci, in pairs or grape-like clusters; Gram-positive; grow freely, aerobically, on common culture media, giving in 24 hours at 37° C. medium size, low, convex, smooth, glistening colonies with an even edge; color of colony variable with species; some strains produce hemolysis on blood agar.

c. Staphylococcus aureus.—Golden-yellow colony; usually hemolytic; frequently found in boils, carbuncles, and other skin lesions; sometimes in blood cultures in the event of septicemia.

d. Staphylococcus albus.—Porcelain-white colony; feebly pathogenic.

e. Staphylococcus citreus.—Lemon-yellow colony. A nonpathogenic saprophyte.

f. Identification.—(1) *Microscopy.*—Gram + staphylococci on direct or culture-stained spread.

(2) *Culture.*—Blood agar plate, 24 hours at 37° C. gives colony features of staphylococcus, species determined by color of colony. Note also presence or absence of hemolysis.

266. Genus Streptococcus.—*a. Habitat.*—Common pathogenic forms; also frequently on skin and body orifices without invasive tendency. Some species are the specific cause of infectious diseases. A number of saprophytic species are commonly present in dairy products and elsewhere.

b. Characteristics.—Gram-positive cocci of medium size, in pairs or short chains, never in packets; grow best on blood or serum agar, aerobically, at 37° C., the 24-hour colony being small, circular, slightly raised, and surrounded at times by zone of hemolysis. Killed at 55° C., in 30 minutes.

(1) Hemolytic group (beta type) have clear zone of hemolysis around colony on blood agar.

(2) Viridans group (alpha type) have greenish zone around colony on blood agar.

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(3) Nonhemolytic group (gamma type) have no area of hemolysis or green zone around colony.

c. Streptococcus pyogenes.—Colonies have beta zone of hemolysis 2 to 3 mm wide. Grow in long chains. Found in man in acute inflammations, including septicemia, cellulitis, wound infections, middle ear or sinus disease or elsewhere. Tend to be more severe and generalized than *Staph, aurens* infections. The cause of scarlet fever; transmitted by nose, throat, and skin contaminations from cases or carriers, and of erysipelas.

d. Streptococcus salivarius (*S. viridans, S. mitior*).—This species is a parasite of the normal nose and throat, also encountered in dental abscesses, in endocarditis, and in some blood cultures. Grow in short chains. This colony readily recognized on a blood agar plate by its greenish zone of hemolysis. Usually not pathogenic for small animals. Distinguished from *Diplococcus pneumoniae* by inability to ferment inulin and by not being bile soluble.

e. Streptococcus lactis.—Is nonpathogenic. Occurs in milk and milk products and in mouth and intestinal tract of man. Colonies on blood plates produce no hemolysis or only trace of green.

f. Streptococcus faecalis.—Is feebly pathogenic, found in feces of man and other animals. Sometimes found in inflammatory exudates and subacute endocarditis. No hemolysis on blood agar.

g. Identification.—(1) *Microscopy.*—Gram stain of direct or culture spreads will show Gram + cocci, singly, in pairs or in chains of varying length. The chain form is best seen in spreads made from liquid culture, or in liquid body fluids.

(2) *Culture.*—Blood agar plates at 37° C. for 24 hours will give the small colony type and form of hemolysis classifying roughly the species. For routine clinical work the examination is usually limited to the study of colonies on blood agar and the results reported as the case may be:

(*a*) Streptococcus, hemolytic.
(*b*) Streptococcus, nonhemolytic.
(*c*) Streptococcus viridans.

267. Diplococcus pneumoniae (pneumococcus).—*a. Characteristics.*—Large lancet-shaped cocci, usually occurring in pairs; sometimes found singly or in short chains. When in pairs, the adjacent ends of the cocci are usually bluntly rounded, and the opposite ends acutely pointed. In films from sputum, blood, and cultures on serum containing media, a definite capsule can be seen. Gram-positive stains well with aniline stains and special capsule stains. Poor growth on plain agar; grows best on blood or serum agar with pH

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7.6 to 7.8. Colonies on blood agar plate, surface flat and smooth with edge sharply raised from the medium, surrounded by a narrow zone of alpha-hemolysis (green discoloration); some stains (types III and VIII) give characteristic mucoid colonies. Killed in 20 minutes or less at 55° C. Bile soluble and ferments inulin. Thirty-one distinct serological types have been identified; called *D. pneumoniae* type I, II, etc., to XXXIII; however, types 26 and 30 apparently are identical with types 6 and 15, respectively.

b. Habitat.—The principal cause of lobar pneumonia (over 90 percent); also may cause bronchitis, bronchopneumonia, conjunctivitis, otitis media, brain abscess, meningitis, endocarditis, and arthritis. Frequently present in normal mouths. Highly pathogenic for mice and slightly less so for rabbits.

c. Identification.—(1) *Direct microscopy.*—Make spreads of specimen on slide, fix, and stain by Gram's method and/or Hiss' capsule stain. Examine for diplococci showing typical morphology; if present confirm by procedures below.

(2) *Typing by Neufeld reaction.*—This is the rapid method of choice for identification of type on materials direct from the patient, giving the type within 30 minutes. It is less applicable to typing of cultures or to detection of type in patients who have received one of the sulfanilamide compounds. Only after pneumococci have been shown by stained spread to be present in appreciable numbers, is this typing effort to be attempted.

(*a*) *Collection of specimen.*—Small sample of sputum, carefully coughed up by the patient from deeper air passages, as free as possible of saliva, is collected in a Petri dish or wide-mouthed bottle and should be typed within 1 hour of collection. Older specimens may be examined only if they have been kept on ice. Preferably, the sample is to be collected before beginning treatment with sulfapyridine (which interferes with this test). Samples with few pneumococci, or which otherwise give poor results by this test may be inoculated intraperitoneally into a mouse, and the mouse's peritoneal washings 6 to 18 hours later used for this or other typing effort. Specimens of spinal fluid and cultures in blood or serum broth also may be typed directly.

(*b*) *Materials.*

1. Platinum loop, 1-mm, for transferring sputum.
2. Platinum loop, 4-mm, for serum and dye.
3. Loeffler's alkaline methylene blue (not required if dye is already in type serum).
4. Glass slides and cover glasses.

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5. Typing sera (rabbit): types I to XXXIII monovalent serum and group mixtures; mixtures A (types 1, 2, and 7); B (3, 4, 5, 6, 8); C (9, 12, 14, 15, 17); D (10, 11, 13, 20, 22, 24); E (16, 18, 19, 21, 28), and F (23, 25, 27, 29, 31, 32). These sera may be in capillary tubes, each with enough for one test, or in small bulk bottles.

(*c*) *Technic of test.*

1. Divide three clean slides into halves by wax pencil and label halves "A, B, C, D, E, and F."

2. Place tiny fleck of sputum in center of an area, with small loop.

3. Add typing serum, about five times as much as specimen used.

4. Add large loopful of methylene blue. Mix thoroughly and apply cover slip.

5. Let stand for 5 minutes (prepare other slides while waiting).

6. Examine under oil immersion objective for dark-blue diplococci surrounded by unstained area with definite outline. Only a small indistinct capsule can be seen around the pneumococci mixed with heterologous antisera. Large distinct halo surrounds the pneumococci which have been mixed with their type antisera. If none of the mixtures are found positive at first examination, yet pneumococci have been shown to be present by stained spread, reexamine these slides from time to time over a period of 30 minutes.

7. Positive mixtures having been determined, repeat the test procedure with each serum contained in that mixture, until the positive type or types have been determined.

(3) *Culture.*—Streak specimen (sputum, pus, peritoneal washings, or mouse's heart blood) on blood plate and incubate 24 hours at 37° C.; then select well-isolated, typical greenish colonies and examine on stained slide for morphology. Transfer suspect colonies to calcium carbonate broth, incubate for 24 hours, and examine by confirmatory tests.

(4) *Animal inoculation* (applicable when other tests are incomplete).—(*a*) Select small piece of tenacious sputum, wash through three changes of sterile saline, and emulsify in mortar with sterile saline.

(*b*) Inject mouse intraperitoneally with 1 cc of suspect emulsion

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or culture. The pneumococci grow rapidly in the peritoneum while most other bacteria die off.

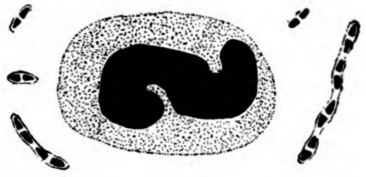
(*c*) When mouse appears sick, or after 6 to 8 hours, withdraw a drop of peritoneal exudate by a capillary pipette or hypodermic needle punctured through the abdominal wall. Examine microscopically.

(*d*) If diplococci are numerous, kill the mouse and use peritoneal washings for typing and culturing; also make culture from heart's blood.

(*e*) If diplococci are not numerous on preliminary trial, retest in 24 hours or when mouse becomes definitely sick.

(5) *Confirmatory tests.*—When indefinite results are obtained by the above tests, the identity of a pure culture of the suspect organism can be confirmed by the following examinations:

(*a*) *Bile solubility test.*—Add 0.2 cc of sterile bile to 0.8 cc of culture; prepare a control by adding 0.2 cc of sterile saline to 0.8 cc



① Type I pneumococcus in sputum mixed with type II antiserum; no swelling of capsule.



② Type I pneumococcus in sputum mixed with type I antiserum; swelling of capsule (Neufeld reaction).

FIGURE 16.—*D. pneumoniae* (showing Neufeld reaction).

of culture; incubate 2 hours and note clearing of the turbidity due to solubility in bile of the pneumococcus. Streptococci and other organisms are not bile soluble.

(*b*) *Inulin fermentation.*—Inoculate a tube of inulin-serum water and incubate 37° C. for several days. This medium is usually fermented and coagulated by pneumococci but not by streptococci.

(*c*) *Agglutination tests.*—Macroscopic tests with suspensions of the organisms and antipneumococcus sera.

268. Neisseria.—*a. Characteristics.*—Gram-negative cocci of variable growth vigor and pathogenicity. All members give a positive oxydase reaction.

b. Habitat.—*N. gonorrhoeae* (gonococcus) is the cause of gonorrhoea; *N. intracellularis* (meningococcus) is the cause of a specific meningitis. Both organisms may be readily demonstrated in the exudates from involved tissues. *N. catarrhalis* and several other species, which are found in the nose and throat of normal individuals,

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are sometimes associated with certain epidemics of respiratory or eye infections.

269. Neisseria gonorrhoeae (gonococcus).—A strict parasite of man. Found in discharges from the genito-urinary system in acute or chronic gonorrhoea, and in the pus from gonorrhoeal conjunctivitis; rarely in the blood stream.

a. Characteristics.—Oval or spherical cocci of moderate size, frequently arranged in pairs with adjacent sides flattened or slightly concave, resembling a pair of kidney beans side by side. In exudates, the cocci are fairly regular in size and shape and are usually inside the pus cells; in cultures, the cocci show variations in size. They are noncapsulated and Gram-negative. The cocci will not grow on plain agar, enrichment of media is needed; grow on moist chocolate agar at 37° C. in 24 hours to small, round, convex, grayish-white colonies. Growth is aerobic, favored by atmosphere of 10 percent CO_2. Highly susceptible to inimical agencies; when dried the cocci die in 2 hours; moist heat at 55° C. kills in 5 minutes; quickly killed by 1:4,000 silver nitrate; cultures kept at room temperature die in a few days, but at 37° C. they may survive several weeks.

b. Identification.—Microscopic examination only is generally done; cultural confirmation done only under special conditions.

(1) *Microscopy.*—Make direct spreads of the infected urethral, cervical, or conjunctival discharges on glass slides, fix with heat, and stain by Gram's method. Examine the stained preparation for Gram-negative, coffee-bean shaped, intracellular or extracellular diplococci having the typical morphology of gonococci. Report whether diplococci are intra- or extra-cellular, or both. Also report any other bacterial forms present, noting for each whether Gram-negative or Gram-positive and whether coccus or bacillus; also the relative numbers and kinds of tissue cells present.

(2) *Ordinary culture methods.*—These methods, especially in chronic urethral or cervical infections, will reveal only the secondary organisms which may occur. A special culture program is needed for growing *N. gonorrhoeae.*

(3) *Special culture program.*—The cultural demonstration of the gonococcus is superior to direct spread examinations in cases of chronic gonorrhoea in both sexes and in all cases in the female, especially when material for examination is taken from the cervix.

(*a*) *Cultivation of gonococcus.*—The cultivation of gonococcus, mixed with freer-growing micro-organisms, requires observance of the following special procedures:

1. Take specimens of representative material and apply directly to plate media.

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2. Use a medium such as moist chocolate agar, which will readily grow the gonococcus in mixed culture.
3. Grow in 10 percent CO_2.
4. Identify the gonococcus-meningococcus group by colony form and oxydase reaction.
5. Confirm the identification by carbohydrate fermentation tests.

(*b*) *Specimen taking and transmission* (optional methods listed in order of preference).

1. Platinum loop is touched to drop of pus, to urethra, or to cleansed cervical os, and is immediately stroked broadly over a warm-culture plate at the bedside or clinic chair.
2. Sterile swab is similarly contaminated with the suspected material at the bedside or clinic chair and immediately placed in a tube containing 1 cc of nutrient broth for prompt transmission to laboratory and inoculation of warm-culture plate (broad spread of 0.1 cc of this broth).

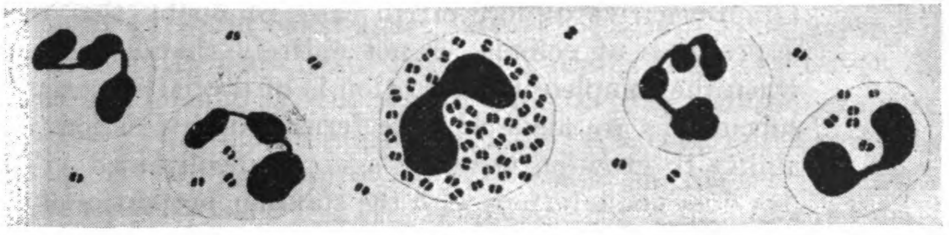


FIGURE 17.—Gonorrhoeal pus. (Film from urethra showing intracellular and extracellular *N. gonorrhoeae*.)

3. For delayed inoculation (up to 8 hours), the swab-broth tube No. 2 is stored in icebox until culture-plate inoculation is made.

(*c*) *Culture media.*

1. Chocolate agar, soft, moist, and warm.
2. The media of McLeod is elsewhere described.
3. Difco "Proteose No. 3 Agar" and "Bacto Hemoglobin" may be combined.

(*d*) *Incubation.*—At 37° C. in 10 percent CO_2 in closed jar, 24 to 48 hours.

(*e*) *Examination of culture.*—Observation made of two features:

1. Colony form: convex, slightly opaque colonies, 1 to 3 mm in diameter, with undulated margins. Their slight opacity and characteristic undulated margins serve to differentiate them from colonies of streptococci and diphtheroids.

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2. Oxydase reaction: Flood a segment of the plate with 1 cc of 1 percent aqueous solution of dimethyl paraphenylene diamine hydrochloride. (The McLeod program similarly uses 1 percent tetramethyl paraphenylene diamine hydrochloride, giving the colonies a bright purple color, is more expensive, but has the advantages of a more rapid reaction and not killing the cocci in 30 minutes, as does the dimethyl.) Gonococcus colonies develop a pink color which on further oxidation becomes maroon and finally black. Streptoccus and diphtheroid colonies fail to undergo this color change. Caution is indicated not to be mislead by a mere darkening of the surrounding media. Spreads made and stained from the oxydase-positive colonies must verify the tinctorial and morphological properties of the micro-organisms, as this stain is not entirely specific for the *Neisseria* group. Medium-sized, convex and translucent colonies which give the oxydase reaction may be accepted as gonococci if they consist of Gram-negative diplococci; in cases of doubt, that is, if appearance of colonies is not entirely characteristic or when the complete identification is of special importance, subcultures are made and the fermentation reactions and ability to grow on ordinary agar are determined. (The dye does not interfere with the staining properties of the gonococcus, though it does interfere with its cultivation, if it has proceeded beyond the pink stage.)

270. Neisseria intracellularis (meningococcus).—*a. Characteristics.*—Similar to the gonococcus, but found in different locations and possessed with different invasiveness; distinguishable by serological tests. Divided into five types by serological behavior, types I and II and less commonly types III, IV, and V; types I and III, and II and IV, respectively, are very closely related. Responsible for endemic and epidemic cerebrospinal meningitis in man; may be found in and isolated from infected spinal fluid, blood, or nasopharyngeal secretions of patients suffering with cerebrospinal meningitis and from the nasopharyngeal secretions of carriers. Highly susceptible to inimical agents; cocci die in less than 3 hours when dried and kept at room temperature; killed by moist heat at 55° C. in less than 5 minutes; cultures die in a few days when kept at room temperature.

b. Identification.—(1) *Macroscopic.*—Macroscopic appearance of the spinal fluid is to be noted and reported. Normal fluid is water-

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clear and colorless. Meningitis fluid is more or less turbid. Color, turbidity, blood, and clot are to be noted. Blood, if fresh, may have come from the spinal puncture and make examination of the fluid difficult.

(2) *Microscopy.*—An immediate presumptive diagnosis of meningococcic meningitis may be made by direct study of cerebrospinal fluid.

(*a*) *Stained films of suspected spinal fluid.*—Centrifuge the fluid, prepare spreads of the sediment on glass slides, fix, and stain by Gram's method. Examine for typical Gram-negative, coffee-bean shaped, intracellular diplococci. If present, they should be considered as meningococci and tentatively reported as such, to be confirmed by culture and agglutination tests. The presence of other organisms and the relative number and kind of tissue cells are also reported.

(*b*) *Cell counts of spinal fluid.*—Make total and differential counts, comparable to the counting of blood cells. The relative number of polymorphonuclear and mononuclear leucocytes are to be noted; the former are usually enormously increased in cerebrospinal meningitis.

(3) *Culture of sediment of spinal fluid.*—Plant several loopfuls of sediment on warm blood agar plate. Inoculate tube of warm serum dextrose broth with 1 cc. Incubate cultures at 37° C. for 18 to 24 hours and observe for the typical Gram-negative, coffee-bean shaped diplococci. Cultures are generally pure; if mixed, pure growth is to be obtained by subcultures on solid media (as for the gonococcus). Pure cultures are used for fermentation tests to rule out *N. gonorrhoeae*, and for tube-agglutination tests.

(4) *Culture of blood.*—This is not a routine procedure. The meningococcus may be recovered from the blood by routine methods, in anomalous infections with septicemia, with or without meningitis.

(5) *Culture of nasopharynx.*—This is done for the detection of carriers only. The nasopharynx of convalescents and of potential carriers are touched with a sterile applicator or inoculating needle, and this inoculum is spread diffusely onto warm blood agar or chocolate agar plates. After incubation at 37° C., suspect colonies are fished to warm serum dextrose broth for confirmation of identity.

(6) *Agglutination tests of pure cultures.*—A presumptive slide agglutination may hasten the procedure and cast out atypical organisms. A macroscopic tube-agglutination test with polyvalent meningococcic antiserum is used for final proof of identity. Occasionally type determination will be indicated. Most of the saprophytic *Neisseria* are salt or serum sensitive; to rule out nonspecific clumping, it is necessary, in all agglutination tests for meningococci, to run controls using normal horse serum (diluted 1:10) and saline.

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(*a*) *Presumptive test.*—Place a drop each of polyvalent antimeningococcic serum (1:10), normal horse serum (1:10), and sterile saline on separate areas of a slide; emulsify bacteria (portion of suspected colony) in each drop; observe for clumping of organisms.

(*b*) *Macroscopic test tube agglutination test.*—Add 0.5-cc amounts of each serum, diluted to one half of titer shown on vial, into labeled tubes. Use separate tube for polyvalent and for each type antimeningococci serum (usually I and II only) to be tested. Another tube receives 0.5 cc of normal horse serum (diluted 1:10) and last tube receives 0.5 cc of saline. To each tube add 0.5 cc of suspension of cocci being tested and incubate overnight at 45° to 55° C. or for 2 hours at 37° C. and overnight in icebox.

(*c*) *Result.*—If the organism is a meningococcus, it should agglutinate in tube containing polyvalent and homologous type serum and not in other tubes. If clumping occurs in either control tube, the test is unsatisfactory.

(7) *Fermentation reactions.*—With material from a pure culture, inoculate tubes of serum water media containing four pivotal sugars (see table XI) and incubate at 37° C.

271. Neisseria catarrhalis.—*a. Characteristics.*—Gram-negative diplococci. In sputum, the organisms are shaped like coffee beans and may be both intra- and extracellular; in cultures, they are generally larger and are found in pairs and tetrads. Grow freely, forming large colonies in 24 hours. They are normally found in the nose and throat; have meager pathogenicity; and may be found, incidentally, in inflammatory secretions especially of respiratory area. A number of closely related *Neisseria*, also found in respiratory area, are included on differential chart (table XI).

b. Identification.—(1) *Microscopy.*—Make Gram-stained spreads of the infected secretions and examine for Gram-negative cocci. These organisms are larger than meningococci, may not be arranged in pairs, and may be intracellular.

(2) *Culture.*—(*a*) Inoculate plain agar and incubate at 22° C.; *N. catarrhalis* will grow, gonococcus and meningococcus will not.

(*b*) Pure culture is inoculated into sugar series in serum water media. (See table XI for results.)

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TABLE XI.—*Differentiation of various species of Neisseria*

Species	Dextrose	Maltose	Levulose	Sucrose	Agar growth	22° C. growth	Agg. with meningococci serum	Special colony feature
N. gonorrhoeae	A	—	—	—	—	—	—	Small, round, convex.
N. intracellularis	A	A	—	—	±	—	+	Small, round, bluish gray.
N. catarrhalis	—	—	—	—	+	+	—	Large, grayish white.
N. sicca	A	A	A	A	+	+	—	Large, wrinkled, impossible to emulsify.
N. perflava	A	A	A	A	+	+	—	Greenish yellow, adherent to medium.
N. flava	A	A	A	—	—	—	—	Yellow.
N. subflava	A	A	—	—	±	±	—	Greenish yellow, adherent to medium.
N. flavescens	—	—	—	—	?	?	—	Golden yellow.

A indicates formation of acid.

272. Chromogenic bacteria.—*a.* A large number of bacteria, when cultivated upon suitable media, give rise to characteristic colors which aid in their identification. For each species the color is usually constant and dependent, to some extent, on the conditions of cultivation. Examples:

(1) *Staphylococcus aureus* and *citreus.*

(2) *Serratia marcescens* (*Bacillus prodigiosus.*)

(3) *Pseudomonas aeruginosa* (*Bacillus pyocyaneus*) and other bacteria producing water-soluble bluish-green or yellowish-green pigment, frequently found in soil, water, and feces.

(4) Sulfur bacteria, red, purple, etc., found in decomposing organic matter.

(5) Yellow to orange pigment-producing bacilli (*genus Flavobacterium*) found in water.

b. Most of the chromogenic bacteria found in the dust, soil, water, cesspools, and other sites of decomposing organic matter are nonpathogenic. Many of them, being in dust, may contaminate laboratory cultures. Most of the Gram-negative chromogenic bacilli, *Flavobacteria* and related chromogens, are incapable of producing disease in man or animals. The one species here described has feeble pathogenicity and is frequently associated with infected wounds.

273. Pseudomonas aeruginosa (Bacillus pyocyaneus—Bacillus of blue pus).—*a. Characteristics.*—Small, slender, motile

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rods; Gram-negative, non-acidfast, noncapsulated; aerobic and facultative anaerobic (pigment formed only in presence of oxygen); grows readily on simple media at 37° and also at 25° C. Colony on agar: large, spreading colony, grayish with dark center and translucent edge, irregular, fluorescent yellowish-green color, surrounding media taking a greenish color.

b. Habitat.—Widely distributed in nature, on skin and in upper respiratory tract. It is often a secondary invader of inflammatory areas in the wake of another incitant; occasionally is the primary cause. It may be found in the stools of children suffering from diarrhoea, in purulent discharges, and in wound infections.

c. Identification.—(1) Culture is made on plain agar, incubated at 30° C. for several days.

(2) Observe colony and depth of media for color change.

(3) Confirm microscopic morphology of the resultant cultured growth.

274. Pasteurella (hemorrhagic septicemia group).—Small, Gram-negative rods showing bipolar staining. Litmus milk not coagulated. Aerobe, facultative anaerobe; nonmotile or motile; frequently pathogenic, producing characteristic hemorrhagic infections in man and animals. Includes—

a. P. pestis, causing plague in man and rodents.

b. P. tularensis, causing tularemia in man and rodents.

c. P. avicida
d. P. muricida
e. P. cuniculicida
} associated with fowl cholera or hemorrhagic septicemia of birds or lower animals.

275. Pasteurella pestis.—*a. Habitat.*—A parasite of rats and other rodents causes plague in man. Transmitted by the bite of infected rat flea, or by contact or contamination with rodent, human case, or carrier.

b. Characteristics.—Short, thick bacillus; pleomorphic, especially in 3 percent salt agar; bipolar staining; grows readily on agar at 37° C. with raised, translucent, grayish-yellow, glistening, viscid growth. May live for months in bodies of dead animals. Agglutinated by plague antiserum. Infections by inoculation for small animals; subcutaneous injection into guinea pigs provokes local edema followed by inflammatory swelling of regional lymph nodes, and a generalized infection to death in 2 to 5 days. Post mortem appearance: glands enlarged, surrounded by hemorrhagic exudate; small grayish, necrotic areas in liver and spleen; bacilli found in local lesions, bubo, internal organs, especially spleen, and blood.

c. Collection of specimens.—(1) Pus or gland fluid from bubos,

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aspirated by syringe or collected after incision. (May be forwarded to distant laboratories on agar slants.)

(2) Portions of affected tissues, removed at operation, to be forwarded in sterile bottles.

(3) Blood specimens, taken during period of septicemia.

(4) Autopsy materials, preferably bubo, lung, liver, and spleen.

(5) Sputum, in cases of pneumonic plague.

(6) Rodent: the whole rodent, shipped in fruit preserving jar, sealed.

d. Microscopic examination.—(1) Stain films from suspect materials by Gram's method, and with methylene blue or dilute carbol-fuchsin (for bipolar staining).

(2) The presence of typical Gram-negative, short, ovoid, polar-staining bacilli, including many degenerated and poorly stained forms, is suggestive but not conclusive evidence of *P. pestis* infection.

e. Culture.—(1) Inoculate surface of blood agar, glycerol agar, and 3 percent NaCl agar plates.

(2) Plant blood specimen into nutrient broth and incubate before plating.

(3) Incubate cultures at 30° to 35° C. for 36 to 48 hours.

(4) Observe growth and transfer to agar, broth, litmus milk, gelatin, tryptone broth, lead acetate medium, and sorbitol broth for further study. (See par. 261*b*.)

f. Agglutination.—Macroscopic method is preferred, to avoid the spontaneous clumping confusing the microscopic test.

(1) Make suspension of young agar culture in normal saline, using only the fine supernatant emulsion remaining after period of settling.

(2) High-titer agglutinating serum (horse) is generally used.

(3) Test is of greatest value in identifying suspect cultures, positive titer being interpreted in comparison with the titer of same serum tested with a known plague antigen.

(4) Test is of little value as applied to patient's serum, for agglutinins do not appear in patients suffering from plague until about ninth day.

(5) Salt solution controls are necessary in all tests, to detect auto-agglutination.

g. Animal inoculation.

Caution: Animals should be freed of all ecto-parasites, prior to use, by dipping in an antiseptic solution. Then place in glass jars covered with fine mesh gauze to prevent access or escape of any parasites. When handling animals, living or dead, protect the hands and arms by wearing rubber gloves and long-sleeved gown.

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(1) Inoculate guinea pigs or mice subcutaneously with small amount of the original specimen, or with a loopful of suspected culture. Putrefied materials may be applied to the freshly shaven abdomen of a guinea pig (plague bacilli penetrate the abraded skin, contaminants do not).

(2) If *P. pestis* is present, the animals will develop characteristic lesions and die in 2 to 5 days with characteristic post mortem appearance. Cultures of *P. pestis* may be isolated from the lesions.

276. Diagnosis of plague in rodents.—*a.* Post mortem appearance will usually evidence the natural infection in rodents.

(1) Bubo, with hemorrhagic spots and areas of gray necrosis.

(2) Subcutaneous and general congestion.

(3) Granular liver, with punctate hemorrhage and gray-yellow spots.

(4) Congested spleen.

(5) Pleural effusion.

b. Bacilli may be found in bubo, liver, spleen, and blood, and isolated from thence for study in pure culture by methods used for clinical materials.

c. Shipment to a distant laboratory for examination: The entire carcass is placed, without any preservative, in a tightly sealed container which is packed in a second container to avoid breakage and escape of contents. The package must be shipped by express; federal laws prohibit the shipment of plague-infected materials by mail. Decomposition may be avoided by surrounding the inner container with ice or "dry ice." Label package "Perishable—for bacteriological examination—please expedite."

277. Pasteurella tularensis.—*a. Characteristics.*—Small, Gram-negative, nonmotile rods; pleomorphic, bacillary and coccoid forms; stained best with carbol-fuchsin and crystal violet, show bipolar staining; fail to grow on ordinary media; aerobic; require specially enriched media for growth. An organism which grows on plain agar or in broth is not *P. tularensis.* Growth on serum-glucose-cystine agar, 2 to 5 days at 37° C.; minute, grayish-white colonies. Fairly susceptible to inimical agencies; killed by moist heat at 56° C. in 10 minutes. Agglutination tests of great value in diagnosis of disease by serum study, or in identification of cultures; agglutinins may persist for 20 years after recovery and a positive serum agglutination does not necessarily mean active infection. *P. tularensis* antiserum also agglutinates *Brucella* antigens to about one-fourth of its titer. *P. tularensis* is the cause of "tularemia," a plague-like infection of rodents, especially rabbits, and occasionally in man.

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Generally transmitted from rodents to man by infected, blood-sucking insects such as flies, ticks, lice, fleas, and bedbugs, or by direct handling of infected rabbits or squirrels. Accidental laboratory infections occur, due to its ability to invade unbroken skin.

b. Microscopic examination.—Of value—

(1) To study morphology of organisms.

(2) To rule out *M. tuberculosis* by observing acidfast stain of spreads made from pathological materials.

c. Culture.—(1) Piece of infected tissue, pus, fluid, or blood is planted on slants of glucose-cystine agar or blood cystine agar. Incubate at 37° C. for 3 to 5 days.

(2) Blood agar plates also are planted to detect other organisms.

(3) Observe cystine slants for characteristic colonies. If negative, continue observation for 21 days; if growth occurs, identify organism by stained spread, pure culture transplants, and macroscopic agglutination tests with high-titer immune serum.

(4) Cultures made from blood and lesions of man are usually unsatisfactory. Cultures should be made from heart's blood, spleen, lymph nodes, and liver of guinea pigs following inoculation with material from patient.

d. Animal inoculation.—(1) Inoculate guinea pigs, rabbits, or mice with suspected materials from glands, ulcers, or blood—

(*a*) Subcutaneously; and

(*b*) Rubbed on the recently shaven, abraded abdomen, if other bacteria are present.

(2) Result: death in 5 to 10 days (generally) with characteristic lesions:

(*a*) At site of inoculation, hemorrhagic edema, no pus.

(*b*) Bubos, cervical, axillary, or inguinal.

(*c*) Glands enlarged and filled with dry, yellow, caseous material.

(*d*) Spleen enlarged, dark.

(*e*) Liver contains discrete, white, caseous granules.

(*f*) Organisms can be seen in spreads and be cultured from spleen, liver, bubo, and blood.

e. Agglutination reaction.—Macroscopic tube method preferred.

(1) Set up agglutination tests of patient's serum against *P. tularensis* and *Brucella* (*abortus* or *melitensis*) antigens. Incubate in water bath at 45° to 55° C. for 12 to 18 hours.

(2) Agglutination of *P. tularensis* by serum in dilutions of 1 to 80 or higher is considered diagnostic of tularemia, provided there is no cross agglutination with *Brucella*. Agglutinins appear in the patient's blood after the first week of the disease and usually increase rapidly.

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(3) Identity of a suspect culture may be established by a similar test, using a suspension of the organisms and serial dilutions of a *P. tularensis* antiserum of known titer. The resultant agglutination to be significant, must be present in dilutions approaching the known titer of the serum.

278. Malleomyces mallei (glanders bacillus).—*a. Characteristics.*—Slender, nonmotile, noncapsulated rods, with rounded ends. Gram-negative; bipolar staining common. Litmus milk slowly coagulated and sometimes digested. Grows readily into round, moist, grayish-white, glistening, convex colonies on agar at 37° C. in 24 hours. Ferments no carbohydrates.

b. Habitat.—The cause of glanders in horses, asses, and occasionally man.

c. Identification.—(1) Male guinea pig may be given intraperitoneal injection of a small amount of infected material from a lesion, or a suspension of a culture. Result: Orchitis starts in 2 to 3 days; later there is tumefaction and pus formation (the "Straus reaction"). Post mortem findings in addition to the testicular lesions: subcutaneous abscesses and small miliary grayish-white nodules in liver, spleen, pancreas, and lungs; bacilli may be recovered from these lesions.

(2) Complement fixation test of blood serum, using special glanders antigen.

(3) Culture of aseptically removed specimens of pus from suspicious lesions, lymph gland, or other material onto 3 percent glycerol agar of pH 6.6 to 7.0 and potato medium given prolonged incubation at 37° C. Result: After several days or a week, round, whitish or yellowish colonies, characteristic on potato media as yellowish, semitransparent honey-like growth becoming brownish or amber colored and tenacious, the medium becoming green or greenish brown.

(4) Histopathological slide of suspect tissue lesion to show typical glanders tubercle.

279. Genus Brucella.—*a. Description.*—Minute rods with many coccoid cells; 0.5 by 0.5 to 2.0 microns; Gram-negative; do not show bipolar staining; all species pathogenic to man are nonmotile; do not liquefy gelatin; and fail to ferment any carbohydrates.

b. Habitat.—Strict parasites, invading animal tissue, producing infection of the genital tract, the mammary gland or the lymphatic tissues, and the intestinal tract. *Br. melitensis*, *Br. abortus*, and *Br. suis* primarily infect goats, cows, and hogs, respectively, causing abortion and systemic infection; infectious to other domesticated animals; may infect man causing undulant fever (brucellosis).

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280. Br. melitensis, Br. abortus, and Br. suis.—*a. Description.*—Gram-negative, nonmotile coccobacilli as for genus. *Br. melitensis* and *Br. suis* grow aerobically; *Br. abortus* requires 10 percent CO_2 for initial isolation and early culture transplants. Growth on all media is slow; grows best on liver infusion agar with pH 6.6; 48-hour colonies on plate are small, circular, convex, amorphous, smooth, glistening, and entire. Agar cultures turn media brownish after 7 days. The three species are very closely related; may be separated with difficulty on basis of—

(1) CO_2 requirement for growth.
(2) Growth on media containing certain dyes.
(3) H_2S production.
(4) Agglutinin absorption tests.

b. Habitat.—Found in blood, urine, feces, exudates, and occasionally sputum and nasal drainage of human cases; also in milk, cheese, and other dairy products from unpasteurized milk from infected animals.

c. Laboratory examination of clinical material.—(1) *Microscopic.*—Indistinguishable morphologically. However, Gram-stained smears from pathological lesions should be examined for the small Gram-negative rods described above.

(2) *Cultural.*—While the organisms may be found in the blood early in the disease and during the febrile periods and in urine and milk specimens at irregular intervals, the percentage of positive cultures, even from proven cases is low.

(*a*) Obtain specimen consisting of 10 to 12 cc of blood or 50 cc of urine or milk. Other body foci such as contents of ovarian cyst, synovial fluid, or excised glands may also be subjected to cultural study.

(*b*) Inoculate two flasks containing 100 cc of veal infusion broth pH 6.6 with 5 cc of blood, several loopfuls of sediment from catheterized urine specimen, or several loopfuls of sediment and of cream layer from milk. Also streak specimen on two infusion agar plates.

(*c*) Incubate one set of media in incubator at 37° C. for growth of *Br. melitensis* and *Br. suis;* place other set of media in jar containing 10 percent CO_2 and incubate at 37° C. for *Br. abortus.*

(*d*) Examine plates and Gram-stained films from broth after 24 to 48 hours and at frequent intervals thereafter for growth. Streak new plates from broth at least once per week, even if no evidence of growth is discernible. Observe cultures for at least 4 weeks before reporting as negative.

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(*e*) Identify any positive culture as belonging to this group by agglutination with antisera prepared against either *Br. abortus*, *Br. melitensis*, or *Br. suis*.

NOTE.—Although not usually required, the species of young cultures can be determined by agglutinin absorption tests, by tests for H_2S production and by ability of the organism to grow on media containing certain dyes (basic fuchsin and thionin).

TABLE XII.—*Differential characters of the three related species of genus Brucella*

Species	10 percent CO_2 required for primary isolation	H_2S formation (days)	Growth on media containing	
			Thionin	Basic fuchsin
Br. melitensis	0	±1	+++	+++
Br. abortus	++	2	0	+++
Br. suis	0	4	+++	0

(3) *Animal inoculations.*—*Br. melitensis* and *Br. suis*, and less constantly *Br. abortus*, may be isolated from infected material by subcutaneous inoculation into guinea pigs (preferably males). After 4 weeks, kill the animal; examine Gram-stained smears from the lymph glands, spleen, and liver; and make cultures from the liver, spleen, blood, and lymph nodes. This test is seldom used because of the great danger of laboratory infection.

(4) *Serological.*—(*a*) *Identification of pure cultures.*—There is complete cross agglutination to titer between an antigen prepared with either species and antisera prepared against any other species. However, *Br. abortus* and *Br. suis* can be differentiated from *Br. melitensis*, but not from each other, by agglutinin absorption tests.

(*b*) Serum from a patient taken after the fifth day of disease will usually contain agglutinins. Set up macroscopic agglutination tests in dilution of 1/20 to 1/640 or higher against a *Brucella* antigen (*abortus*, *melitensis*, or *suis*) and against *Pasteurella tularensis* antigen. Agglutination of *Brucella* antigen in dilution of 1/100 or higher is considered to be significant. Cross agglutination in serum from patients with brucellosis or tularemia is frequently present, but is less marked with the heterologous antigen. Agglutinins may persist for years after recovery. This is the most valuable test for diagnosing *Brucella* infections and is the only one routinely used.

281. Genus Hemophilus.—Minute rods, sometimes almost coccoid, sometimes threadlike and pleomorphic; Gram-negative; not

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acidfast; nonmotile, nonsporing, nonencapsulated. Strict parasites; do not grow on common media but require for their cultivation accessory substances present in the blood and fresh vegetable tissue. *H. influenzae*, *H. ducreyi*, and *H. pertussis* are the three most important species.

282. Hemophilus influenzae.—*a. Characteristics.*—Very small, short rod; stains faintly, best by dilute carbol-fuchsin or Giemsa stain. Grows bests on media containing hemoglobin; subcultures on plain or serum agar fail to grow. Chocolate agar plate colony, 24 hours at 37° C.: small, pinpoint, transparent, smooth, raised. Tendency to grow best near colony of other aerobic organism, i. e., "satellite" colonies. Not subject to agglutination test, and does not have conspicuous biochemical activities.

b. Habitat.—Commonly found in cultures of upper respiratory tract, their significance there questionable; probably not as name implies, related to the disease influenza. Occasionally found in pathological spinal fluids. "Koch-Weeks" bacillus, formerly called *H. conjunctivitidis*, found in eye fluids in acute infectious conjunctivitis ("pink eye") is now classified as *H. influenzae*.

c. Identification.—(1) *Koch-Weeks bacillus.*—(*a*) Make slide spread from conjunctiva and stain by Gram's method and with dilute carbol-fuchsin.

(*b*) Observe for minute Gram-negative bacilli; often intracellular.

(*c*) Culture is not informative except to reveal other organisms.

(2) *Spinal fluid, respiratory tract, and other suspect materials.*—(*a*) Make culture on chocolate agar and incubate at 37° C. for 2 days.

(*b*) Suspect colonies are identified by colony appearance, microscopic morphology of organisms, and failure of subcultures to grow on plain agar. Colony may be confused with a streptococcus colony.

(*c*) Specific identification considers source of the specimen, hemolytic properties, and requirements of accessory growth factors.

283. Hemophilus pertussis.—*a. Characteristics.*—Like *H. influenzae*, except bacilli, are more uniform in size, with less pleomorphism, ferment no carbohydrates, and do not require accessory factors for growth, but cannot be distinguished on morphology alone. Colony on potato-glycerin-blood medium (pH 5.0) at 37° C. barely visible in 24 hours; plainly visible after 48 to 72 hours as small, grayish, raised, pearl-like growth. After several generations growth is freer, grayish, and glistening, becoming in a few days heavy, almost like the growth of typhoid bacilli; then transplants will grow on plain agar.

b. Habitat.—Constantly present in the respiratory secretions of whooping cough.

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c. Identification.—Cough plate method for isolating *H. pertussis* is preferable to sputum cultures: Open Petri dish, containing potato-glycerin-blood medium, is held in front of the mouth during a cough paroxysm. The organisms, sprayed on the plate with droplets of secretion appear in colonies after 37° C. for 48 hours. Colonies are larger, more opaque and whiter than those of *H. influenzae.*

284. Hemophilus duplex (Morax-Axenfeld bacillus).—*a. Characteristics.*—Short, stumpy, moderate-size bacillus, often in diploform and chains. Cultivated only on media containing blood, serum, or ascitic fluid. On Loeffler's blood slant colonies appear after 24 to 36 hours at 37° C. as small indentations which indicate a liquefaction of the medium.

b. Habitat.—Found in eye and subacute infectious conjunctivitis. Not pathogenic for animals.

c. Identification.—(1) Prepare slide spreads from conjunctival sac and stain with dilute carbol-fuchsin or Gram stain.

(2) Short, stumpy bacilli in direct spreads are presumptively Morax-Axenfeld bacilli.

(3) Culture on Loeffler's blood slant or other special media may confirm.

285. Hemophilus ducreyi.—*a. Characteristics.*—Very small, ovoid rod, nonmotile, tendency to be in short chains and parallel rows; Gram-negative; tendency to be more deeply stained at the poles. In pus, the bacilli are often found within leucocytes. Difficult to cultivate; coagulated blood which has been kept for several days in sterile tubes (fresh blood will not do unless heated to 55° C. for 15 minutes) has been found to be a favorable medium.

b. Habitat.—The cause of chancroid, the soft chancre, and found in the pus of ulcerating chancroidal ulcers, mixed with secondary infection, and in purer state in the chancroidal bubo. Not inoculable to lower animals.

c. Identification.—Examination of spreads or cultures for *H. ducreyi* is seldom practiced because of the technical difficulties of identification, and the fact that chancroid lesions are usually distinguishable as such without laboratory confirmation.

(1) *Direct diagnostic cultivation from chancroidal lesion.*—(*a*) Media: 1 cc of sterile rabbit blood (freshly drawn) is placed in each of several small tubes, allowed to clot, then heated to 55° C. for 15 minutes, and kept in ice box until used.

(*b*) Thoroughly cleanse lesion with sterile water or salt solution.

(*c*) Scrape material from bottom of ulcer or from beneath its edges, with a stiff platinum loop and plant in a tube of clotted blood by passing the wire around the clot.

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(*d*) After 37° C. for 24 hours, the serum around the clot is stirred with the platinum loop and a spread is made and examined by Gram method.

(*e*) Characteristic chains of Gram-negative bacilli, sometimes in pure, sometimes in mixed culture, will sufficiently identify the organism.

(*f*) Transfer onto soft, moist blood agar of pH 7.2 may give in 48 hours pinhead size, transparent, gray colonies with a firm, finely granular consistency.

(2) *Culture from unruptured bubo.*—Pus is withdrawn by aspiration with a sterile hypodermic syringe and needle. Cultured as in (1) above.

286. Gram-negative, aerobic, nonspore-forming enteric bacilli (family Enterobacteriaceae).—Gram-negative rods, widely distributed in nature. Grow aerobically. Many species are parasitic for man, several of which cause typical disease; other species are saprophytes, or parasites on plants and animals. Grow well on ordinary culture media. All species attack certain carbohydrates forming acid, or acid and visible gas. May be motile or nonmotile. Nonspore forming. Has been divided into five tribes, only three of which (*Eschericheae*, *Proteae* and *Salmonelleae*) contain species of interest in medical bacteriology. All of these bacteria are morphologically similar. They have many other characteristics in common, and serological as well as cultural methods may be required to definitely identify a member of the group.

287. Coli-aerogenes group (tribe Eschericheae).—Motile or nonmotile rods, commonly occurring in the intestinal canal of normal animals, in the respiratory tract of man, or widely distributed in nature. All ferment dextrose and lactose with the formation of acid and visible gas. Do not liquefy gelatin except slowly by one species (*Aerobacter cloacae*). Separated into three genera on basis of results of methyl red test, Voges-Proskauer test, and ability to utilize citric acid as sole source of carbon.

TABLE XIII.—*Coli-aerogenes differentiation*

Genus and species	Methyl red test	Voges-Proskauer test	Indol test	Citrate utilization	Gelatin liquefaction	H_2S formed
Escherichia coli	+	−	+	−	−	−
E. freundii	+	−	(+)	+	−	+
Aerobacter aerogenes	−	+	(−)	+	−	(−)
A. cloacae	−	+	−	+	+	(−)
Klebsiella pneumoniae	(+)	(−)	−	(+)	−	−

NOTE.—Some species give variable results; (+) or (−) indicates usual reaction.

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288. Escherichia coli.—*a. Characteristics.*—Coccoid to long rods, occurring singly, in pairs, and in long chains. Gram-negative. Motile or nonmotile. Not usually capsulated. Ferments many carbohydrates, including dextrose and lactose, with formation of acid and gas. The large number of species formerly identified on basis of motility and carbohydrate fermentation are now included within this species as varieties.

b. Habitat.—Occurs in normal intestinal tract of animals; frequently found in soil and water as a result of fecal contamination. Sometimes acquires pathogenic power and may cause local or general infections; frequently causes infections of the genito-urinary tract; and invades the circulation in agonal stages of diseases.

c. Identification.—(1) For isolating *E. coli* from water and sewage see section III, chapter 10.

(2) For *E. coli* in feces, urine, etc., follow the procedure outlined under examination of feces for *E. typhosa* (par. 295) and identify according to the reactions in table XIII above.

289. Aerobacter aerogenes.—*a. Characteristics.*—Short rods with rounded ends, usually shorter and plumper than *E. coli.* They are aerobic, Gram-negative, nonspore-forming and frequently capsulated. Ferment many carbohydrates, including dextrose, lactose, and glycerol with formation of acid and gas. Do not liquefy gelatin. Colonies on solid media are large and very viscid.

b. Habitat.—Widely distributed in nature; normally found on grains and plants; sometimes found in the intestinal canal of man and animals. It has been reported as the cause of cystitis.

c. Identification.—(1) See section III, chapter 10, for method of isolating *A. aerogenes* from water.

(2) Isolate organism from feces, food, and soil by plating on eosin methylene blue agar or other media as described under *E. typhosa* and identify by characteristic biochemical reactions shown in table XIII.

290. Klebsiella pneumoniae.—*a. Characteristics.*—Short, plump, nonmotile, Gram-negative rods; aerobic, growing well on ordinary media; produces a large, mucoid colony on solid media. It has a large capsule which can be demonstrated readily in spreads from sputum, animal exudates, and other pathological material. Ferments dextrose, levulose, galactose, saccharose, and usually lactose with production of acid and gas.

b. Habitat.—Common commensal in respiratory tract; occasionally found in soil, dust, and water. Associated with pneumonia and other inflammations of the respiratory tract. Occasionally found in various suppurative lesions of the body and may give rise to septicemia.

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c. Identification.—(1) Examine stained spreads from pus, sputum, or fluid from lesions for Gram-negative encapsulated bacilli.

(2) Inoculate eosin methylene blue agar plates or other media. Examine for mucoid colonies consisting of bacilli with typical morphology. Identify suspected colonies through cultural and biochemical tests.

(3) Blood culture may be made by usual methods. Identify any suspect colonies as above.

291. Genus Proteus.—*a. Characteristics.*—(The only genus in tribe *Proteae*). Consists of highly pleomorphic, Gram-negative rods; filamentous and curved rods and involution forms are common. Generally actively motile. Characteristically produce ameboid colonies on moist media and decompose proteins; gelatin is rapidly liquefied by most species. Ferment dextrose and generally sucrose, but not lactose, with formation of acid and small amount of gas. Usually Voges-Proskauer test is negative. Genus consists of eight species; type species is *Proteus vulgaris.*

b. Habitat.—Putrefying animal and vegetable materials; found in feces, soil, and gunshot wounds. Certain *Proteus* strains, identified as X19, X2, and X Kingsbury, originally isolated from typhus fever cases, are used as antigens in the Weil-Felix test (see sec. V, ch. 10). One species, *P. morgani*, has been reported as the cause of mild enteritis.

c. Identification.—(1) Most laboratories roughly identify any Gram-negative, motile bacillus that produces an ameboid colony on moist agar at 37° C. as belonging to the *Proteus* group and do not classify further.

(2) However, one species of the genus, *Proteus morgani*, produces the typical ameboid colony only when grown on 1 percent agar at 21° to 28° C. Isolate pure cultures of this organism as described under *E. typhosa* (par. 295) and identify on basis of fermentation of dextrose and other hexoses only, with formation of acid and slight amount of gas.

292. Typhoid-dysentery and paratyphoid-enteritis groups (tribe Salmonelleae).—Motile or nonmotile, Gram-negative rods; grow aerobically; nonspore-forming; Voges-Proskauer test negative; gelatin not liquefied; and no spreading growth. Attack many carbohydrates with formation of acid or acid and gas. Certain species of genus *Shigella* and genus *Eberthella* attack lactose with gas formation only. Tribe consists of three genera; genus *Salmonella* organisms ferment dextrose with the formation of acid and usually gas; genus *Eberthella* and genus *Shigella* organisms ferment dextrose with

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formation of acid, but no gas, *Eberthella* being motile and *Shigella* nonmotile.

293. Genus Salmonella.—*a. Characteristics.*—Usually motile, but nonmotile forms occur. Attack numerous carbohydrates with the formation of acid, and usually gas; lactose, saccharose, and salicin are never attacked. Do not form indol or liquefy gelatin. Differ from coli-aerogenes group in failing to ferment lactose; and from typhoid-dysentery group in forming gas from dextrose. Can be separated into 37 species, several of which are pathogenic for man, causing a typhoid-like fever, food poisoning, or an acute gastro-enteritis. All species pathogenic for man .are motile.

b. Important species.—(1) *S. paratyphi*, the cause of paratyphoid. A fever in man. Characteristic reactions: never ferments xylose, rarely able to produce H_2S, and fails to utilize citrate and d-tartrate.

(2) *S. schottmuelleri*, the cause of paratyphoid B fever in man. Characteristic reactions: ferments xylose and usually attacks inositol; H_2S formed; citrate + and tartrate usually −.

(3) *S. typhimurium*, (formerly *S. aertrycke*) a natural pathogen of rodents, especially mice, and many other animals; causes food poisoning in man. Characteristic reactions: very difficult to distinguish from *S. schottmuelleri*, by means of either biochemical or serological reactions, most reliable tests for separating them being—

(*a*) *S. typhimurium* is usually tartrate +.

(*b*) Agglutination reactions with "H" antigens in the specific phase.

(4) *S. enteritidis* and its varieties are widely distributed among animals; sometimes the cause of food poisoning in man. Characteristic reactions: ferments xylose, but never attacks inositol; H_2S +, citrate +, and tartrate +.

(5) *S. hirschfeldii*, the cause of a typhoid-like fever in man, sometimes referred to as paratyphoid C bacillus. Found principally in Europe. Characteristic reactions: biochemical reactions similar to those of *S. enteritidis;* serologically, closely related to *S. choleraesuis.*

(6) *S. choleraesuis*, two varieties, causing American and European hog cholera, respectively; occasionally infect man. Characteristic reaction: fails to ferment arabinose, a carbohydrate attacked by other *Salmonella.*

c. Identification.—(1) Isolate paratyphoid fever group from feces, urine, or blood as described under *E. typhosa* (par. 295).

(2) Food poisoning group. Isolate pure cultures from feces or food (see sec. I, ch. 10).

(3) Identify pure cultures by means of carbohydrate fermenta-

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tions and other biochemical tests (sec. I) and by agglutination reactions.

(4) The *Salmonella* group, including *E. typhosa*, is very complex, serologically. Each species possesses from one to three distinct antigenic components in the body of the bacillus ("O" antigens) and other distinct components in the flagella ("H" antigens), the latter occurring in many species in two alternate phases, the specific phase and the group phase, each possessing different antigens. The same antigenic components may be found in several different species in various combinations. However, most strains of the pathogenic species listed above can be definitely classified on basis of—

(*a*) Source of specimen.

(*b*) Biochemical reactions.

(*c*) Series of agglutination tests.

294. Genus Eberthella.—The organisms of this genus are defined as Gram-negative, motile rods, generally occurring in the intestinal canal of man, usually in different forms of enteric inflammation. Attack dextrose and several other carbohydrates with the formation of acid, but no gas; certain nonpathogenic species may attack lactrose, saccharose, and/or salicin with formation of acid, but no gas. *E. typhosa* is the only species regularly pathogenic for man.

295. Eberthella typhosa.—*a. Characteristics.*—Actively motile, Gram-negative rods, possessing the general features of the tribe and genus. Never attack lactose, saccharose, or salicin. The normal smooth, motile form has one somatic and one flagellar antigen, thus producing both "H" and "O" agglutinins; nonmotile variants are rare. The somatic antigens are related to those of *Salmonella enteritidis* and a number of other species of *Salmonella*. Colonies on plain agar, after 24 hours' incubation at 37° C., are smooth, round, domed, grayish in color, transparent to opaque, with entire edge; after cultivation on artificial media, rough-type variants may develop. See paragraph 261*b*(2)(*d*) for biochemical characteristics and table XIV for reaction on Russell's double sugar tubes, and for type colonies on differential plate media.

b. Habitat.—Found in feces and blood, and occasionally in bile and urine, of patients ill with typhoid fever of which it is the causative agent; also present in feces, urine, and bile of carriers.

296. Laboratory examination of specimens for typhoid.—The specimens to be examined will usually consist of blood, feces, urine, or bile of suspected cases of typhoid or paratyphoid fever and of bile, feces, and urine of carriers for cultural study; also, of serum from patients for agglutination (Widal) test.

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a. Microscopic examination.—This is of no value.

b. Culture.—(1) *Feces, urine, bile, etc.*—(*a*) Spread the material, suspended in broth or saline if solid feces, over the dry surface of eosin-methylene blue agar, Leifson's desoxycholate-citrate, or other special differential media in Petri dishes, in such a manner as to insure the growth of well-isolated colonies. Also, inoculate specimen into tube of selinite-F broth or bile broth.

(*b*) Incubate 18 to 24 hours at 37° C.

(*c*) Study the plate cultures carefully, select several well-isolated colonies of the type desired (see table XIV), and from each inoculate Russell's double sugar (R. D. S.) tube and plain agar slant.

(*d*) After 24 hours' incubation examine cultures for type reaction on R. D. S., motility, and Gram-staining properties.

(*e*) Identify any suspected pure culture by—

1. Inoculating various carbohydrate media and media for the other biochemical tests.

2. By setting up macroscopic agglutination tests against known type antisera (*E. typhosa*, *S. paratyphi*, and *S. schottmuelleri;* other antisera may be used, if indicated).

(*f*) If at the end of 24 hours, plate cultures show no colonies of the type produced by pathogenic organisms, streak new set of plates from the broth culture and reincubate old plates for an additional 24 hours before discarding as negative.

(2) *Blood.*—Blood for culturing should be taken early in the disease, preferably during first week.

(*a*) Obtain 10 to 15 cc of citrated or defibrinated blood; whole blood can be used for immediate inoculation of media at bedside.

(*b*) Inoculate—

1. Flask containing 100 cc of bile broth, 1 percent dextrose infusion broth, or brilliant green broth with 2 to 5 cc of blood.

2. Two agar pour plates with 1.0 cc of blood each.

3. Streak two or three loopfuls of blood on eosin-methylene blue agar plate.

(*c*) Incubate at 37° C. and make daily transfers to blood agar and E. M. B. agar plates. If colonies develop, transfer to Russell's double sugar and identify by the procedure outlined above.

c. Serological examination.—(1) *Macroscopic tube-agglutination test.*—This test, as indicated above, should be used to confirm the identity of an organism isolated from cultures. Use suspension of suspected culture as antigen along with known type antisera.

(2) *Widal test.*—After the first or second week, demonstrable anti-

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bodies, including agglutinins, develop in the blood of patients with an enteric fever. These may be demonstrated by Widal test. This test consists of a macroscopic (preferred) or microscopic agglutination test, using the patient's serum, and stock *E. typhosa* "H", *E. typhosa* "O", *S. paratyphi*, and *S. schottmuelleri* antigens.

297. Genus Shigella.—*a. Characteristics.*—Small, Gram-negative, nonmotile rods. Attack a number of carbohydrates with formation of acid but no gas.

b. Habitat.—Several species are pathogenic for man, causing bacillary dysentery. Other species may be found in the normal human intestinal tract. Several species are pathogens of fowls and other small animals.

298. Dysentery group.—*a. Shigella dysenteriae* (Shiga).—A cause of dysentery in man and monkeys. Produces acid but no gas from dextrose, levulose, and a few other carbohydrates. Never attacks mannitol, maltose, lactose, or sucrose. Indol not formed. Serologically homogenous and different from the other species of *Shigella.*

b. Shigella sp. (Newcastle type).—A cause of human dysentery. In peptone water solution, dextrose, maltose, and occasionally dulcitol are fermented with acid production; lactose, mannitol, and saccharose usually not fermented. Peculiarities of the organism are: Occasionally a slight bubble of gas is produced from dextrose and dulcitol; when dissolved in beef extract broth, dextrose, dulcitol, and maltose are always fermented to acid and gas. Indol not formed. Serologically homogenous and not agglutinated by antisera prepared against *S. dysenteriae* or *S. paradysenteriae.*

c. Shigella paradysenteriae.—A cause of dysentery in man, and of summer diarrhoea in children. Produces acid but no gas from dextrose and mannitol; some strains attack maltose or saccharose; dulcitol and lactose never fermented. Indol formation is variable. Has been divided into five races (V, W, X, Y, and Z) by agglutination tests based upon the preponderance of one or another of four antigenic components, V, W, X, and Z; considerable cross agglutination between races; serologically distinct from *S. dysenteriae* and Newcastle's bacillus; slight cross agglutination with *S. sonnei*, *S. alkalescens* and *S. madampensis.*

d. Shigella alkalescens.—Isolated from human feces and intestines; pathogenicity doubtful. Ferments dextrose, mannitol, maltose, dulcitol, and sometimes saccharose; never attacks lactose. The most characteristic reaction is an initial and lasting, intense alkalinity produced in litmus milk. Serologically homogenous and distinct except minor cross agglutination with *S. sonnei*, *S. paradysenteriae*, and *S. madampensis.*

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TABLE XIV.—*Colony characteristics of Gram-*

Medium	Eosin-methylene blue agar plate	Desoxycholate agar plate
Mechanism	Coli-acrogenes group ferment lactose and grow into large, opaque colonies; also absorb dye to give color to colony. The nonlactose-fermenting pathogenic species develop as small, colorless, translucent colonies.	Lactose-fermenting organisms produce large, reddish colonies. Nonlactose-fermenting organisms grow into small, clear, colorless, translucent colonies.
Genus *Escherichia*	Large colonies with large, dark (almost black) centers, and with greenish metallic sheen.	Large, opaque, reddish colonies; occasionally have colorless rim.
Genus *Aerobacter* and genus *Klebsiella*.	Large pinkish mucoid colonies with small, dark-brown or black centers; rarely show metallic sheen.	Similar to *Escherichia* colony except larger and mucoid.
Genus *Salmonella*	Translucent, colorless, or pinkish colonies, usually slightly larger than typhoid; later have bluish tint.	Large translucent colonies, domed, shiny, smooth, and colorless.
Shigella dysenteriae and *S. paradysenteriae*.	Small, translucent, colorless colonies.	Same as *Salmonella* colonies, except smaller.
Shigella sonnei and *S. madampensis*.	Small, translucent, colorless colonies;	Same as *S. dysenteriae* colonies during first 24 hours; later may show reddish daughter colonies or entire colony may become red.
Eberthella typhosa	Translucent, colorless colonies.	Translucent colonies, domed, shiny, smooth, and colorless.
Genus *Proteus*	Translucent, colorless, spreading colonies.	Same as *Salmonella*, spreading inhibited.

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negative intestinal bacilli on differential media

Desoxycholate-citrate agar plate	Bismuth sulfite medium (Wilson & Blair)	Russell's double sugar medium (Phenol red indicator—alkaline is red; acid, yellowish)	
		Slant	Butt
Same as for desoxycholate agar, but with greater inhibition of coli-aerogenes group.	Genus *Shigella* and coli-aerogenes group organisms are inhibited. *E. typhosa* and some *Salmonella* grow well, reducing sulfite to sulfide in presence of glucose.	The small amount of acid produced by dextrose (0.1 percent) is diffused, leaving alkaline slant. The larger amount of acid from lactose (1 percent) gives acid slant.	Organisms producing acid from either dextrose or lactose give acid butt. *Salmonella* give acid and gas (bubbles in medium.)
Much inhibited; if any growth, it is pink and opaque, opacity spreading to surrounding medium.	Usually completely inhibited. Growth, when present, gives small black, brown, or greenish glistening colony without surrounding zone; no metallic sheen.	Acid.	Acid and gas. (++++)
Same as *Escherichia* shown above.	Usually completely inhibited. May form raised, mucoid colonies exhibiting a silvery sheen.	Acid, returning to neutral or alkaline after several days.	Acid and gas. (++++)
Same as on desoxycholate agar plates.	*S. schottmuelleri* and *S. enteritidis*, black colonies similar to those of *E. typhosa*. *S. paratyphi*, *S. typhimurium*, and *S. choleraesius*, flat or slightly raised green colonies.	Alkaline.	Acid and gas. (++)
Same as on desoxycholate agar plates.	*S. dysenteriae*, inhibited. *S. paradysenteriae*, see *S. sonnei*.	Alkaline.	Acid.
Same as on desoxycholate agar plates.	*S. sonnei* and *S. paradysenteriae* may produce brownish, raised colonies with depressed, crater-like centers. Other *Shigella* inhibited.	Alkaline. Small acid-producing daughter colonies may be formed after several days.	Acid.
Same as on desoxycholate agar plates.	Black colony surrounded by black or brownish-black zone several times its size; zone surrounding colony shows metallic sheen.	Alkaline.	Acid.
Same as on desoxycholate agar plates.	Small, shiny, green colonies; no spreading.	Alkaline.	Acid and gas. (+)

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e. Shigella sonnei.—A cause of mild dysentery in man, or of summer diarrhoea in children. Ferments dextrose, mannitol, maltose, lactose, saccharose, and several other carbohydrates with formation of acid, but no gas; dulcitol is never, and xylose seldom, attacked; fermentation of substances other than the monosaccharides may require days or weeks. Indol not formed. Serologically divisible into two types; some cross-agglutination with *S. paradysenteriae*, *S. alkalescens*, and *S. madampensis.*

f. Shigella madampensis (*S. dispar*).—Isolated from human feces; apparently not pathogenic. Fermentation reactions similar to those of *S. sonnei.* Indol is formed. Antigenically heterogeneous; may show slight cross agglutination with *S. paradysenteriae*, *S. alkalescens*, and *S. sonnei.*

299. Laboratory examination of specimens for dysentery.—*a. Microscopic examination.*—In bacillary dysentery, especially in infections with *S. dysenteriae* (Shiga), an early presumptive diagnosis can usually be made by direct microscopic examination of fresh fecal discharges.

(1) Select portions of a very fresh specimen containing bits of mucus, bloody feces, or shreds of the exudate. Prepare first, thin films on slide and second, cover slip preparations, both unstained and stained with Loeffler's methylene blue or 1 percent aqueous solution of brilliant cresyl blue, in order to study the cells present.

(2) If the disease is the bacillary type of dysentery, microscopic examination will show blood in varying amounts, but usually abundant early in the disease; polymorphonuclear neutrophiles form about 90 percent of the exudate, and many of these show nuclear degeneration (ringing), while the cytoplasm frequently contains fat; endothelial macrophages, which are present in varying numbers, are actively phagocytic and frequently contain engulfed bacteria, erythrocytes, and leukocytes; these undergo degeneration and form "ghost cells;" plasma cells are present and are more abundant early in the disease; bacterial content is scanty.

(3) For characteristic findings in amoebic dysentery stools, see chapter 12.

b. Cultural examination.—*Shigella* may be isolated from the feces of patients and carriers by the methods indicated under *Eberthella.* However, both eosin-methylene blue agar plates and desoxycholate-citrate agar plates should be inoculated routinely, since the latter is an especially favorable culture medium for *Shigella.*

c. Serological examination.—(1) The suspected organisms may be identified by agglutination tests using polyvalent and species specific antisera; *S. dysenteriae*, *S. paradysenteriae*, *S. sonnei*, and polyvalent

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(Shiga, Sonne, and paradysentery) antidysenteric antisera are generally used.

(2) Agglutination tests, using serum from patient against known antigens, are of limited value.

300. Vibrio comma.—*a. Description.*—Slightly curved rods with rounded ends, often resembling a comma; occur singly, in S-shaped pairs, short chains, or spirals; actively motile; grow readily aerobically on simple media at 37° C. Agar plate colony: 1 to 2 mm diameter, grayish yellow, translucent, low convex, with smooth or finely granular glistening surface and an entire edge, and butyrous consistency. Broth growth: abundant, with powdery deposit and thick surface pellicle.

b. Identifying characteristics.—(1) Their power to grow on solid media which are so alkaline (pH 8.0 to 8.4) that other organisms cannot develop.

(2) Their initial growth at surface of liquid media, while accompanying organisms grow throughout the liquid.

(3) Cholera red reaction + (also given by two saprophytic species).

(4) Indol +, M. R. −, V. P. −; acid, no gas in glucose, levulose, galactose, maltose, mannitol, and sucrose; lactose may become acid after 14 days; litmus milk alkaline at top, slightly acid at bottom, not coagulated, slowly peptonized; nitrites produced from nitrates.

(5) Gelatin stab growth: good filiform growth, confluent at top, discrete below, funnel-shaped liquefaction, with thick yellowish-brown pellicle on surface.

(6) Agglutination with cholera-immune serum.

c. Choleralike Vibrios.—There are several classified and probably many unclassified vibrios isolated from feces or water and differentiated on serological and biochemical characteristics.

d. Examination of clinical material.—*Vibrio comma* may be isolated from the stools or intestinal contents of cases or carriers, and from contaminated water or foods and identified by microscopic, cultural, and serological methods.

(1) *Specimen collection.*—(*a*) The "rice water" stool of cases or the feces of carriers are transmitted without the addition of glycerol or other preservative.

(*b*) Surface water transmitted in a sterile liter flask.

(2) *Microscopy.*—A presumptive diagnosis of suspected cases, not of carriers, may be quickly made by examining stained spreads of flakes of mucus from the "rice water" stool; stain by Gram's method and with dilute-carbol-fuchsin; if Gram-negative, comma-shaped or-

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ganisms are present, examine a hanging-drop preparation. Presumptive positive report may be made if large numbers of typical, actively motile, vibrios are found. This finding must then be confirmed by cultural and serological examination.

(3) *Cultural.*—(*a*) *Feces.*—Specimens of feces from suspected cases or carriers should be planted, using two or more loopfuls of intestinal mucus or liquid feces, with the least possible delay and incubated at 37° C. Use the following media:

1. Alkaline peptone water, pH 8 to 8.4 (several tubes).
2. Alkaline nutrient agar, pH 8 to 8.4.
3. Dieudonne's agar.

(*b*) *Water.*—Water under test is placed in 100-cc amounts in sterile flasks. To each flask are added 10 cc of 10 percent peptone water. After 6 to 12 hours' incubation at 37° C., transfer a portion of the surface growth to the three media above.

(4) *Rapid presumptive test.*—After 6 to 8 hours at 37° C., examine hanging-drop and stained-film preparations made from the surface growth of peptone water. If Gram-negative, motile vibrios in large numbers are noted, test as follows:

(*a*) *Microscopic agglutination test.*—Deposit, near one end of a slide, a drop of agglutinating serum of a dilution of 1:200 (titer not less than 1:4,000) and near the other end a drop of saline; also place a third drop consisting of normal serum (diluted 1:10) near center of the slide as a control. Then touch the suspected surface growth with point of the inoculating needle and rub up in the drop of saline solution; flame the point, again touch the surface pellicle with the point and rub it in the drop of serum dilution; flame the point of the platinum needle again and add bacteria to the serum control in the same manner. Agglutination will almost instantly appear in the anti-cholera serum (if cholera). The drops may be allowed to dry; then fix and stain; if agglutination has taken place, it will be evident, in the stained specimen, to the naked eye or on slight magnification with the hand lens.

(*b*) *Cholera red test.*—A few drops of concentrated sulfuric acid are added to a 24-hour peptone water culture; a resulting red color depends upon the nitroso-indol reaction from the production of indol and the reduction of nitrite in the peptone.

(5) *Confirmatory tests.*—If either of the presumptive tests is positive, obtain pure cultures for confirmation by selecting isolated colonies from plate media and transferring to:

(*a*) *Gelatin tube.*—To note characteristic type of liquefaction.

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(*b*) *Alkaline peptone water.*—For cholera red test.

(*c*) *Agar slant.*—For macroscopic tube-agglutination test.

301. Family Bacillaceae (spore-forming bacilli).—*a. Definitions.*—(1) This family consists of rods producing endospores; usually Gram-positive; may be motile or nonmotile. Often decompose protein media actively through the agency of enzymes. Consists of two genera:

(*a*) Aerobic forms, mostly saprophytes, genus *Bacillus.*

(*b*) Anaerobic forms (occasionally microaerophilic), often parasitic, genus *Clostridium.*

(2) Bacterial spores (endospores) are round or oval, highly refractile structures which may be formed within the body of certain bacilli by a process of condensation and dehydration of the cell cytoplasm. They are nonreproductive bodies, being simply a resting stage of the bacterial cell, in which it is far more resistant to adverse environmental conditions than it is in the ordinary vegetative stage. Spores may be located centrally, subterminally, or terminally in the body of the bacillus and may have a diameter less or greater than that of the parent bacillus; the size, shape, and location of spores are characteristics of great value in classification.

b. Remarks.—Genus *Bacillus* and the food-poisoning group (*Cl. parabotulinum* and *Cl. botulinum*) of anaerobes will be discussed here. For a description of the other pathogenic species of genus *Clostridium* see section II, chapter 10.

302. Genus Bacillus.—Aerobic, spore-bearing rods, generally liquefy gelatin. Often occur in long chains. Form of rod usually not greatly changed at sporulation. Young organisms are Gram-positive, old forms may be Gram-ambophilic or negative. Widely distributed in nature, including 145 or more described species; only one of particular medical importance (*B. anthracis*). Mostly saprophytes and frequently contaminate culture media.

303. Bacillus subtilis (hay bacillus).—*a. Characteristics.*—Large, straight rods; occurs singly and in pairs, sometimes in short chains; spores are usually slightly nearer one pole than the other; Gram-positive. Actively motile in young cultures. Gelatin is liquefied. Grows freely aerobically forming dry, corrugated pellicle; colonies are large, rough, and irregular with fringed edges.

b. Habitat.—Found in soil, water, dust, laboratory contaminations, and as a saprophyte in old sinuses and infected wounds. Its significance is its close resemblance to *B. anthracis* when found in wounds.

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304. Bacillus anthracis.—*a. Characteristics.*—Large, nonmotile, sporulating rods; in blood and body fluids of affected animals, occur in pairs or short chains; in cultures, occur in long, segmented, parallel, or interwoven chains. Ends of the bacillus are square-cut or concave. Capsules are formed in animal body and on serum media but are lost on agar. Spores are centrally placed, formed only in the presence of oxygen and not formed in the animal body. Gram-positive. Agar plate colony: large, raised, dull, opaque, grayish-white, irregular borders, uneven surface; "Medusa-head" appearance under low power microscope. Gelatin stab: crateriform liquefaction, inverted pine-tree appearance. Aerobe, facultative anaerobe. Spores killed by boiling for 10 minutes, but may survive in dry state for years.

b. Habitat.—Naturally pathogenic to man and large herbivorous animals, causing anthrax. When injected subcutaneously into guinea pigs, mice, and rabbits, produce death in 12 to 72 hours with hemorrhagic local exudate, enlarged spleen, and bacilli in blood.

c. Identification.—(1) *Material.*—(*a*) Pus or fluid from skin lesion (malignant pustule).

(*b*) Blood in septicemic stage of diseased or infected animal.

(*c*) Sputum in pulmonary infection.

(*d*) Spinal fluid in meningeal infections (rarely).

(2) *Microscopic study.*—Make film preparations with the infected material, stain by Gram's method, and examine for the characteristic, large, Gram-positive bacilli. Spores may be present only if the bacilli have been exposed to atmospheric oxygen. In blood or animal tissues, the organisms are encapsulated.

(3) *Culture.*—(*a*) Plant portion of specimen in nutrient broth and agar plates. Incubate at 37° C. for 24 hours or more, and observe colonies.

(*b*) For pure culture isolation, heat the broth culture to 60° C. for 20 minutes to kill the associated organisms and transplant on agar plates.

(4) *Animal inoculation.*—This is an important diagnostic procedure. Inoculate white mice, a guinea pig, or rabbit subcutaneously with a small portion of the broth culture or suspension of agar growth, or, for a rapid diagnosis, the original suspected material. If anthrax bacilli are present, the animal will die with a fatal septicemia in from 12 to 72 hours. The organisms may then be isolated from the heart's blood, liver, and spleen of the animal.

(5) *Result.*—The diagnosis of anthrax is warranted if the specimen contains a Gram-positive, square-ended, chain-producing, spore-

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forming, nonmotile bacillus which produces characteristic Medusa-head colonies on agar and, when injected subcutaneously into small animal, produces a fatal septicemia.

305. Cl. parabotulinum and Cl. botulinum.—*a. Habitat.*—These are primarily saprophytes of the soil but may occasionally be found in the intestinal tract of domesticated animals and on various foods contaminated by soil or dust. They are not infective to man or animals, but do produce disease by means of the violent poison, toxin; they may produce in foods which act as a culture media for their saprophytic growth. This toxin is not formed within the body. This poisonous toxin, variously applied, produces "botulism" or food poisoning in man, "forage poisoning" in animals, or "limberneck" in poultry. The living organism may be sought for in the infected food, but not in the poisoned man or animal, for it is not an infection. Botulism may be associated with meat or meat products, fruits, vegetables, canned goods, and various pickled and preserved foodstuffs. Broth cultures, injected subcutaneously in mice, guinea pigs, rabbits, cats, or monkeys, prove fatal in 1 to 4 days.

b. Morphology and staining.—Large sporulating rods with parallel sides and rounded ends, occurring singly or in chains; slightly motile; not capsulated; Gram-positive. Spores are oval, larger than the bacilli, and usually situated at or near the end. Spores form best in sugar-free media at a temperature of 20° to 25° C.

c. Metabolism.—Strict anaerobe, growing well in ordinary media with neutral or slightly alkaline reaction. Optimum temperature 35° C. (growth poor at 37° C.). Hemolysis produced on erythrocytes (human and horse). *Cl. parabotulinum* cultures are generally proteolytic; *Cl. botulinum*, only slightly proteolytic. Optimum pH, 7.4 to 8. Above 37° C. toxin formation is impeded, below 20° C. toxin formation stops. Different strains of *Cl. parabotulinum* produce type A or type B toxin; *Cl. botulinum* produces type C toxin.

d. Cultivation.—(1) *Agar (4-day growth).*—Flat, irregular, grayish yellow, filamentous colonies with alternately smooth and granular surface, and indefinite fringed periphery.

(2) *Deep glucose agar shake (4-day growth).*—Colonies, thin, semiopaque disks with biconvex, brownish centers, and translucent edges; abundant gas formation.

(3) *Blood agar plate (horse) (3-day growth).*—Irregular, round, 2- to 3-mm colonies with smooth center and fimbriate periphery; alpha type hemolysis.

(4) *Cooked meat mediums (brain) (4-day growth).*—Abundant growth with turbidity and gas formation; brain digested and blackened; butyric acid odor.

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(5) *Broth (4-day growth).*—Dense turbidity and rancid odor.

e. Biochemical reactions.

	A	B	C
Glucose	AG	AG	AG
Maltose	AG	AG	AG
Salicin	AG	AG	—
Glycerol	AG	AG	AG
Lactose	—	—	—
Inositol	—	—	AG

f. Serology.—Types A and B are identifiable by agglutination and toxicity tests. Their toxin is specific only for type; the antitoxin of one is not neutralized by the toxin of another. Type C, forming another separate specific toxin, is distinguished chiefly by lack of proteolytic powers.

g. Resistance.—The bacilli without spores are readily killed by heat and chemicals. Spores withstand dry heat of 180° C. for 15 to 30 minutes and moist heat at 100° C. for 3 to 5 hours. Toxin is destroyed by 80° C. in 5 to 15 minutes.

h. Identification.—See section I, chapter 10.

306. Corynebacterium diphtheriae (diphtheria bacillus).—*a. Description.*—Slender rods, straight or slightly curved, of medium size; often lie at various angles to one another forming V or Y shapes, or clumped as Chinese letters; generally not uniform in thickness, exhibiting rounded, pointed, or swollen ends or enlargements along the length of the cell; usually stain unevenly, showing barred and granular large forms and solid-staining short forms; Gram-positive, non-acidfast, and nonmotile; grow readily at 37° C., preferably on Loeffler's serum or blood agar, as small, circular, smooth, moist, grayish to creamy-white colonies, some strains giving narrow zone of hemolysis on blood agar; pathogenic to man and to guinea pigs.

b. Habitat.—The cause of diphtheria, usually found in the mucous membranes of the nose, throat, and larynx of cases and carriers; occasionally found as cause of conjunctivitis, wound infection, middle ear infection, and broncho-pneumonia. It is not found in the blood stream, the generalized symptoms being caused by the powerful toxin formed at the local site of infection.

c. Identification characteristics.—(1) Shape, size, irregular staining, and V or Y arrangements as seen in a direct spread, or spread from Loeffler's medium, stained by Loeffler's methylene blue or Neisser's stain.

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(2) Growth freer on Loeffler's serum medium than that of other organisms.

(3) Colony form on blood agar. Colony on tellurite medium becomes black.

(4) Pathogenicity for guinea pig (see virulence test *h* below).

(5) Carbohydrate fermentation (see chart in *j*(2) below).

d. Collection and transmission of specimens for examination.—Cotton swab may be applied to the involved area (throat, nose, or wound) or to the membrane or exudate from that area, with care to gather a considerable amount of the exudate on the swab and with caution not to contaminate the swab by it touching the tongue or other noninvolved areas. Use this swab for—

(1) Immediate inoculation of Loeffler's serum slant for 18 to 24 hours' incubation at 37° C., or for shipment to distant laboratory.

(2) Immediate inoculation of blood agar plate for incubation at 37° C. for 24 hours.

(3) Spread on slide for direct Neisser stain examination.

(4) (Optional.) Plant on tellurite media.

e. Microscopic examination (direct spread, Neisser's or Loeffler's stain).—An immediate presumptive diagnosis can sometimes be made on the basis of morphology and staining features of what few diphtheria bacilli may be observed in the direct smear, but here they will be confusedly mixed with the many other micro-organisms of mouth or wound flora. Vincent's organisms and diphtheroids may give confusion and should be noted on report if found. Negative finding by direct method cannot be given value. Presumptive positive finding should be confirmed by cultural and virulence tests.

f. Cultural examination.—(1) Loeffler's tube, after 18 to 24 hours' incubation at 37° C., is examined by broad needle drag along its surface; this then is spread on a slide and planted on blood agar and tellurite media for later pure colony isolation. The slide spread is stained by Neisser method and observed for diphtheria bacilli; the irregularity of staining and shape and the metachromatic granules may be noted. If typical diphtheria bacilli are found and the culture is from a suspected case, a presumptive diagnosis should be made at once. If the culture is from a suspected carrier, diphtheria-like bacilli should be further identified by fermentation and virulence test before reporting.

(2) Blood agar plate will provide information on general bacterial flora, particularly streptococci, and will give opportunity for notation of colony form, and for single colony isolation of diphtheria-like bacilli.

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(3) Tellurite media will point out the diphtheria-like colonies by black color.

(4) (Optional—rapid.) Apply a sterile serum-swab to involved area, return to serum tube, and incubate for a few hours; transfer to other media and examine slide made by gently rolling swab out into thin film. Sterile serum swabs are prepared by placing sterile swabs into sterile tubes containing a few cubic centimeters of serum. Some such swabs are made to contain 2 percent potassium tellurite to attain blackening from growth of diphtheria bacilli.

g. Fermentation reactions.—*C. diphtheriae* can be differentiated from related organisms by their fermentation reaction in dextrose, saccharose, and dextrin. The absence of power of a particular organism to ferment glucose or its ability to ferment saccharose is usually sufficient to exclude the organism from being a diphtheria bacillus.

h. Virulence test.—This is the only certain method by which the identity and virulence of *C. diphtheriae* can be confirmed or distinguished from nonvirulent variants. No other known species of this genus, occurring in man, produces a fatal toxemia in guinea pigs. Pure cultures are preferred for this test, but for speedy test the suspension of a heavily positive Loeffler's tube may be substituted.

(1) *Subcutaneous method.*—Inject 2 cc of a pure culture grown for 48 hours in infusion broth, or 1 cc of a Loeffler's slant suspension in 2.5 cc of saline, subcutaneously into a 250-gram guinea pig. At the same time a similar injection of the culture is made into a control guinea pig which had been given 250 units of diphtheria antitoxin, intraperitoneally, 24 hours previously. If the organism is a virulent diphtheria bacillus, the unprotected animal will die within 3 to 5 days and on post mortem will show a gelatinous edema around point of injection and enlarged hemorrhagic adrenals.

(2) *Intracutaneous method.*—Two guinea pigs of 250 grams are used, one of which has been injected intraperitoneally with 250 units of diphtheria antitoxin 24 hours previously. The growth from a 24-hour Loeffler's slant is suspended in 20 cc of normal saline and 0.15 cc is injected intracutaneously at corresponding site of each pig. Six cultures may be tested at the same time on two animals. Virulent strains of diphtheria bacilli produce a definitely circumscribed local infiltrated lesion which shows superficial necrosis in 2 to 3 days. In the control pig the skin remains normal. If a mixed culture was used for test, and contained streptococci or staphlococci with sufficient virulence, local lesions will occur in both animals; the test would then be considered inconclusive and repeated using a pure culture.

i. Schick test.—This is an intracutaneous skin test to evidence the

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presence or absence of immunity to *C. diphtheriae.* The injection consists of 0.1 cc of diphtheria toxin (1/50 mld). A control test uses the same material which has been made inert by heat (75° C. for 5 minutes). Results are noted daily for 4 days and recorded as positive, negative, positive combined, or negative combined reactions.

j. Differentiation of similar types.—(1) *C. pseudodiphthericum (Hoffman's bacillus).*—This organism is shorter and thicker than *C. diphtheriae*, usually straight and clubbed at one end, rarely at both. When Loeffler-stained, it occasionally shows unstained transverse bands which, unlike those in *C. diphtheriae*, hardly ever exceed one or two. Sometimes the transverse band gives the bacillus a diplococcoid appearance. No polar bodies are demonstrable by Neisser's stain. It grows more luxuriantly and colonies are larger, less transparent, and whiter than are those of true diphtheria bacilli. A positive means of distinction is its inability to form acid on sugar media. It is not pathogenic to guinea pigs or to man. It is a common mouth commensal and may be found in 42 percent of normal throat cultures. Diphtheria-like bacilli which prove to be avirulent generally are found to be *C. pseudodiphthericum.*

(2) *C. xerose (Xerosis bacillus).*—This is a harmless saprophyte, commonly found in the normal or inflamed conjunctiva of the eye. It closely resembles *C. diphtheriae*, and is indistinguishable morphologically and culturally though generally shorter. Polar bodies may occasionally be seen. It differs in its acidifying action on sugar media and its nonpathogenicity to guinea pigs.

	Hiss serum water plus 1 percent			Virulence
	Dextrose	Saccharose	Dextrin	
C. diphtheriae	+	−	+	Virulent.
C. pseudodiphthericum	−	−	−	Nonvirulent.
C. xerose	+	+	−	Nonvirulent.

(3) *Diphtheroid bacilli.*—There is a large group of ill-defined organisms given this general name because of their morphological resemblance to the diphtheria bacillus. They often show metachromatic granules, and are not virulent when tested by the guinea pig virulence test. They are common saprophytes of the throat, skin, and other body areas and are so ubiquitous that any association of them with specific disease must be avoided. They must be distinguished from virulent and therefore significant diphtheria bacilli.

307. Mycobacterium tuberculosis (tubercle bacillus).—*a. Characteristics.*—Slender rods, straight or slightly curved with rounded

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ends; occur singly, in threads or in clumps; may stain evenly or irregularly, showing granular, beaded, or banded forms; stain with difficulty, but when once stained are acidfast; growth on media slow, wrinkly, aerobic, aided by glycerin, coagulated egg albumen, and other enrichments; growth on glycerin agar in 4 weeks at 37° C.; colonies minute, crumblike, irregular, whitish-yellow, later brownish, ridged, becoming dry; pathogenic to guinea pigs.

b. Habitat.—A strict parasite, causing tuberculosis of man, cattle, and other animals. Human and bovine varieties are distinguishable, both infectable to man. There are other species of this genus causing avian tuberculosis, or infecting fish, snakes, turtles, and other cold-blooded animals. There are a number of acidfast bacilli which are strictly saprophytic but confusable with *M. tuberculosis.*

c. Collection and transmission of specimens for examination.—Sputum, exudates, urine, spinal fluid, and tissues may be examined for tubercle bacilli. They should be collected under as sterile precautions as feasible (not possible with sputum) and transmitted in suitable container to laboratory. Sputum collection should be so guided as to provide bronchial material rather than the fluids from the mouth or nose.

d. Microscopical examination (directly applied to specimen).—A presumptive diagnosis can be made by applying an acidfast stain, such as Ziehl-Neelsen carbol-fuchsin to a slide spread of selected (caseous) fragments of the specimen. The red acidfast bacilli will be readily noted in contrast to the blue of the counterstain of all other bacteria, cells, and debris. Stained spreads may be made from the centrifuged sediment of urine or spinal fluid, using small film of sterile egg albumin on the slide to prevent the sediment being washed off during the staining process.

e. Concentration method.—If tubercle bacilli are too few to be found by above method, they may be concentrated by digesting mucus with sodium hydroxide, sulfuric acid, or antiformin and examining the centrifuged sediment by direct spread, by culture, or by guinea pig inoculation.

f. Sodium hydroxide-alum flocculation method.—(1) *Reagents.*—(*a*) *Digester.*

Sodium hydroxide	40.0 gm	(4 percent).
Potassium alum	2.0 gm	(0.2 percent).
Bromthymol blue	0.02 gm	(0.002 percent).
Water to		1,000 cc

NOTE.—Range of indicator 6.0 to 7.6 (yellow to blue); pH 7.0 equals light bluish green.

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(*b*) *Acid* (about 2.5 N)

Hydrochloric acid, concentrated	250 cc (25 percent)
Water to	1,000 cc

(2) *Test.*—(*a*) Mix sputum (3 to 5 cc) with 1 to 4 parts of digester. Shake well. Incubate at 37° C., 30 minutes for culture or animal inoculation or to homogeneous mass for microscopic examination.

(*b*) Adjust to pH 7 with acid-digester adjustment.

(*c*) Centrifuge at top speed for 5 minutes.

(*d*) Remove supernatant fluids.

(*e*) Spread on slides—heat fix, culture, or inject animals with saline suspension of sediment.

(3) *Result.*—The sodium hydroxide digests the organic matter. Flocculation occurs when acid is added. This flocculation carries into sediment the organisms, including tubercle bacilli, not killed or dissolved by the alkali.

g. Animal inoculation.—This is the most certain method of establishing the specific diagnosis of tuberculosis. Centrifuge the NaOH digested sputum or the urine or spinal fluid, suspend the sediment in sterile saline, and inject this subcutaneously or intramuscularly into the thigh of a young guinea pig (250 gm). Autopsy of animal at its death several weeks later, or if it lives, at 6 weeks, will reveal generalized tuberculosis, apparent particularly by caseation of glands, spotted liver, and large, spotted spleen, which may be confirmed by finding acidfast bacilli by direct spread or by special culture of these tissues.

h. Cultural examination.—This is not employed as a routine procedure. Several loopfuls of the sediment in the sodium hydroxide concentrate, or tissue fragment from guinea pig tissue, are planted on the surface of tubes of Petroff's (or other suitable) medium. Incubate the cultures for 2 days, then seal by dipping cotton plugs in melted paraffin. Incubate at 37° C. for 6 weeks and examine for colonies of *M. tuberculosis.*

308. Mycobacterium leprae (leprosy bacillus).—*a. Description.*—Small, slender rods resembling tubercle bacilli, straight, rarely bent or curved, pointed ends; acidfast; tend to be arranged in package bundles; cannot be cultivated; not pathogenic to guinea pig.

b. Habitat.—Found in the various lesions of leprosy, except the anaesthetic areas of nerve leprosy; especially demonstrable in nasal and skin lesions.

c. Identifying characteristic.—(1) Acidfast bacilli occurring in packets.

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(2) Not recoverable in culture or guinea pig.

d. Collection of specimen.—(1) *Nasal lesions.*—As the initial lesion of leprosy is often an ulcer at the junction of bony and cartilaginous septum, swabs or scrapings from this or other nasal lesions are spread onto glass slides.

(2) *Skin lesions.*—With a sterile safety razor blade, quickly make a small incision through the thickened area and, without removing it, depress the upper edge so that a scraping is made of the cut skin from below upward. Prepare slides from this scraped material. The deep, not the surface skin scraping, is desired for the spread.

e. Microscopic examination.—Spreads made as above are fixed, stained by Ziehl-Neelsen method, and observed for acidfast bacilli. They are more easily decolorized than are tubercle bacilli, though decolorization must not be carried too far. Typical packet bundles of lepra bacilli, or, in the skin nodules, lepra bacilli packed in "lepra cells" or in endothelial cells, are conclusive of leprosy.

309. Actinomyces.—Organisms growing in the form of a much-branched mycelium, which may break up into segments that function as conidia; sometimes parasitic, with clubbed ends of radiating threads conspicuous in lesions of the animal body. Some species are microaerophilic or anaerobic. They are nonmotile.

Bergey's Manual places genus *Actinomyces* in the same order as *Mycobacterium* and *Corynebacterium.* However, the general belief now is that they should be classified as true fungi and placed in class *Fungi Imperfecti.* The genus *Actinomyces* consists of 62 species, only 8 of which are described as animal parasites, the remaining species being plant parasites or nonpathogenic saprophytes of soil origin. The species parasitic for animals generally belong to the anaerobic or microaerophilic groups, while the plant parasites and the saprophytes are aerobes. All are Gram-positive. See section VII, chapter 10.

310. Actinomyces bovis.—Strictly parasitic organism causing actinomycosis in cattle and swine; may be transferred to man. Grows only at or near 37° C. and requires lowered oxygen tension. Pus from lesions in the animal body show sulfur granules consisting of club-shaped forms radiating at the outer surfaces of globular clusters of mycelium. Gelatin is liquefied slowly; milk is peptonized after 40 days. (See par. 398.)

311. Actinomyces hominis.—The cause of actinomycosis in man. Very similar in every respect to *A. bovis;* possibly not a true species but a variant of *A. bovis.*

312. Actinomyces madurae.—An aerobic or facultative anaerobic organism associated with one type of Madura foot in man. The

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lesions are most common on the foot, characterized by swelling, suppuration, and sinus formation; pus from lesions contains white to yellow granules. In cultures grows best at 37° C.; litmus milk is rapidly peptonized. See *Madurella mycetoma*, paragraph 398.

313. Examination of clinical materials for Actinomyces.—*a. Microscopic.*—Pus from the suspected lesion, diluted with sterile saline if necessary, may be poured in a thin film in the bottom of a sterile Petri dish and searched carefully for minute granules. The organisms are usually confined to the granules. Fish the granules with a capillary pipette, note color, and examine an unstained preparation microscopically under a cover glass without crushing, using a subdued light, for the detection of the peripheral fringe of club-shaped forms and the characteristic corrugated surface of the granule.

b. Cultural.—Obtain pus from a closed lesion, if possible, and wash in sterile saline; material containing many bacterial contaminants is unsatisfactory for culturing. Place the granules between two sterile slides, crush, and examine for presence of filaments. If these are present, inoculate several tubes of glucose agar, freshly boiled and cooled to 42° C. Incubate at 37° C. If growth occurs, it first appears in 2 to 4 days in a zone 1 or 2 cm below the surface of the medium as minute white specks.

314. Spirochetes.—*a. Characteristics.*—Slender, undulating, corkscrewlike, flexible, filamentous organisms. They have short or long spirals with the twists in three dimensions. The number, depth and relative length, and sharpness of angle of spirals have diagnostic importance, though somewhat variable. Motile by sinuous, rotating movement of the body, not by flagella as in the case of bacteria. Stain with difficulty by ordinary stains though some (genus *Borrelia*) stain readily; the polychrome methylene blue stains of Wright and Giemsa are most used; silver impregnation method is applicable to the more resistant forms, Fontana stain for spreads, Levaditi stain for tissues. Most readily demonstrable, to reveal their characteristic motility, in the fresh state, by dark ground illumination. Cultivation difficult and generally not practical. Animal inoculation significant in a few pathogenic species.

b. Habitat.—Ubiquitous, occurring in nature in soil, water, decaying organic materials, and on and in the bodies of man, animals, and plants. Some are saprophytes, others are commensals; a few are pathogenic, causing such severe diseases as syphilis, yaws, relapsing fever, and infectious jaundice.

315. Borrelia recurrentis (relapsing fever spirochete).—*a. Characteristics.*—Spirochetes having large, wavy, inconstant spirals, usually about five; when seen under darkfield illumination, the

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organisms are very active, in length several times the diameter of an erythrocyte, rapidly progress in either direction, disturbing the red cells by their motion; stain readily and uniformly by polychrome stains (Wright's or Giemsa's) and by simple stains; difficult to cultivate; inoculable into mice and rats, there causing periodicity of spirochetemia but no demonstrable clinical symptoms or tissue lesions.

b. Habitat.—The cause of relapsing fever. Found in blood and tissues of patients suffering from relapsing fever and in the body and intestinal contents of the infected vectors, ticks, and lice. The name applies to the spirochete of European relapsing fever; a number of other species—names have been given for the spirochetes of the United States and Mexico (*B. turicata*), Central and South America (*B. venezuelensis*) and others, differentiation of which is based only on specific immunological reactions. Some lower animals may serve as reservoirs of infection; in the United States, the armadillo and the opossum.

c. Identification.—Fresh or citrated blood, taken during febrile paroxysm, is examined:

(1) Darkfield illumination of fresh, thin slide-cover glass preparation, for the characteristic motility and morphology.

(2) Slide spread, stained by Giemsa's method or by dilute carbolfuchsin for morphology. Here the forms are much distorted, the spirals often obliterated, so that the characteristic morphology cannot be found. These spirochetes may sometimes be detected and the diagnosis suggested, in a routine Wright's stain for differential blood count.

(3) White mouse or rat inoculation, intraperitoneal, of 0.2 to 0.5 cc of blood; examine fresh tail blood from the second to fourteenth day for spirochetes.

316. Fusospirochetal disease (Vincent's angina).—*a. Definition.*—Vincent's angina is an inflammatory lesion in the mouth, pharynx, or throat, most often affecting gum margins and tonsils. An acute inflammation may lead to the formation of a pseudomembrane, suggesting that of diphtheria; later there are punched-out ulcers, suggestive of those of syphilis. The disease is localized, generally mild with minimal systemic disturbances. Two micro-organisms are almost always found together, in great numbers, in films from these lesions; the two forms apparently living in symbiosis. They are rarely present alone, being usually accompanied by other micro-organisms, such as staphylococci, streptococci, even diphtheria bacilli; the latter finding being more significant than the Vincent organisms alone.

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b. Fusobacterium plauti-vincenti (fusiform bacillus of Vincent).—Large bacilli, thick in middle, tapering toward ends to blunt or sharp points. Readily stained by Loeffler's methylene blue, carbol-fuchsin, of Giemsa stain, with characteristic unequality in the intensity of the stain, being more deeply stained near the end; banded alteration of stained or unstained areas in the central body, not unlike the metachromatic granules of diphtheria bacilli.

c. Borrelia vincentii.—Spirochetes somewhat like those of relapsing fever, longer than the fusiform bacilli; made up of variable numbers of undulations, shallow and irregular in their curvatures, unlike the more regularly steep waves of *Treponema pallidum.* They stain more evenly and less distinctly than the fusiform bacilli.

d. Identification.—(1) Make slide spreads from the ulcerative lesions, fix in flame, and stain deeply with dilute carbol-fuchsin,

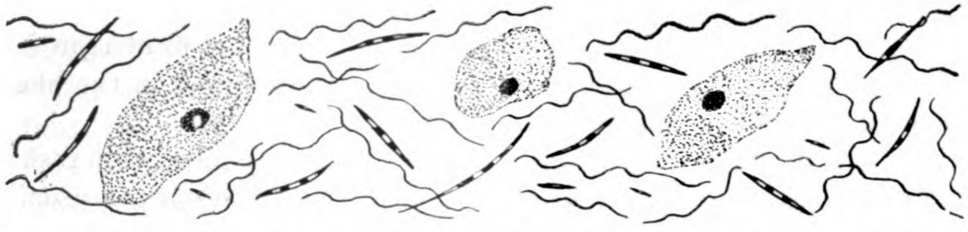


FIGURE 18.—Vincent's angina. (Note presence of *Borrelia vincentii,* fusiform bacilli, and epithelial cells.)

crystal violet, or Wright's stain and examine for fusiform bacilli and spirochetes.

(2) Positive results will be evidenced by finding great numbers of both fusiform bacilli and spirochetes. A few forms of either type is not significant.

317. Treponema pallidum.—*a. Characteristics.*—Delicate spirochete coiled in 8 to 14 regular, rigid, sharp spirals; spirals equal or greater in depth than in length, with acute rather than obtuse angles. As seen under darkfield illumination, it appears as a highly refractile, long, slender, spiral, silvery form with serpentine, corkscrew-like movement; motile, but does not progress rapidly or far; motion rotational with undulations.

Made visible most effectively by darkfield illumination. Difficult to stain with analine dyes other than the Giemsa stain; body stained pink by Giemsa stain or black by silver impregnation method (Fontana stain in spreads, Levaditi in tissues).

May be cultured by special methods and inoculated into some lower animals, neither procedure being practical for diagnostic purposes.

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b. Habitat.—Strict parasite of humans, causing the infectious disease syphilis, with protean manifestations; transmitted only by direct contact, generally through sexual intercourse, occasionally through intimate contact of other mucous membrane or skin sites. Syphilis, one of the most prevalent and important of all infectious diseases, usually progresses through a number of stages, irregular and varied:

(1) *Incubation period.*—Four to six weeks. Spirochetes then cannot be demonstrated.

(2) *Primary stage.*—Hard chancre at site of inoculation. Starts as a papule, enlarges, becomes hardened, and then ulcerates, forming an ulcer with a firm base and hard edge in typical form, but atypical lesions frequently occur, especially if mixed with secondary infection or coexistant with chancroid. Spirochetes can be found in fluid expressed from this chancre. The spirochetes will not necessarily be on the surface, rather in the deeper tissues and in the serum exuding from scarified lesion. At this stage they have already become disseminated to a general infection, can be demonstrated in fluid aspirated from satellite lymph gland, but cannot readily be found in the blood or other areas though potentially there.

(3) *Secondary stage.*—Characterized by mucous patches, skin rashes, and a variety of superficial lesions. *Treponema pallidum* can usually be found in material from these lesions.

(4) *Tertiary stage.*—Lesions of viscera, bones, central nervous and cardiovascular systems; tendency to deep rather than superficial lesions. Spirochetes are usually scanty, not readily demonstrated in these lesions.

c. Identification.—Different procedures are applicable to different lesions and stages.

(1) *Darkfield examination.*—(*a*) Lesions are cleansed of surface crust, detritus, pus, and surface organisms by gauze or cotton applicator. If lesion has received any germicidal agent, examination is deferred until all germicide has been removed and the lesion has had applied to it only a saline pack for a day or two.

(*b*) Primary lesions are then given some trauma, to provoke exudation of serum, by gently rolling the lesions between the gloved finger and thumb or by rubbing its surface with a dry cotton applicator; avoid hemorrhage (though a few erythrocytes or pus cells are desirable to aid in obtaining proper illumination).

(*c*) Secondary lesions are merely cleansed and abraded.

(*d*) Slide-cover glass, fresh preparation may be made from accessible lesions by merely touching the slide to tissue juice and immediately placing the cover glass over this moist drop. Vaseline placed around edge will prevent drying. If lesion is less accessible, the fluid

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may be collected in a capillary pipette and placed on slide from this.

(*e*) Examine immediately on darkfield microscope for characteristic morphology and motion of *Treponema pallidum.* Exercise caution not to misinterpret observation. There are many saprophytic spirochetes which are easily distinguished; there are a few spirochetes, especially in the mouth, which are more difficult to distinguish.

(*f*) "Artifact spirochetes" provoke mistakes to those unfamiliar with the appearance of blood, pus, and cultures under darkfield illumination. Wavy filamentous structures may occur there which give a false impression of spirochetes; forms given off by red corpuscles in a drop under a cover glass may falsely suggest spirochetes.

(*g*) Report findings with qualifying data, such as notation of location of lesion examined, the occurrence of conditions making examination unrepresentative, etc.

(2) *Delayed darkfield method.*—This is a scheme of forwarding lesion fluids to a distant laboratory for darkfield examination; employable when facilities for darkfield examination are not locally available, or when local examiners desire confirmation of their own findings by a consultant. A tissue fluid from a suspected lesion is allowed to flow into a capillary tube 8 cm long by 1 mm in diameter; the two ends of this tube are sealed by pressing into a soft paraffin-vaseline mixture (50 percent of each) and these tubes forwarded for the darkfield examination. At examining laboratory the serum may be transferred to a slide by pressing one end of the capillary tube into a paraffin-vaseline mixture until the opposite end plug is forced out.

(3) *Nigrosine method.*—This is not strictly a staining method for it leaves the unstained spirochete in a black field. A loopful of the fresh tissue fluid is mixed with a loopful of 5 percent aqueous solution of nigrosine (plus 0.5 percent formalin as a preservative); this mixture is spread on a glass slide, dried, and examined by ordinary illumination with oil immersion objective. A remote examination may be made, by forwarding an air-dried drop of the exudate on a slide; the laboratory adds a loopful of water to this to dissolve the exudate and proceeds with the nigrosine preparation. Results are far inferior to the darkfield method, for the characteristic motility is absent and the spirochetes, by distortion, have lost much of their characteristic morphology.

(4) *India ink method.*—Like the nigrosine method, a drop of material is mixed with a drop of drawing ink and the mixture spread on a slide. When dry, examine for white spirals against a dark background.

(5) *Stained spread examination.*—By Giemsa or Fontana methods.

(6) *Local Wassermann.*—Considerable amount of serum is col-

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lected from the local lesion and used for complement fixation test.

(7) *Blood serum and spinal fluid serology.*—Applicable to later stages. It is customary to subject all venereal patients, even after repeated negative darkfields, to follow-up blood tests for several months.

318. Leptospira icterohaemorrhagiae (Weil's disease—infectious jaundice).—*a. Characteristics.*—Spirochetes of many fine coils, so fine as to be difficult to distinguish; one or both ends may be bent into a hook. Rapid, spinning motion with intermittent active lashings. Difficult to stain; stained reddish by Giemsa method. Cultivated only by special methods. Inoculable into guinea pig with distinctive lesions.

b. Habitat.—The blood and kidneys of infected wild rats. The blood, urine, kidney, and biliary tract of patients with infectious jaundice (Weil's disease).

c. Identification.—(1) Guinea pig inoculation: inoculate 3 to 5 cc of fresh blood, fresh urine sediment, or tissue suspension, intraperitoneally into white guinea pig; observe it daily for fever, jaundice in the ears, eyes, and about genitalia and for *leptospira* in the blood (usually found after the fourth day). After the animal dies, large numbers of *leptospira* can be demonstrated in emulsions of the liver, kidneys, and adrenals.

(2) Darkfield examination of tissue emulsions, occasionally of urine or biliary sediment, for the motile *leptospira*.

(3) Stained spreads and cultures have limited application.

SECTION III

STOCK CULTURE MAINTENANCE

	Paragraph
Purpose	319
Type cultures required	320
Rules for maintenance	321

319. Purpose.—Stock cultures of known bacterial species are required for use in—

a. Checking growth properties of newly prepared media.

b. Checking stains.

c. Training technicians.

d. Preparing antigens and antisera.

e. For special investigations.

320. Type cultures required.—The number and type of cultures to be maintained in any particular laboratory depend upon its size and the type of work being done. Corps area, general hospital, and

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larger station hospital laboratories doing bacteriological work should have most of the following cultures:

a. For checking stains.—Gram-positive and Gram-negative cocci and bacilli.

b. For checking media.—(1) Eosin methylene blue plates—*E. coli*, *A. aerogenes*, and organisms of typhoid-dysentery group.

(2) Blood plates—*Staphylococcus aureus*, *Streptococcus salivarius*, and *Streptococcus pyogenes.*

c. For preparing antigens.—*E. typhosa*, *S. paratyphi*, *S. schottmuelleri*, *S. typhimurium*, *S. dysenteriae*, *Proteus* OX_{19}, *Proteus* OXK, and *Br. abortus* or *Br. melitensis.*

d. Special cultures.—Any other culture required for instruction purposes or laboratory use.

321. Rules for maintenance.—*a.* Before adding a new strain to the stock culture collection, unless it has been received from a central laboratory, check the culture very carefully, culturally and serologically, and keep complete record of results.

b. The transfer period for each species must be learned from experience. Roughly, most cocci and Gram-positive nonsporing bacilli require transferring every 1 to 3 months; the strictly parasitic Gram-negative bacilli (family *Parvobacteriaceae*), every 3 to 6 months; other Gram-negative bacilli (enteric group), every year or two; and spore-forming bacilli, every 1 to 5 years.

c. Transfer to simple, solid medium, free from carbohydrates. A too luxuriant growth is not desirable. Plain extract agar slant is satisfactory for most cultures. Others may require a blood agar slant.

d. Check cultures from time to time for variation, especially for the smooth to rough change, by plating on infusion agar or other media and selecting desired typical strain for transfer.

e. Before use, make two or three daily serial transfers to obtain rapidly growing organism.

f. Seal stock cultures carefully with paraffin-petrolatum mixture and store at room temperature.

g. Make one man responsible for handling cultures and making transfers.

h. The Army Medical School preserves many of its cultures by the lyophile process (rapid freezing of culture and drying in vacuum); this is impractical in small laboratories. Such ampoules received at a distant laboratory may be continued as a stock culture by transfer onto culture media.

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SECTION IV

PREPARATION OF AUTOGENOUS VACCINES

	Paragraph
Selection of culture	322
Preparation of suspensions	323
Killing of bacteria	324
Determining bacterial content	325
Preparation of vaccine	326

322. Selection of culture.—An autogenous vaccine is one prepared from a culture isolated directly from the patient who is to be treated with that vaccine. With the infected materials, such as pus or tissues, prepare Gram-stained films and examine them for type organisms present. Inoculate blood agar and infusion agar plates and tube of infusion broth and incubate for 24 to 48 hours at 37° C. Examine the plates for predominating types of colonies and make Gram-stained preparations from each type. Also examine stained preparations from the broth culture for presence of any organism not found on plate cultures and inoculate upon plate media if indicated. If a single type organism is found in pure culture, transplant to the desired media for the vaccine. When mixed cultures are obtained, select isolated colonies of the type desired and transfer to plates to isolate pure cultures; incubate for growth; and again check for purity of cultures. If several organisms are present, presumptive evidence as to which organism is concerned in the infection can sometimes be gained, first, by agglutination of the organism by patient's serum or second, by positive skin reaction on intradermal injection of the vaccine. Do not use spore-forming bacilli or any organisms which are obviously contaminants. Staphylococci and streptococci are the organisms most frequently used for production of autogenous vaccines.

323. Preparation of suspensions.—*a. Agar slant cultures.*—Most bacteria are best grown on infusion agar slants for 24 to 48 hours; blood or serum agar is required for growing other organisms. Add 2 or 3 cc of sterile saline to each tube and emulsify bacteria by shaking or by agitation with a platinum loop. The suspension should be quite heavy. If any clumps are observable in the bacterial suspension, transfer to a sterile flask or bottle containing beads and shake thoroughly; then filter aseptically through several layers of gauze, held in a small funnel, to break up any remaining clumps or to remove any particles of culture media.

b. Broth cultures.—If broth cultures are used, sediment the bacteria by centrifuging and resuspend in sterile saline; centrifuge a

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second time and resuspend in sufficient saline to give a heavy suspension.

NOTE.—It is sometimes desirable to prepare a vaccine containing both bacteria and their exogenous toxins; in this case, do not centrifuge or wash in saline.

Break up clumps of bacteria and continue as above.

324. Killing of bacteria.—Place the bacterial suspension in a sterile ampoule (standard item No. 40074) and seal. Weigh the ampoule with sheet lead and immerse in a water bath at 56° to 60° C. for 1 hour. Remove from the water bath and culture for sterility by inoculating 0.25-cc portions into suitable media and incubating aerobically and anaerobically for 2 or 3 days.

325. Determining bacterial content.—*a. Wright's method.*—Prepare a capillary pipette with long capillary section; mark about ½ inch from tip. Draw up bacterial suspension to the mark, then a small air bubble, and then blood from the finger tip to the same mark. Mix quickly on a slide; make thin smear and stain by Wright's method. Count the number of red blood cells and the number of bacteria in several areas. Use the following formula to determine the number of bacteria per cubic centimeter of suspension:

$$\frac{\text{Bacteria counted}}{\text{Red cells counted}} \times 1{,}000 \times 5{,}000{,}000 = \text{number of bacteria per cc.}$$

b. Nephelometric method (McFarland).—This is the preferred method, when the suspension contains no coloring matter. It consists in comparing the opacity of the bacterial suspension with that of various densities of barium sulfate in a series of test tubes.

(1) *Preparation of standards.*—Prepare 1 percent aqueous solutions of CP sulfuric acid and of CP barium chloride. To a series of 10 pyrex glass test tubes of uniform size add increasing amounts of the BaCl solution, starting with 0.1 cc in first tube, increasing the quantity by 0.1 cc in each succeeding tube, to add 1.0 cc in tenth tube. Then add to each tube enough H_2SO_4 solution (9.9 cc to 9.0 cc, respectively) to bring the total volume to 10 cc. Seal hermetically and label serially from 1 to 10.

The density of the suspensions in these tubes corresponds approximately to from 300 million organisms per cubic centimeter for first tube to 3,000 million per cubic centimeter for tenth tube, increasing by 300 million bacteria for each succeeding tube from 1 to 10.

(2) *Technic.*—Place a measured quantity (1.0 cc or more, depending on density) of the bacterial suspension in a test tube of the same diameter and color as those used for the standard. Dilute

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by adding a measured amount of sterile saline to the density of one of the standards; shake well during process.

(3) *Calculation of bacteria per cc.*—The approximate number of bacteria per cubic centimeter of suspension corresponds to the tube matched, times the dilution. For example, if 1.0 cc of suspension was diluted to 4 cc to match tube No. 4, it contains 1,200,000,000×4, or 4,800,000,000 organisms per cubic centimeter.

NOTE.—A bacterial standard containing 1,000 million organisms per cubic centimeter can be prepared by adding 4 cc of sterile saline to 8 cc of triple typhoid vaccine (1,500 million bacterial per cubic centimeter) and sealing in pyrex glass test tube.

326. Preparation of vaccine.—Most autogenous vaccines for treatment are prepared in a concentration of 1 billion organisms per cubic centimeter. To prepare a definite quantity, say 30 cc, of vaccine of this strength from the bacterial suspension above, find the number of cubic centimeters of suspension required by use of this formula:

$$\frac{30 \text{ (No. of cc of vaccine required)} \times 1{,}000{,}000{,}000 \text{ (desired strength)}}{4{,}800{,}000{,}000 \text{ (strength of suspension)}} = 6.25 \text{ cc}$$

After standardization, add phenol to a final concentration of 0.5 percent (1 cc of stock 5 percent phenol to 9 cc of vaccine) for preservation. Dispense in a properly labeled, sterile vaccine bottle, closed with a rubber stopper.

SECTION V

PRODUCTION OF DIAGNOSTIC ANTIGENS AND ANTISERA

Paragraph
Bacterial antigens 327
Production of antisera 328

327. Bacterial antigens.—*a. Definitions.*—(1) An antigen is any substance which, when injected parenterally into a suitable animal, can stimulate the production of specific antibodies.

(2) Bacterial antigens are suspensions of living or dead bacteria grown in broth cultures, or saline suspensions of cultures from solid media. They are required either for the production of specific diagnostic antisera in animals or for use in testing the sera of patients for specific antibodies.

b. Selection of cultures.—Antigens are usually prepared from normal smooth-type cultures; at times, however, it is necessary to make antigens from variant strains. Having decided upon the species and type of bacteria to be used, considerable knowledge of its normal and

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dissociative state, especially of possible variations in antigenic content, are required to enable one to make an intelligent selection of the culture to be used. Study the culture carefully to determine—

(1) Purity of culture.

(2) Typical morphology of organism, especially shape and motility.

(3) Biochemical and other cultural characteristics, especially noting smoothness or roughness of culture.

(4) Agglutination reactions using homologous and closely related antisera.

c. Preparation of antigen.—(1) *Flagellar "H" antigens.*—These can be prepared as broth cultures, which are usually slightly more sensitive, or as saline suspensions of 24-hour growth on infusion agar, which can be prepared and stored in more concentrated form.

(*a*) First day: Streak culture on infusion agar plate or other suitable media. Incubate at 37° C. for 18 to 24 hours.

(*b*) Second day: Select several colonies of the type desired (usually smooth) and transfer to infusion broth. Incubate 24 hours at 37° C.

(*c*) Third day: Study the 24-hour broth cultures and select the most actively motile, typical growing culture for further transfer.

(*d*) Third to sixth day, inclusive: Make daily transfers to infusion broth.

(*e*) Seventh to tenth day, inclusive: Make twice daily transfers to the medium to be used for final antigen, either infusion broth or infusion agar.

(*f*) Eleventh day: Inoculate desired amount of media, using either infusion broth in flasks or infusion agar in "Kolle" flasks. Incubate 18 to 24 hours at 37° C.

(*g*) Twelfth day: Harvest growth from agar cultures, in about 30 cc of sterile saline per flask or take broth cultures; pool in stock bottle, filtering growth from agar through sterile gauze; and add 0.2 per cent CP formalin to kill the bacteria and preserve sterility. Store in icebox.

(*h*) After 5 days in icebox, remove and take 1 to 5 cc from bottle. Dilute this with sterile saline until the density is similar to that of tube No. 3 of the nephelometer (McFarland) standard, noting exact proportion of antigen and saline required. Label stock bottle with all pertinent data including preservative used and dilution factor. Store in icebox. Prepare small bottles of antigen for use from time to time, diluting stock antigen with sterile saline containing 0.2 per cent formalin.

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(2) *"O" antigens from motile organisms.*—Use same process as *c*(1) above except—

(*a*) Select nonmotile or less motile variant strains.

(*b*) Usually grown on infusion agar.

(*c*) Any flagellar antigenic substance must be destroyed by heating the pooled stock antigen at 90° C. for 1 hour.

(*d*) Kill and preserve stock antigen with 0.5 percent phenol. Also prepare antigen for use by diluting with sterile saline containing 0.5 percent phenol.

(3) *Antigens from nonmotile bacteria.*—Prepare as in *c*(1) above, but grow on infusion agar and use 0.5 percent phenol as preservative.

328. Production of antisera.—*a. Definition.*—Serum from an animal, such as horse, goat, or rabbit, containing specific antibodies produced by the injection of an antigen, is known as an antiserum.

b. Preparation.—(1) Prepare antigen and dilute to 1,000 million organisms per cc.

(2) Inject animal, usually rabbit, intravenously, subcutaneously, or intraperitoneally with increasing dosages of the antigen. Dosage will vary with animal used, bacterial species, frequency of inoculation, and route of inoculation.

(*a*) For most nontoxic bacteria, inject rabbit intravenously with dosages of 0.5 cc, 1.0 cc, 1.5 cc, and 2.0 cc at intervals of 5 to 7 days.

(*b*) For toxic organisms, such as *S. dysenteriae* and the *Salmonella* group, inject rabbit intravenously with smaller doses (25, 50, 100, and 250 million organisms, etc.) at intervals of 3 to 5 days.

(3) Observe rabbits carefully for evidence of toxic poisoning (loss of weight, weakness, etc.); if present, decrease dosage or lengthen period between injections.

(4) Six days after last injection, withdraw 1.0 or 2.0 cc of blood from ear vein and check for satisfactory agglutination titer. If necessary, give rabbit 2 or 3 more injections and recheck.

(5) If titer is satisfactory, bleed animal to death from the heart. Separate serum, sterilize, clarify by filtration, and preserve with 0.5 percent phenol. Check titer with homologous and closely related antigens.

SECTION VI

HANDLING OF BACTERIOLOGICAL SPECIMENS

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329. General.—Specimens of pathological materials to be used for bacteriological examination should be collected from locations where the suspected organisms are most likely to be found and must be handled aseptically in order to avoid contamination with extraneous micro-organisms. Care must also be exercised to avoid collecting specimens at a time when the infecting organisms have probably been killed by the use of antiseptics. Closed, sterile, glass containers are required, but the type of container will depend on whether the specimen can be examined immediately or must be shipped some distance. As a rule, small specimens are shipped in sealed glass tubes, protected with absorbent cotton, in tightly closed tin containers which are enclosed in larger mailing cases. Methods suitable for collecting a variety of pathological materials from different locations are briefly indicated in the paragraphs below.

330. Materials from eye.—Collect specimens from the infected eye during the period in which the disease is progressing, as at a later time one may obtain only such saprophytes as *C. xerosis* or staphylococci. The secretions may be collected either with a cold sterile platinum loop or with a cotton swab, avoiding contamination due to contact with the margins and angles of the lids. Sufficient material should be taken to prepare at least two stained smears in addition to the cultures. Prepare cultures on blood agar in Petri plates and in infusion broth or Robertson's broth. Specimens from the infected cornea, the anterior chamber, or the iris should be collected by the ophthalmologist in charge of the case. Pus from sties should also be collected by the attending surgeon. Enucleated eyes may be sterilized externally by dipping them in boiling water or an antiseptic solution, after which they can be opened aseptically and the desired structures removed for bacteriological examination.

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331. Materials from ear and mastoid.—In infections of the external auditory canal, the excretions may be collected on an ordinary sterile cotton swab. In otitis media or in mastoid infections, the specimens should be collected by the attending surgeon. Both smears and cultures should be prepared, using for the latter, blood agar plates and either infusion or Robertson's broth.

332. Materials from nose, sinuses, and nasopharynx.—Collect specimens from either the nasal passages or from the nasopharynx on a small sterile cotton swab, tightly wrapped on a sterile wooden applicator. Tilt the tip of the nose upward and insert the applicator gently, pushing it backward with a rotary motion until the posterior wall is reached. Withdraw the applicator and prepare cultures on blood agar plates, Loeffler's coagulated serum slants, and in infusion broth, or any other medium desired. Material from infected sinuses should be collected by the rhinologist responsible for the case.

333. Materials from throat and tonsils.—*a.* Inflammatory exudates from the throat are usually collected on sterile cotton swabs slightly larger than those recommended for use in the nose. The throat should be well illuminated so that material can be taken directly from inflamed areas, which must be swabbed rather deeply to insure getting a sufficient number of the pathogenic micro-organisms. Prepare smears and stain by Gram's method; or, if indicated, by Neisser's differential method for *C. diphtheriae*, or with dilute carbol-fuchsin for the organisms of Vincent's angina. Make cultures on blood agar, Loeffler's coagulated serum, and if desirable, in infusion broth.

b. When searching for focal infections, or for diphtheria carriers, it may be desirable to make cultures of materials from the crypts of the tonsils. First collect material from the entire tonsillar surface on a cotton swab. Then carefully insert into the crypts a platinum loop bent at right angles. If desirable the contents of the crypts may be squeezed out by pressure or drawn out with a special glass tube attached to a suction pump. As a rule such specimens should be collected by the attending laryngologist.

c. Tonsils removed surgically may be sterilized externally by dipping them into boiling petrolatum or water, or by exposure to antiseptic solutions, after which materials from their internal structure may be obtained aseptically and used for examination.

334. Sputum.—Sputum may be collected in a sterile Petri plate, wide-mouthed bottle, or vial, preferably soon after the patient awakes in the morning. Prior to the collection, the teeth should be brushed

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with a boiled tooth brush, and the mouth washed with boiled water. The sputum is then coughed up and expectorated directly into the sterile container, avoiding unnecessary introduction of saliva. The use of paper boxes or other containers which are not sterile is not recommended even for specimens which are to be examined microscopically for tubercle bacilli. Specimens should be kept cold and, if possible, examined within 24 hours. The procedures followed will differ considerably depending on the pathological conditions suspected. If the specimen is to be examined for pyogenic bacteria, prepare films and stain by Gram's method; also make cultures on blood agar and in infusion broth. If tubercle bacilli are suspected, the films should be stained by the Ziehl-Neelsen method; cultures may be prepared by the Petroff or Corper method and animals may be inoculated. Sputum to be examined for pneumococci may be inoculated on blood agar and infusion broth and may also be inoculated into white mice, if direct typing by Neufeld's method is unsatisfactory.

335. Blood.—Blood for bacteriological examination must be collected under aseptic conditions, by an experienced technician or preferably by a physician. The specimens are usually collected from the median basilic or median cephalic veins. Withdraw 8 to 10 cc of blood by venipuncture as described in paragraph 40. Place 2 to 3 cc of the blood into each of two flasks containing 100 cc of glucose infusion broth or other suitable media, and place the remainder in a large sterile test tube containing 1 cc of a 0.2 percent sterile solution of sodium citrate in physiological salt solution. Measured amounts of the citrated blood can be placed in plates and mixed with melted agar or other media as desired. For shipment, blood is collected as above and placed in bottles of sterile glucose broth, or, if typhoid is suspected, bottles of sterile bile may be used. Such culture bottles are labeled, packed, and shipped in double containers.

336. Blood serum.—Blood to be used for the collection of serum, for agglutination, complement-fixation, or precipitation tests may be obtained with a large sterile needle and a sterile tube. Allow from 5 to 10 cc of blood to flow directly from the needle into the tube. Stand the blood specimen in an upright position, protected from dust and sunlight, until coagulation is complete. Loosen the clot from the side of the tube with a sterile wire or glass rod, and centrifuge until the serum is free from red blood cells. With a sterile pipette, transfer the clear serum to sterile vials. Cork securely, label, pack, and ship at once. Contaminated specimens deteriorate rapidly in warm weather and become unsuitable for satisfactory examination.

337. Cerebrospinal fluid.—Immediately after collection prepare films, stain by the Gram and Ziehl-Neelsen methods, and inoculate

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0.5- to 1.0-cc amounts on plates of blood or serum agar and on slants of Loeffler's coagulated serum. Also inoculate tubes of infusion or Robertson's broth. If a coagulum forms in the fluid, this should be selected for bacteriological examination. If only a few organisms are present, they may be concentrated by centrifuging.

338. Pleural, pericardial, or other fluids from serous cavities.—These may be collected by the attending physician by aspiration with a sterile syringe and a moderately large needle. The films and cultures are prepared as with cerebrospinal fluid.

339. Bile.—Bile collected by the surgeon at operation may be placed in a sterile tube and later inoculated on blood agar, eosin methylene-blue agar and infusion broth (pH 7.4 to 7.6), using 1 cc to 100 cc of the medium. However, specimens are more commonly obtained by nonsurgical drainage through a sterile duodenal tube. The different fractions of bile obtained may be inoculated on duplicate sets of culture media. Such cultures may be very helpful in the isolation of typhoid bacilli from suspected cases or carriers.

340. Feces.—*a.* Fecal materials to be examined bacteriologically should be passed by the patient directly into a sterile container, such as a bedpan, basin, fruit jar, or Petri plate. A small portion may be collected with a sterile spatula and placed in a sterile wide-mouthed bottle or vial. In the United States Army, special vials and metal spoons (medical supply items Nos. 44710 and 44000) are furnished for the collection of feces. The specimens may be preserved by adding a small portion of feces to a vial containing 2 cc of glycerol saline solution (glycerol 30 cc, NaCl 0.42 gm, sterile water 100 cc). The vial also may be obtained ready for use (item No. 18270).

b. Specimens may be collected directly from the individual as follows: After cleansing the perianal skin with soap, water, and dilute alcohol, introduce into the anus a sterile cotton swab moistened with sterile broth or salt solution.

c. If it is desirable to collect culture materials from the lesions in ulcerative colitis, this should be done by a proctologist, and the specimen should be inoculated on blood agar and infusion broth.

341. Urine.—Specimens should be collected by catheterization under aseptic conditions, but in dealing with infants, specimens must be collected directly from the cleansed urinary meatus. It should be remembered that urine samples collected by the latter method are often contaminated with colon bacilli and staphylococci. However, because of the striking differential features of certain organisms including those of the typhoid-paratyphoid group, contamination may not seriously interfere with their recognition in cultures, and therefore, specimens to be used in carrier surveys may be passed by the patients

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directly into sterile containers. Such specimens can also be used in preparing films to be examined for various organisms including gonococci, streptococci, colon bacilli, and tubercle bacilli, although the results are insufficient for anything more than a "presumptive" identification. In suspected tuberculosis of the urinary tract, it is safer to base the diagnosis on the examination of urine collected aseptically through a catheter and inoculated into animals. All urine specimens should be centrifuged and the sediment used for examination.

342. Materials from urethra and prostate.—*a.* In gonorrheal urethritis, collect pus from the urethra on a sterile cotton swab and with this prepare two or more films and stain them by Gram's method. If desirable, inoculate cultures on chocolate blood agar or other special media.

b. In chronic infections, urethral and prostatitic secretions may be collected subsequent to emptying the bladder. The meatus is washed with soap and water, and while the patient constricts the urethra just behind the glands, the prostate is massaged until a small amount of fluid is expressed. Collect this in a sterile Petri plate and use for preparing films or cultures as desired.

343. Materials from wounds.—Pus or fluid in infected wounds may be collected on sterile cotton swabs or with a platinum loop. If it is desirable to estimate the number of bacteria present on different days, use the same size loop for each collection and make the films as uniform as possible. Such preparations should be stained by Gram's method. Two sets of cultures should be prepared on nutrient agar, blood agar, and Robertson's broth, one set to be incubated under aerobic, the other under anaerobic conditions. If it is suspected that the patient may have gas gangrene, this information should accompany the request in order that special examinations may be immediately made for the detection of pathogenic anaerobes. Wound specimens should be collected by the attending surgeon.

344. Autopsy materials.—Due to the rapidity with which bacteria invade the tissues after death, all diagnostic cultures to be made on autopsy materials should be collected within 1 or 2 hours, if the results are to be of value. As embalming destroys most bacteria, the cultures should be collected before this is done. In taking cultures from the various organs, it is customary to first sear the surface with a flat cautery and, after making a small incision with a sterile knife or needle, to remove sufficient material with a pipette, swab, scalpel, or platinum loop.

345. Disinfection of discarded specimens.—In many instances it is advisable to keep specimens until the bacteriological examination

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has been completed, in order to have on hand sufficient material to repeat the tests if necessary. Specimens or cultures to be discarded should first be sterilized. The contaminated glassware which accumulates from day to day may be placed temporarily in a large bucket or crock filled with a 5 percent solution of cresol. Pipettes are usually kept in separate containers. At convenient intervals remove the glassware, place it in a metal pan or pail, and sterilize it in the autoclave. Thereafter it may be safely washed and prepared again for use.

346. Data to accompany specimens.—Each specimen sent to a laboratory should be accompanied by W. D., M. D. Form No. 55 L–15 or other appropriate slip in duplicate, signed by the requesting officer. State specifically the examination desired. Show tentative diagnosis and any other information that might be of value to the laboratory officer in selecting tests to be run. If two or more different type examinations, such as for a Wassermann test and for a Widal test for typhoid, are desired on the same specimen from a patient, submit, if possible, separate specimens, each accompanied by appropriate request slips.

347. Precautions.—Pack the specimen carefully to avoid breakage and leakage. Do not ship a liquid specimen or a specimen in liquid media in a cotton-plugged container. Submit specimen sufficient for all tests required and, after specimens have been prepared, mail or deliver to the laboratory promptly to prevent spoilage.

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CHAPTER 10

SPECIAL BACTERIOLOGICAL METHODS

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SECTION I

BACTERIAL FOOD POISONING

	Paragraph
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348. General.—Food poisoning herein considered is that due to the ingestion of foods contaminated with certain types of bacteria or their products, provoking clinical manifestations. A variety of bacterial species have been suspected in outbreaks of food poisoning, of which three main types of bacteria have been definitely incriminated and must be considered in analyzing an outbreak.

a. Botulinus group (*Cl. parabotulinum* and *Cl. botulinum*).—These are saprophytic anaerobes of the soil which produce botulism only when their powerful performed exotoxin is ingested in foods.

b. Salmonella (*paratyphoid*) *group of bacilli.*—Chiefly *S. schottmuelleri*, *S. enteritidis*, *S. choleraesuis*, and *S. typhimurium* which cause food poisoning either by preformed thermostable toxins or by rapid multiplication in the body after ingestion.

c. Staphyloccocus group.—Various species, contaminating such food items as cream fillers in pastry, cakes, incubated tenderized ham, and others.

349. Investigation program.—It is essential that the clinician, the epidemiologist, and the bacteriologist work in close cooperation in the difficult program of determining the cause and source of an outbreak of food poisoning. The clinician recognizes when and where food poisoning is occurring and reports the cases and their features. The bacteriologist aids in the diagnosis of the cases and identifies suspect agencies presented by the study of the epidemiologist. The

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latter assembles the data, traces the source and agency, and determines corrective measures. The following general procedures are to be followed by the clinician, epidemiolgist, or bacteriologist in the study of a food-poisoning outbreak.

a. List of all cases and their clinical features is prepared.

b. History of all cases is obtained, especially itemizing foods and associations prior to onset of symptoms.

c. Food responsible for the illness is determined or suspected by comparing histories of the cases.

d. History of implicated food is studied, sample reclaimed if possible, the names of food handlers listed, and method of preparation and storage determined.

e. Contamination source is estimated from the history of food.

f. Suspect materials and diagnostic materials are presented for test.

(1) Left-over portions of the suspected foods (packed in ice, examined promptly).

(2) Specimens of vomitus, feces, blood serum (later) from the cases.

(3) Feces and blood from suspected food handlers (for *Salmonella* studies only).

(4) Specimens of blood, spleen, liver, and intestines of fatal cases.

g. Bacteriological examination for the three groups of organisms or their bacterial toxins.

350. Bacteriological program.—*a. First day.*—(1) Morphological features of predominating organisms are determined in Gram-stained films prepared from liquid portions of the foodstuffs or from suspensions of solid foods.

(2) Cultures are planted.

(*a*) Eosin methylene blue plates for the detection and isolation of organisms of the genus *Salmonella*, 37° C. incubation.

(*b*) Infusion agar and broth for *Staphylococci*, 37° C. incubation.

(*c*) Anaerobic media for *Cl. parabotulinum* (if suspected, otherwise omit). Heat half of the inoculated tubes at 70° C. for 20 minutes. Incubate at 35° C., preserving anaerobiasis.

(3) Test for toxin: The samples of suspected foods may be tested for preformed toxins or for pathogenic (to animals) bacteria by oral or subcutaneous administration to guinea pigs or white mice.

(4) Test for botulinus toxin (used only when suspected): Suspected food is suspended in saline solution, centrifugalized or filtered until clear, and 1 cc of this is injected subcutaneously into each of three guinea pigs. At the same time one animal receives (ip) 1 to 1.5 cc of type A antitoxin, another receives a like amount of type B

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antitoxin, and the third receives the same amount of both A and B antitoxin. If botulinus toxin in fatal dosage is present in the food, the type is indicated by the following table of expectancy:

Guinea pigs protected with antitoxin	1 cc botulinus toxin subcutaneously		
	A	B	A+B
No. 1, type A	Live	Die	Die.
No. 2, type B	Die	Live	Die.
No. 3, types A and B	Live	Live	Live.

That is, if No. 1 dies, it is type B; if No. 2 dies it is type A; if both die, there are both A and B toxins.

b. Second day.—(1) Examine agar plates for *Staphylococci.*

(2) Examine eosin methylene blue plates for *Salmonella.* If present, isolate pure colonies and identify (see par. 293).

(3) Note the presence of other organisms on either media.

(4) Examine anaerobic tube. If growth is noted, prepare Gram-stained slide films. If organisms of the morphology of *Cl. parabotulinum* are found, transfer to fresh blood agar plates and incubate these at 35° C. anaerobically for 24 to 48 hours. Isolate in pure culture and identify species and type toxin by test for toxin production, as above, for suspected foods.

c. Third and following days.—Determinative work on isolated micro-organisms.

Section II

BACTERIOLOGY OF WAR WOUNDS

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351. General.—*a.* A war wound, in fact any traumatic wound, presents problems quite different from those encountered in aseptic surgery.

(1) Bacteria, aerobic and anaerobic, are carried by the projectiles or secondary missiles from the soil, clothing, or skin, into the tissues.

(2) Tissues are destroyed to a variable degree and such dead or devitalized tissues furnish excellent culture media for bacterial growth.

(3) Latent period, or interval, between contamination of the wound and definitive surgical aid, if beyond 6 hours, permits proliferation and penetration of the contaminating organisms.

b. The immediate aim of treatment, to be followed by such surgical restoration as indicated, is the prevention or limitation of infection. Bacteriological control, by examination of wound smears and cultures, determines these procedures.

c. Debridement is the first step by the surgeon in limiting infection in such wounds, by removing all of the devitalized tissues and foreign bodies which would provide nidus of infection. Bacteria are greatly diminished in number but not eradicated by this procedure; at least most of the culture materials for bacterial growth are removed.

352. Smear or culture control.—*a. Smear method.*—(1) The smear method is simple, can be carried out by an average technician without elaborate procedures, and yields results of value. Smears are made of the wound secretions every other day, or daily as the time of secondary closure occurs, in such a way that an approximate estimate of the number of bacteria contained in the wound can be made. The examination need not begin earlier than 12 hours after the infliction of the wound, since up to that time few bacteria will be found. Smears are taken with a platinum loop from different parts of the wound, choosing points most likely to harbor bacteria, such as crevices, necrotic bone, foreign bodies, or deep sinuses; do not take from bleeding points, smooth muscle, or clean areas, and also avoid the skin adjacent to the wound. With a small platinum loop, small amounts of secretion are picked up and smeared upon slides in such a way that about the same area is covered by the different loopfuls of secretions—with practice a uniformity of technic is attained to provide comparable bacteria counts. These smears are allowed to dry, stained by Gram's method, and examined under the microscope to gain an approximate estimate of the number and relative proportions of various types of organisms.

(2) The number of bacteria per field (oil immersion objective) are counted. If the average number exceeds 50 or more to the field, more accurate counting is not necessary for the wound still contains

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too many bacteria to warrant closure or relaxation of local therapy. Gradually as the wound improves, fewer and fewer bacteria will appear in the daily series of slides, and, when the number has dropped below 50 per field, careful counting may give an index to daily variation. Eventually the number will decrease to only one micro-organism per 5, 10, or 20 fields. The daily counts may be charted to provide a curve which will show the surgeon at a glance the numerical progress of the bacterial infection.

(3) No smears are taken while hemorrhage exists. Smears should not be taken within 2 hours after the application of Dakin's or other antiseptic solution. When no bacteria can be found on smear, it does not mean that the wound is completely sterile; cultures may yet reveal organisms; and, when the period of secondary closure approaches, especially when streptococci have been present previously, cultures are to be taken, aimed particularly at the demonstration of hemolytic streptococci, before the secondary closure is done.

b. Culture method.—Cultures are made at the beginning by planting the selected wound secretions onto fresh blood agar plates (without glucose). This is aimed at determining if pyogenic cocci, especially if hemolytic streptococci or staphylococci, are present. If the smears show a great many bacilli resembling the ordinary anaerobes, anaerobic cultures also may be planted. However, because of the long time required for working out the anaerobes in the laboratory, the surgeon is not concerned about this as a guide for his program of therapy. Suture of the wound is not carried out if hemolytic cocci of any kind are present, hence frequent cultures upon blood plates are made during the progress of the treatment.

353. Surgical procedures.—The surgeon's principles of procedure in considering these bacteriological problems are as follows:

a. Debridement is done as soon as possible after infliction of the wound.

b. Primary suture is not done except in quiet periods of warfare and in hospitals where the patient may be retained for careful observations, otherwise wound suture may lead to enclosure, in an imperfectly debrided wound, of harmful micro-organisms, especially of the gas gangrene group.

c. Delayed primary suture may be done if the cultures, taken 18 to 48 hours after debridement, show no organisms; if hemolytic cocci are present, suture is not considered. The presence of one other organism per two fields (including a few anaerobes) does not counterindicate suture. Considerable number of organisms of any kind indicates delay of suture.

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d. Secondary suture is undertaken when the organisms on two successive counts are few and the culture has shown an absence of hemolytic cocci.

354. Genus Clostridium.—*a. Characteristics.*—Rods; anaerobic or microaerophilic; usually Gram +; form endospores in the so-called clostridium forms; often decompose proteins or ferment carbohydrates actively through the agency of enzymes; often parasitic and pathogenic.

b. Nomenclature.—The war wound infections of the World War (1914–18) activated study of this group, adding many names to lists of those previously described, some known species being described under several different names. In the past two decades this group has been further studied and classified, the genus *Clostridium* now including 51 described species, those of greatest importance in human medicine being *Cl. tetani*, *Cl. parabotulinum*, *Cl. botulinum*, and the gas gangrene group.

355. Clostridium tetani.—*a. Habitat.*—The tetanus bacillus is found in the intestinal tract of man and animals, in cultivated soils, road dirt, etc., and is therefore potentially present in wounds contaminated with these materials. Being a strict anaerobe, it tends to grow in the tissues as it does on culture media, in the depths remote from oxygen, therefore it will seldom infect surface wounds, but will infect deep punctured or lacerated wounds. It produces in the tissues, as it does in the culture media, a powerful toxin which, on dissemination, produces the clinical features of tetanus. This is strictly a local infection which becomes a general intoxication. The micro-organisms can be found only in the local site, not in the blood stream or secretions.

b. Morphology and staining.—Slender, moderate-sized rods having round terminal spores, thicker than the cell, giving drumstick appearance; no capsule: slightly motile; stain readily and are moderately Gram-positive, but even young cultures may have many Gram-negative forms.

c. Cultivation.—Being a strict anaerobe, special facilities are required to attain growth. Grows well on ordinary media; growth greatly improved with blood or serum. Produces hemolysis of blood cells. Optimum temperature of growth, 37° C. Optimum pH, 7.4 to 7.8. Has slight proteolytic powers; gelatin slowly liquefied, coagulated albumin not liquefied. No acid or gas formed from carbohydrates. On litmus milk gives no change, or slow precipitation of casein.

d. Colony form.—Irregularly round, 2 to 5 mm in diameter, effuse glistening, translucent colonies with irregularly granular surface, and

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ill-defined edge, showing filamentous, curled projections. Organisms tend to spread over entire surface of plate, making it difficult to obtain isolated colonies.

e. Resistance.—Spores are very resistant to environmental influence; retain their vitality for years in a dried condition, resist boiling for 15 to 70 minutes, resist 5 percent phenol or 1:1,000 mercuric chloride for weeks. All spores are killed by exposure to dry heat, 160° C. for 1 hour, or to steam under pressure, 120° C. for 20 minutes.

f. Serology.—While *Cl. tetani* has been divided into seven seriological types, the toxin of all types is identical and is neutralized by antitoxin prepared from any member of the group.

g. Immunology.—A powerful exotoxin is produced both "in vivo" and "in vitro" which is thermolabile, being destroyed at 65° C. in 5 minutes. An antitoxin, prepared from horse serum immunized by toxin injection, has long been in use and is of high proven value in prophylaxis and treatment of tetanus. Recently active immunizations against tetanus by the injection of tetanus toxoid has received favor and is often given, parallel with typhoid inoculations, as an aid to protection against war wound infection, or comparable peacetime infections.

h. Examinations of clinical materials.—The identification of *Cl. tetani* in infected wounds is usually quite difficult. While it may be demonstrated by miscroscopic or cultural methods, the most practical method available is animal inoculation.

(1) *Collection of specimens.*—Pus and tissue fragments taken from suspected wounds by surgical removal, on sterile cotton swab or on platinum loop, may be placed in a tube of sterile saline solution and this used for microscopic examinations, cultures, or toxicity tests. Spinal fluid from clinical cases may be tested for toxin content.

(2) *Microscopic examination.*—Make film preparations of the suspected material. Stain and examine for the characteristic drumstick spores of *Cl. tetani.* If present in small numbers, they may be overlooked. If nonvirulent anaerobic or aerobic bacilli with round terminal spores are present, differentiation from the *Cl. tetani* cannot be made. Therefore this method of diagnosis is of very little practical value.

(3) *Cultural examination.*—Inoculate the specimen into cooked meat media, into the water of condensation of an agar slant, and on blood agar plate; incubate these at 37° C. for 72 hours in anaerobic jar and observe for tetanus bacilli. The agar slant, so inoculated, may give a pure culture by the growth of an effuse, tenacious proteus-like growth over the surface of the slope; subcultures from the edge of this fern-like growth into the water of condensation of a fresh agar

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slant will yield the *Cl. tetani* in pure culture after several transfers. If spores are present in the cooked meat medium, heat the culture to 80° C. for 30 minutes to kill any nonsporulating organisms and then inoculate blood agar plates for the isolation of pure colonies. *Cl. tetani* cultures attain a foul odor resembling burnt horn.

i. Demonstration of virulent Cl. tetani by animal inoculation.—Mix a portion of the original material, of the heated culture or, preferably, of a broth suspension of a pure culture, with sterile emory dust and inject 1.0 cc subcutaneously into the thigh of a guinea pig. A control pig receives the same injection plus an intraperitoneal inoculation of tetanus antitoxin. If *Cl. tetani* is present, the unprotected animal will develop tetanus and die in 1 to 4 days.

j. Demonstration of tetanus toxin.—Inject, subcutaneously, 0.5 cc of filtrate of 10-day broth culture into each of two mice or guinea pigs, one of which has been given a prophylactic dose of antitoxin (intraperitoneal). Similar toxicity tests may be made by injecting animals with spinal fluid from cases of tetanus.

356. Organisms associated with gas gangrene.—*a.* The anaerobic organisms associated with gas gangrene may be divided, on the basis of pathogenicity, into three groups:

(1) *Pathogenic.*—(*a*) *For man.*
  1. *Cl. perfringens* (welchii).
  2. *Cl. septicum* (vibrion septique).
  3. *Cl. novyi* (oedematiens).
  4. *Cl. bifermentans* (oedematoides; sordelli).

(*b*) *For animals.*—*Cl. chauvoei.*

(2) *Lesser pathogenicity.*—(*a*) *Cl. histolyticum.*

(*b*) *Cl. fallax.*

(3) *Nonpathogenic.*—(*a*) *Cl. sporogenes.*

(*b*) *Cl. aerofoetidum.*

(*c*) *Cl. lento-putrescens* (putrificum).

(*d*) *Cl. tertium* and others.

b. On the basis of their biochemical reactions, they may be separated into a saccharolytic group and a proteolytic group. There is not a strict demarcation of these properties, for most members have some properties of the other group; that is, some are both saccharolytic and proteolytic but are so classified as to the property which is most prominent. It may be noted that most of the pathogenic group are saccharolytic and most of the proteolytic are nonpathogenic (except *Cl. histolyticum*). The organisms of the proteolytic group are not in themselves pathogenic but complicate wounds by their intense proteolytic action; they are saprophytes, have no power of invading

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the tissues and, if present without members of the saccharolytic group, usually do not interfere with the healing of the wound.

357. Clostridium perfringens (Cl. welchii).—*a. Description.*—Short, thick, nonmotile, Gram+ rods with rounded ends, of moderate size, occur singly, in pairs, seldom in chains: form capsules in the animal body and at times in culture media. Spores are large, oval, central, or subterminal, formed only in alkaline sugar-free media and not in animal tissues. Rods are not distinctly swollen at sporulation. Spores resist heating to 80° C. for 1 hour. Grows best anaerobically, some growth microaerophilically; blood agar colony: round, domed, gray-white with smooth glistening surface, surrounded by zone of beta hemolysis. Ferments all common sugars with production of large amount of gas, and lactic and butyric acids, latter giving characteristic odor. Produces at least four different types (A, B, C, and D) of thermolabile exotoxin, sometimes all four being produced by one strain; each type of toxin is neutralizable by a specific antitoxin; type A is usually utilized for preparation of therapeutic antitoxin which may not protect against types B, C, or D, if present in the wounds. Pathogenic for man and small animals, the latter dying in a day following I. M. injection, with extensive blood-stained fluid necrosis of tissue and marked gas formation; the muscles are friable, pale pink color, the wound giving a foul acid odor, but there is no putrefaction.

b. Identification.—Can be identified by—

(1) Stormy fermentation of milk.
(2) Morphology and culture features.
(3) Intravenous inoculation of rabbit (Welch-Nuttall test).
(4) Guinea pig protection test.

c. General comments.—(1) The free fermentation of sugars is a prominent characteristic both in its production of gas gangrene and the laboratory identification of cultures. *Cl. perfringens* ferments the muscle sugars, producing gas in the tissues; this is forced along fascial planes and vessels, hence it is called the "gas bacillus," giving the crepitation of gas gangrene. Fermentation in the test tube may be so marked as to blow out the plugs; in milk cultures it is made evident by the stormy fermentation, an acid clot torn by gas bubbles, and separation of the milk into coagulum and whey.

(2) Gas gangrene is essentially a local infection, not invading blood stream until shortly before death. Spores are never formed in the animal body.

(3) The toxin produced is an exotoxin comparable to that of tetanus and diphtheria. An effective antitoxin is used in therapy. This antitoxin is specific only for *Cl. perfringens*, not for other wound anaerobes; therefore, if gas gangrene associated with

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Cl. perfringens and *Cl. novyi* is treated by a monovalent antitoxin, the latter infection would not be influenced. However, most commercial antitoxins are polyvalent, affording protection against several forms of gas gangrene.

358. Clostridium novyi (Cl. oedematiens).—This is a large, Gram-positive, sluggishly motile, spore-bearing, anaerobic bacillus, resembling the anthrax bacillus in appearance; spores central, excentric to subterminal; rods distinctly swollen at sporulation. The lesion in an experimentally infected guinea pig is characterized by a whitish gelatinous exudate, little necrosis, and absence of gas. It is feebly hemolytic, much less so than *Cl. perfringens*. It forms a soluble toxin by which antitoxic serum may be prepared.

359. Clostridium septicum (Vibrion septique of Pasteur).—*a.* This organism differs from previous two in that the rods are more slender and more pleomorphic. Even in young cultures, clubbed, citron, or navicular rods and filaments are present. It is motile, nonencapsulated, a strict anaerobe, and hemolytic. It invades the blood stream, producing a septicemia. The occurrence of long filamentous forms in the livers of guinea pigs dying of this infection is characteristic and is used in identification of this organism. A powerful, soluble toxin is produced, which provokes local necrosis, not death, in guinea pigs inoculated intramuscularly, their death on intravenous inoculation. The antitoxin is specific and does not protect against *Cl. perfringens* or *Cl. novyi*.

b. This bacillus is closely related and very similar to *Cl. chauvoei*, the bacillus of symptomatic anthrax or blackleg of cattle and sheep. The latter has never been isolated from wound cultures and has never been known to cause infection in man.

360. Clostridium bifermentans (Cl. oedematoides and B. sordelli).—This species consists of large, sluggishly motile, Gram-positive bacilli; oval spores are formed centrally or subterminally, without swelling of bacillus. In pathogenicity it resembles closely *Cl. novyi;* however, different strains show varying degrees of virulence and toxicity, from acute to none; the more toxic and virulent strains are commonly referred to as *Bacillus sordelli*.

361. Proteolytic group.—*a.* The organisms of this group can never produce gas gangrene without the presence of one or more bacilli of the saccharolytic group. They digest milk without the formation of a clot and liquefy and often blacken coagulated serum. These characteristics, plus the resultant offensive odor, point to their recognition. None of these organisms are very pathogenic or produce a general picture of toxemia in spite of the great liquefaction of tissue caused by them.

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b. Cultures from wounds may contain both groups of bacilli, with difficulty of separation by culture methods. Such a mixed culture may be purified by animal inoculation, when the more pathogenic organisms of the saccharolytic group may invade the blood stream and be isolated from the heart's blood. This group is a great nuisance to the bacteriologist, for they give great difficulty in separation from the more pathogenic clostridia. *Cl. sporogenes*, the most frequent and active one, also gives confusion by resembling *Cl. septicum* morphologically.

c. Cl. sporogenes, next to *Cl. perfringens*, the anaerobe most frequently found in wound culture, is usually responsible for the foul odor of wounds, but its pathogenicity is neglible. It does not produce a soluble toxin and is not pathogenic for laboratory animals.

d. Cl. histolyticum differs distinctly from *Cl. sporogenes* in that it is more actively proteolytic, digesting living tissues. Injection of a pure culture I. M. into a guinea pig results in rapid complete destruction of the skin and muscle and may expose the bone, the striking and characteristic feature being that in spite of the great local lesion the animal may remain well. The exudate will contain no gas, no putrid odor. A soluble toxin has been reported and antitoxin prepared.

362. Anaerobic cocci.—Charts for the species identification of the anaerobic cocci are given here. They are fairly common contaminants of wounds and are not generally described in bacteriology texts. The aerobic streptococci are not here described in detail, for they are better known and are described in standard texts. Isolate pure cultures by selecting isolated colonies from anaerobic blood plates and identify by planting on the following media:

a. Infusion broth.—Examine for turbidity, sediment, gas, and odor.

b. Ten percent serum broth.—Examine for gas and odor.

c. Veillon's semisolid agar.—Examine for gas.

d. Blood agar plates.—Examine surface colonies for morphology and action on blood.

e. Neopeptone water.—Examine for character of growth and gas production.

f. Litmus milk.—Examine for acid production, coagulation and clot retraction.

g. Gelatin.—Examine for liquefaction.

h. Carbohydrates (1 percent dextrose, levulose, galactose, sucrose, maltose, lactose, and mannite, in sugar-free base).—Test media after 48 hours' anaerobic cultivation for acid or acid and gas. Continue negatives up to 21 days.

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i. Guinea pig.—Examine for pathogenicity.

j. Mice.—Examine for pathogenicity.

363. Key of anaerobic nonhemolytic streptococci.

a. Strict anaerobes:

(1) Gas and fetid odor produced.

(*a*) No general turbidity in broth.

1. Acid from maltose. 1. *Streptococcus anaerobius.*

2. No acid from maltose. 2. *Streptococcus foetidus.*

(*b*) Turbidity in broth.

1. No gas in semisolid agar (Veillon).

No gas in peptone water. 3. *Streptococcus putridus.*

2. Gas in semisolid agar.

Gas in peptone water. 4. *Streptococcus lanceolatus.*

(2) No gas, no fetid odor produced.

(*a*) Milk not coagulated. 5. *Streptococcus micros.*

(*b*) Milk coagulated.

1. Viscous sediment in broth.

Colonies on semisolid agar blacken with age. 6. *Streptococcus parvulus.*

2. No viscous sediment in broth.

Colonies do not blacken with age. 7. *Streptococcus intermedius.*

b. Microaerophilic:

Strictly anaerobic on isolation.

Later microaerophilic. 8. *Streptococcus evolutus.*

364. Wound bacteriology record.—The cultured micro-organism from debridement tissue and post-operative control specimens may be placed in one of the following groups; by so recording it, there may result a uniformity of record for comparison and for tabulation of results of series of cases:

a. Aerobic cocci.

(1) Streptococcus-beta (hemolytic).

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(2) Streptococcus-alpha (viridans).
(3) Streptococcus-gamma (anhemolytic).
(4) *Staphylococcus aureus.*
(5) *Staphylococcus albus.*
(6) Pneumococcus (specify type).
(7) Other cocci (specify).

b. Anaerobic cocci.

(1) Streptococcus, hemolytic, strict anaerobe.
(2) Streptococcus, hemolytic, microaerophilic.
(3) Streptococcus, nonhemolytic, gas forming.
(4) Streptococcus, nonhemolytic, nongas-forming.
(5) Streptococcus, nonhemolytic, microaerophilic.
(6) Other anaerobic cocci (specify).

c. Aerobic bacilli.

(1) Gram-positive, sporulating.
(2) Gram-positive, nonsporulating.
(3) Gram-negative, sporulating.
(4) Escherichia group.
(5) Aerobacter group.
(6) Proteus group.
(7) Pyocyaneous group.
(8) Typhoid-dysentery group.
(9) Others (specify).

d. Anaerobic bacilli.

(1) *Clostridium perfringens* (Welchii).
(2) *Clostridium septicum* (vibrion septique).
(3) *Clostridium novyi* (oedematiens).
(4) *Clostridium histolyticum.*
(5) *Clostridium tetani.*
(6) Others (specify).

365. Bacteriological investigation of war wounds.*—*a. Purpose.*—(1) To provide more precise information about infections so often associated with war wounds, especially the anaerobic infections.

(2) To supplement clinical data as to the effects of various methods of treatment upon these infections.

(3) To secure further data as to the importance of cross infection by streptococci (and other organisms) in hospital wards; and as to how these may be controlled.

b. Collection of specimens.—(1) Swab, mounted on wooden applicator, 5 inches long, wholly encased in a sterile tube plugged with cotton, is used.

*Reference: "Notes on the Diagnosis and Treatment of Gas Gangrene," H. M. Stationery Office, London, 1940, including scheme suggested by Committee of London Sector Pathologists.

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(2) At the operation an attendant can remove the plug and tip out the end of the swab stick, thus allowing the surgeon to take the specimen and replace it into the tube without impairing his sterility. The attendant will then plug the tube, label it, and dispatch it to the laboratory.

(3) Pus may be collected to some advantage in a test tube or a capillary pipette as this allows of a more satisfactory microscopical examination.

(4) Specimens should be taken from the deepest parts of a wound.

c. Time of collection of specimens.—The following program will enable the course of the infection to be ascertained and the incidence of hospital infection to be determined:

(1) Initial specimens should be collected at the commencement of the operation in order to ascertain the nature of the primary infection.

(2) Another specimen should be taken at the time of the first complete dressing.

(3) Further specimens should be taken at weekly intervals.

(4) Specimens should be taken more frequently in cases receiving special methods of treatment (such as by the sulfonamide drugs) and the treatment checked by as detailed bacteriological examination as possible, following the methods outlined below.

d. Procedure.—(1) *Microscopic examination of films of material from wound.*—Gram-stained films should always be examined. They may give immediate information to the surgeon as to the general nature of the infection and also indicate to the bacteriologist suitable special methods of culture.

(2) *Cultural methods.*—It is recommended that the following cultures be made:

(*a*) Blood agar plate of unheated pus, for *aerobic* incubation.

(*b*) Blood agar plate of unheated pus, for cultivation in *anaerobic* jar. These anaerobic plates should be well dried before incubation, to prevent the undue spreading of colonies. An indicator tube should always be placed in the anaerobic jar.

(*c*) Litmus milk, for the detection of *Cl. perfringens.* The milk tubes should be boiled to expel the air, and, on cooling, they should be thickly inoculated from the swab or pus, covered with melted vaseline, and incubated without further anaerobic precautions.

(*d*) Robertson's meat medium, which grows both aerobes and anaerobes and is especially useful in that it provides a culture to which the bacteriologist can return if, for any reason, his plate cultures are unsuccessful. It also allows slowly developing anaerobes to be detected in subcultures made after some days of incubation. These cul-

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tures, if they are to be stored, should be covered with a layer of liquid paraffin to prevent drying.

(*e*) If, in any laboratory, it is impossible to proceed with the isolation of the anaerobes, a meat media tube should be inoculated from the wound swab and sent to a reference laboratory.

e. Observation of anaerobic plate cultures.—(1) These will yield many aerobic organisms, both spore bearers and nonspore bearers, in addition to the obligate anaerobes. Some strains of bacteria which first appear exclusively on the anaerobic plate will prove on subculture to be aerobes. Colonies of some aerobic spore bearers, when growing anaerobically, will often simulate those of *Clostridia.*

(2) The cultures should be examined the day after inoculation and again after 48 hours, as the colonies of the spore-bearing anaerobes may then be more characteristic.

(3) *Spore-bearing anaerobic bacteria.*

Organism	Surface colonies	Deep colonies in agar	Litmus milk	Coagulated serum or egg	Spores
Cl. perfringens.	Large, regular, probably hemolytic.	Lenticular, opaque.	Stormy acid clot.*	-------	Seldom seen in culture.
Cl. novyi-----	Clear, slightly irregular.	Woolly-----	Acid-------	-------	Central and subterminal; very few.
Cl. septicum--	Transparent, spreading.	Transparent, branching.	Little change (? slight acid).	-------	Central or subterminal.
Cl. sporogenes.	Medusa-head (if plate dry); irregular (if plate moist).	Woolly, opaque.	Digestion with foul odor.	Digestion.	Central or subterminal.
Cl. tertium----	Small, clear, circular.	Small lenticular.	Little change.	-------	Terminal oval.
Cl. tetani-----	Very transparent, spreading.	Delicate filaments.	Little change.	-------	Terminal round.

*This appearance may be simulated by certain other microbes.

(4) It is emphasized that the presence of *Cl. perfringens* in a wound is not necessarily an indication of gas gangrene. This organ-

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ism is present in the cavities of many wounds of patients not suffering from that disease.

(5) Fermentation tests may be made after a pure culture is attained. It should be noted that some indicators may be irreversibly decolorized in the anaerobic jar. Tubes showing no apparent increase in acidity after anaerobic incubation should be tested with a drop or two of fresh indicator.

(6) Streptococci may be found in anaerobic cultures which do not appear on aerobic blood agar plates. While some of these are true anaerobes, many are cocci which, when just isolated, will not grow aerobically, but which soon become acclimatized and grow readily in open tubes and plates.

(7) True anaerobic streptococci should be investigated as to cultural reactions, digestion of albumin, production of foetid odor, sugar reactions, and as to pathogenicity for animals. Cultures may be maintained on Robertson's meat medium for future study. It is suggested that a microscopical examination of the original meat culture should be made after some 3 days' incubation (this also gives valuable information as to the spore-bearing anaerobes present) and, if streptococci are seen which did not appear in the culture plates, further cultures should be made on blood agar, anaerobically. Streptococcal colonies which appear may then be subcultured into "sloppy" glucose-agar (glucose-broth containing 0.1 to 0.2 percent agar).

f. Aerobic plate cultures.—Note and study as described elsewhere:

(1) Hemolytic streptococci.

(2) Staphylococci.

(3) Other organisms.

(4) Relative numbers of different bacteria present (as indicated by the primary microscopical examination and from the aerobic and anaerobic cultures).

g. Special procedures.—(1) *Potassium tellurite media.*—(*a*) The bacteriostatic properties of potassium tellurite on nutrient agar are approximately as follows:

1. Haemophilic bacilli and most coliforms are inhibited by a concentration of 1:500,000.

2. Ps. aeruginosa is inhibited by a concentration of 1:50,000.

3. Proteus strains are variable and many are resistant to 1:50,000.

4. Streptococci, staphylococci, and dihptheroid bacilli are resistant to 1:10,000.

(*b*) If, in the direct microscopical examination of the pus, large numbers of coliform organisms are seen, potassium tellurite may be

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incorporated in the medium in a concentration of about 1 in 50,000, in order to inhibit them and to allow the cocci to be easily isolated.

(*c*) A much simpler method is to inoculate a plate in the usual way and then to spread 2 or 3 drops of 1 in 1,000 tellurite over half of the plate. In this way one-half of the plate is an ordinary culture, while on the other half the coliforms are generally completely inhibited. A convenient stock solution is 1 in 1,000.

(*d*) Potassium tellurite may be used in the same way for the separation of anaerobes as there are differences in the sensitivity of different members of this group to the chemical. More work remains to be done in order to determine its exact usefulness in this direction.

(2) *Isolation of organisms from material containing genus Proteus organisms.*—(*a*) Blood-agar plate is inoculated in the usual way. Melted agar at 45° C. is poured over the surface of the plate to a depth of 2 or 3 mm and allowed to set. After incubation it will be found that any colonies of *Proteus*, growing between the two layers of agar, show no tendency to spread and colonies of other organisms can be easily picked out from among them. Some of the *Proteus* may spread around the edge of the agar to the upper surface and this must be killed before any deep colonies are picked out. A satisfactory method is by flooding the surface of the plate with saturated mercuric chloride solution for about 30 seconds; the solution is washed off with tap water and some of the surface growth of *Proteus* is scraped off with the end of a microscope slide to enable the deep colonies to be picked out and subcultured.

(*b*) Potassium tellurite in concentration of 1:20,000 to 1:50,000 can be incorporated in the agar which is poured over the surface. This inhibits most of the coliform bacilli and sometimes prevents *Proteus* group organisms from spreading over the surface.

(3) *Negative staining for detection of clostridial spores in culture.*—(*a*) A small drop of nigrosin solution is placed on a slide and some of the culture is mixed with this and spread out into a thin film by means of a wire or another slide. This film is allowed to dry and can then be examined. In the thicker parts of the film the large spores stand out as clear spots, while the bacillary portions are partly overlaid by the nigrosin. This simple method of demonstrating spores may, if necessary, be confirmed by the usual staining methods.

(*b*) Alternative method: Make a film of the bacteria in water on a slide in exactly the same way as is done preparatory to staining. When this film is dry a small drop of nigrosin is placed on the slide and is spread in a thin film over the bacteria.

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(*c*) Nigrosin solution: Gurr's nigrosin in water about three-quarters saturated.

(4) *Influence of CO_2 on growth of bacteria in wounds.*—(*a*) Some streptococci and other bacteria will not grow, or will grow only poorly, unless there is an increased amount of CO_2 in the atmosphere. Where facilities exist, observations may be made on the effect of an atmosphere of from 2 to 10 percent of CO_2 on the growth of aerobes and anaerobes of war wounds.

(*b*) Cultures, either plates or tubes, are placed in a container of about 3,600 cc volume. There is placed in the container an open tube 8 by 1 inches, containing 8 cc (excess) of 25 percent HCl; a marble chip of about 0.7 gm is dropped into the acid and the lid pressed down; this will give about 5 percent CO_2 at 37° C.

(5) *Indicator tube for anaerobic jar.*—To a tube of 5 cc of 2 percent glucose-broth add 0.1 cc of Loeffler's alkaline methylene blue. This should become decolorized in the jar and it should remain colorless throughout incubation if anaerobic conditions are maintained.

SECTION III

BACTERIOLOGICAL EXAMINATION OF WATER

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366. References.—*a.* Standard Methods of Water Analysis, American Public Health Association, eighth edition, 1936 (for technic).

b. Section XI, AR 40-310, and paragraph 10*b*(3) AR 40-205.

367. Collection of sample.—*a.* Bacteriological examination of specimens of drinking water from all Army stations are made routinely once every month and at more frequent intervals when indicated by local conditions, always supported by sanitary surveys and at times by sanitary chemical examination of the water. These examinations are made locally when laboratory facilities are available, otherwise at the nearest laboratory.

b. Samples, representative of the source, are collected by medical personnel of local station and shipped in item No. 18050 (sterile

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120-cc bottle in double mailing container) to corps area or comparable laboratory. The collection must be carefully made to avoid extrinsic contaminating factors, such as would be added by the use of unsterile containers, tap drippings, dead end water, insects, and other unrepresentative items.

c. All samples should be identified with the essential information as to exact source, time of collection, special circumstances (if any), and the address of the person to whom the report is to be submitted. The laboratory includes these data in its report and adds the time of beginning examination, and the results of test.

d. Specimens, upon receipt in the laboratory, must be stored in icebox and examined as soon as possible; impure waters should be examined within 6 hours of collection, relatively pure waters within 12 hours. Interpretation of results of examination of waters exceeding this shipment interval must consider the elapsed time and the prospect of bacterial changes in interim.

e. Before beginning test observe appearance of water and record as clear or cloudy, also observe and record presence and relative amount or absence of sediment.

368. Equipment required.—The apparatus, materials, and media requirements are laid down in detail in "Standard Methods," being in general those for routine bacteriological work with special emphasis on specific details of content of media used. The media for water analysis are not interchangeable with other bacteriological media, as they differ in several features:

a. Beef extract, not beef infusion, is always used.

b. No sodium chloride is added.

c. Peptone is contained in reduced amount (0.5 percent).

d. The pH is adjusted to the acid side (6.4 to 7.0).

369. Required tests.—Two separate and distinct tests are run on each water sample to determine its potability: first, a total bacterial count and, second, a completed test for the presence of members of coli-aerogenes group.

a. Total bacterial count.—(1) *General.*—(*a*) This consists of determination of the colony count given by 1 cc of water on standard nutrient agar after 24 hours' incubation at 37° C. It is not a true total count for it misses dead bacteria, bacteria that do not grow at 37° C., and bacteria that do not form visible colonies within 24 hours under standard conditions.

(*b*) Only two portions (1 cc and 0.1 cc) of the sample are routinely plated. If an exact count of badly contaminated water is desired, additional plates may be planted with smaller measured amounts of water.

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(*c*) Colony counts of over 200 per cc for treated waters and 500 per cc for raw waters (spring, well, etc.) are arbitrarily considered as evidencing sufficient contamination of the water to render it unfit for drinking uses. Interpretation of any result must also consider the water source, treatment, and sanitary survey.

(2) *Preparation of plate cultures.*—(*a*) Two Petri plates are labeled on lid with sample number and amount.

(*b*) Sample is mixed thoroughly by shaking vigorously 25 times.

(*c*) One cc of water sample is measured, by a sterile pipette, into one plate and 0.1 cc into the second plate.

(*d*) Nutrient agar is added to each plate: 10 cc of liquefied agar, cooled to 42° to 45° C.

(*e*) Mixture of agar and water is effected by tilting and rotating plates.

(*f*) Hardening of agar is then permitted by a few minutes' rest at room temperature, then the dishes are inverted and so kept throughout later observations.

(*g*) Control plate is prepared by same procedure, less the water sample.

(*h*) Incubate all plates at 37° C. for 24 hours (±1 hour).

(3) *Colony counting.*—(*a*) Count the number of colonies on the plates, using a lens of 2½ diameters magnification and standard ruled counting plate or using a "Quebec colony counter."

(*b*) Calculate colonies per cubic centimeter by multiplying the number of colonies on the plate by the fraction of cubic centimeter of sample used in the plate which gives the most practical number of colonies for counting, i. e., less than 300 colonies per plate.

(*c*) Report colony count, exactly in low counts, approximately in higher counts:

Colony count	Reported—
1 to 50	Exactly as counted.
51 to 100	To nearest 5.
101 to 250	To nearest 10.
251 to 500	To nearest 25.
501 to 1,000	To nearest 50.
1,001 to 10,000	To nearest 100.

b. Completed test for presence of members of coli-aerogenes group.—(1) *General.*—(*a*) Completed test is indicated when dealing with drinking water examination; this is the usual program followed in Army laboratories.

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(*b*) Partial tests are used for hasty examinations, for raw water in process of purification, for sewage, and other known polluted waters where complete test is unnecessary, such as in water purification plants and sewage disposal plants.

(*c*) The coli-aerogenes group includes all aerobic and facultative anaerobic, Gram-negative, nonspore-forming bacilli, which ferment lactose with gas formation.

(*d*) Micro-organisms of this group are not pathogenic, not necessarily harmful to the water, but are considered as evidencing fecal pollution and the potential presence of pathogenic fecal organisms of dysentery-typhoid-*Salmonella* or cholera groups which are not so readily detected in routine tests.

(*e*) Positive completed tests require the demonstration, in subcultures made from initially inoculated lactose broth cultures, of one or more aerobic plate colonies of Gram-negative, nonspore-forming bacilli which form gas when again inoculated into a lactose broth fermentation tube (secondary lactose tube).

(2) *Media required.*—(*a*) Lactose broth with broomcresol purple indicator placed in large test tubes (30 cc) and small test tubes (10 cc), each with small, inverted test tubes within to demonstrate gas formation.

(*b*) Eosin-methylene blue agar for water (E. M. B.).

(*c*) Brilliant green lactose bile (B. G. L. B.) fermentation tubes or an authorized substitute.

(*d*) Nutrient agar slant.

(3) *Technic of test.*—(*a*) *First day.*

1. Mix water sample thoroughly by shaking vigorously 25 times.

2. Inoculate two small lactose tubes with 0.1 cc and 1.0 cc portions, respectively, and five large lactose tubes with 10 cc each of the water sample; label tubes with sample number, and number from 1 to 7.

3. Place in incubator at 37° C. for 24 hours.

(*b*) *Second day.*

1. Observe lactose fermentation tubes; record presence and percent, or absence of gas formation.

2. If gas has been formed in any tubes, inoculate the following media:

(a) Streak on E. M. B. plate; plant from the tube inoculated with smallest amount of original water sample and showing gas formation.

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(b) Brilliant green lactose bile tubes; plant from at least three (preferably all) tubes showing gas formation, including tubes inoculated with smallest portions of original water sample.

3. Place original lactose tubes and transplants in incubator at 37° C. for another 24 hours.

(*c*) *Third day.*

1. Make and record 48-hour readings on original lactose tubes; if no gas has been formed in any tube, make negative report; if gas has been formed in tubes inoculated with a smaller portion of water sample than at 24 hours, inoculate E. M. B. plate and B. G. L. B. and proceed as indicated for procedure (*b*), second day.

2. Observe 24-hour E. M. B. plate for typical coli or aerogenes type colonies; if present, select one or more (one of each type present) well-isolated colonies and transfer to small lactose fermentation tube and plain agar slant. The presence of typical colonies within 48 hours is recorded in column D, W. D., M. D. Form 95, as positive. If no typical colonies are found on plate and gas has been formed in corresponding B. G. L. B. tube, a new E. M. B. plate should be streaked from that tube. Reincubate any negative plates.

3. Observe B. G. L. B. tubes for gas formation; gas in any amount is recorded in column C, W. D., M. D. Form No. 95, as positive. Reincubate any negative tubes.

4. Place newly inoculated media and other media as indicated into incubator at 37°C. for 24 hours.

(*d*) *Fourth day.*

1. Observe any 48-hour cultures, E. M. B. plates for typical colonies or B. G. L. B. tubes for gas formation, and record results. If positive, proceed as indicated for procedures *2* and *3*, third day; if no typical colonies are present on plate and no gas has formed in any tube of liquid confirmatory media, report as negative.

2. Observe secondary lactose tubes for gas formation and record results. Reincubate.

3. If gas has been formed in lactose tube and not otherwise, make Gram-stained film from corresponding plain agar slant culture, examine for Gram-negative, nonspore-forming bacilli, and record in column F as "C.-A."

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(*e*) *Fifth day.*

1. Make 48-hour reading of secondary lactose tube and record.

2. If gas formation has occurred in lactose tube that was negative at end of 24 hours, make Gram-stained film and examine as above.

3. Prepare report on W. D., M. D. Form No. 95, using a standard remark wherever applicable.

NOTE.—In most infected water samples, gas formation will occur within 24 hours in all cases and the test can be completed as shown in above outline within 4 days of receipt of specimen. Sometimes, as indicated in procedure *1* for third and fourth days, lactose fermentation is delayed; in this case proceed as if reaction had occurred within 24 hours, but note that 5 or more days will be required to complete test.

370. Standard remarks for use in reporting results of bacteriological examinations.

No. 1. Condition: No gas in any lactose tube. Colony count under 200 per cc.
Remark: Potable bacteriologically. No evidence of fecal contamination.

No. 2. Condition: No gas in any lactose tubes. Colony count over 200 per cc.
Remark: Potability questionable. Colony count is high ("very high" if over 1,000).

No. 3. Condition: Gas due to coli-aerogenes group in one or two large lactose tubes. Colony count low.
Remark: Potability questionable. Coli-aerogenes group organisms present in one (or two) 10-cc samples.

No. 4. Condition: Gas due to coli-aerogenes group in one or two large lactose tubes. Colony count over 200 per cc.
Remark: Not potable bacteriologically. Coli-aerogenes group organisms present in one (or two) 10-cc samples. Colony count high.

No. 5. Condition: Gas in one or more original lactose tubes. Failure to demonstrate presence of coli-aerogenes group (E. M. B. plate and B. G. L. B. tubes negative, no gas in secondary lactose tubes, or gas due to spore-forming bacillus). Colony count under 200 per cc.
Remark: Potable bacteriologically. Gas formation not due to coli-aerogenes group.

No. 6. Condition: Same as No. 5, except colony count is over 200 per cc.
Remark: Not potable bacteriologically. Gas formation not due to coli-aerogenes group. Colony count is too high.

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No. 7. Condition: Gas formation due to coli-aerogenes in three or more large lactose tubes or in a small lactose tube. Colony count high or low.

Remark: Not potable bacteriologically. Tests reveal evidence of fecal contamination.

No. 8. Special remarks—to be added after use of some other remark.

a. Specimen ——— days in transit to laboratory.

b. Evidence of defective packing or collection.

371. Differentiation of members of the coli (genus Escherichia)-aerogenes (genus Aerobacter) group.—A satisfactory identification of a coli-aerogenes group organism as *Escherichia coli, E. freundi, or Aerobacter aerogenes* can be based upon four tests (indol, methyl red (M. R.), Voges-Proskauer (V. P.), and sodium citrate utilization). These tests are not routinely run but are sometimes requested as part of a sanitary survey of a watershed. *E. coli* (indol+; M. R.+; V. P.−; and citrate−) is considered to be of fecal origin; *E. freundi* (indol±; M. R.+; V. P.−, and citrate+) and *A. aerogenes* (indol−; M. R.−; V. P.+, and citrate+) of nonfecal origin.

372. Bacteriological examination of swimming pool water.—This is carried out by the technic described for drinking water. The standard of purity recommended is identical with the standard for a potable drinking water. Since most swimming pools contain residual chlorine, sufficient to kill bacteria in sample between time of collecting and testing, it is required that such samples be collected into sterile water-sample bottles containing approximately 0.02 to 0.05 gm of sodium thiosulfate.

Section IV

BACTERIOLOGICAL EXAMINATION OF DAIRY PRODUCTS

Paragraph
Bacteriological examination of milk 373
Bacteriological examination of cream 374
Determination of number of bacteria in plain ice cream 375

373. Bacteriological examination of milk.—*a. References.*—(1) Standard Methods for the Examination of Dairy Products, seventh edition, 1939, published by American Public Health Association.

(2) Standard Milk Ordinance and Code of the United States Public Health Service.

(3) Sections XII and XIII, AR 40-310.

b. Definitions.—(1) Raw milk is untreated (except for refrigeration) milk.

(2) Pasteurized milk is milk that has been treated with limited

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heat by one of several methods, in order to kill most pathogenic bacteria.

(3) Certified milk is an especially pure raw or pasteurized milk, generally for infant feeding, produced under the supervision of a medical milk commission of the county or state Medical Society, based on requirements of the American Association of Medical Milk Commissions.

(*a*) Certified raw milk: colony count should not exceed 10,000 per cc.

(*b*) Certified pasteurized milk: colony count of not over 10,000 per cc before, and of not more than 500 per cc after pasteurization.

(4) Standard Milk Ordinance and Code classifies and defines milk as—

(*a*) Grades A, B, C, and D raw.

(*b*) Grades A, B, and C pasteurized.

(5) Grade A pasteurized milk is the grade usually sold for drinking purposes; must have colony count of not over 30,000 per cc, and must be prepared from grade A (50,000 per cc), or grade B (200,000 per cc) raw milk in plants meeting strict sanitary requirements.

(6) Other grades of milk are based on definite sanitary requirements for the production, distribution, and bacterial content. The allowable colony counts for raw milks are greater than for the corresponding grade of pasteurized milk; also, the sanitary requirements are progressively less rigid and the allowable colony counts greater for grades B, C, and D milk, respectively.

c. Collection of samples.—(1) Sample selected should be representative of the lot to be tested, free of extrinsic contamination and so preserved by the use of sterile or contamination-free containers, and iced from the time of collection to time of laboratory test, to prevent bacterial growth in transit.

(2) Bottle should be picked at random from distribution channel, kept on ice, and set up in laboratory within 4 hours, if possible.

(*a*) Protect cap and lip of bottle from contamination in transmission by a tight-fitting, waterproof covering.

(*b*) Pack in ice in upright position, keeping its temperature under 45° F. until examined.

(*c*) If sample is to be sent to a distant laboratory by mail for a direct microscopic count, add 2 drops of formalin for each 10 cc of milk, fill a sterile 120-cc glass-stoppered bottle up to the stopper, label "Formalinized," and prepare for mail shipment in a double mailing case.

(3) Bulk milk may be sampled at the plant or in distribution by collection with sterile equipment and handled as in (2) above.

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(4) A plate count at a local laboratory is preferable to a direct count at a distant laboratory.

(5) All samples should be properly identified with the essential information as to name of dairy, time of collection, source and grade of milk, preservative used (if any), and the address of the person to whom the report is to be submitted. The laboratory includes these data on its report and adds the time of the start of examination and results of test.

d. Standard tests.—The following methods are applicable to samples of milk received under differing conditions and according to the laboratory facilities locally available:

(1) *Agar plate method.*—This consists in counting the number of visible colonies of bacteria in a culture made of a measured amount of milk in standard nutrient agar after 48 hours' incubation at 37° C. This gives an estimate of the number of living bacteria present in the milk and is the test routinely used in Army laboratories.

(2) *Direct microscopic method.*—Consists of an examination of stained films of milk and cream dried on glass slides. It is used in the central Army laboratories in the making of estimates of the number of individual bacteria (living or dead) in specimens of formalinized milk from outlying stations where laboratory facilities for agar plate method are not locally available. See "Standard Methods for the Examination of Dairy Products" for technic.

(3) *Methylene blue reduction method.*—This method, usually known as the "reductase test," is based on the fact that color imparted to milk by a small amount of methylene blue will disappear more or less quickly from incubated milk as a result of the consumption of the dissolved oxygen by growing bacteria. The results of this test compare favorably with those obtained by other methods. Due to the small amount of equipment, space, and experience required, it is applicable to small laboratories and in isolated posts.

(4) *Sediment test.*—This depends upon appearance of standard filter disks after passage of 1 pint of milk. It is used as an index to the cleanness of milk. It has little utility in Army laboratories.

(5) *Tests for specific types or groups of bacteria.*—Such tests, as for coli-aerogenes group, hemolytic streptococci, tubercle bacilli, and *Brucella* are described in "Standard Methods for the Examination of Dairy Products." These tests are similar to the routine laboratory examinations for those micro-organisms and are seldom done routinely.

e. Standard plate count.—(1) *First day.*—(*a*) Agitate milk sample thoroughly, preferably by pouring back and forth from one sterile container to another.

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(*b*) Using sterile dilution bottles containing exactly 9.0 cc or 99 cc of distilled water, prepare a series of dilutions depending upon expected colony count (based on grade of milk and results of previous examinations); the plate to be counted should have between 30 and 300 colonies; routinely, dilutions of 1/100, 1/1,000 and 1/10,000 are prepared.

(*c*) Mix each dilution as prepared, by shaking rapidly up and down 25 times in arc of 1 foot. Transfer 1.0 cc of each dilution to properly labeled Petri dish.

(*d*) Add tube of standard nutrient agar (tryptone-glucose-extract-milk agar), previously melted and cooled to 42° C., to each Petri dish and mix with sample by rotating and tilting the dish carefully. Allow to cool.

(*e*) Incubate at 37° C. for 48 hours (plus or minus 3 hours).

(2) *Third day.*—(*a*) Select the plate containing between 30 and 300 colonies and count all colonies, including those of pinpoint size. The use of a Quebec colony counter is recommended. If only a fraction of the plate is counted, determine total plate count by multiplying the average number of colonies per square centimeter by a variable factor depending upon the average inside diameter of the Petri dishes being used (90 mm, multiply by 63.5; 91 mm by 65: 92 mm by 66.5).

(*b*) Multiply the number of colonies found by the dilution factor to find colony count per cubic centimeter of sample.

(*c*) Report: "Standard plate count, ------ per cubic centimeter."

f. Methylene blue reduction method.—This test is most applicable to raw milk; asceptically drawn normal milk from healthy udders seldom, if ever, reduces methylene blue in less than 10 hours.

(1) *Methylene blue reagent.*—Use only certified methylene blue thiocyanate tablets. Prepare fresh reagent weekly by dissolving one tablet of dye in exactly 200 cc (at room temperature) of sterile or freshly boiled distilled water.

(2) *Technic of test.*—(*a*) Mix sample thoroughly and transfer 10 cc to a 12 to 15 by 150 mm test tube, fitted with a rubber stopper.

(*b*) Add 1.0 cc of the methylene blue reagent.

(*c*) Stopper the tubes immediately, label indelibly, and place in water bath at 37° C. Invert the tube once at end of 5 minutes, after which avoid agitation that might disturb the cream layer.

(*d*) Observe tubes at frequent intervals (15 to 30 minutes) and record the end point (disappearance of the blue color from at least four-fifths of the contents of the tube).

(3) *Interpretation of results.*—(*a*) *Class 1.*—Excellent, not decolorized in 8 hours.

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(*b*) *Class 2.*—Good, decolorized in less than 8 hours but not less than 6 hours.

(*c*) *Class 3.*—Fair, decolorized in less than 6 hours but not less than 2 hours.

(*d*) *Class 4.*—Poor, decolorized in less than 2 hours.

g. Tests for presence of coli-aerogenes group.—This tests consists of inoculating five tubes each of various portions (10 cc, 1.0 cc, $\frac{1}{10}$ cc, $\frac{1}{100}$ cc, etc.) of the milk to be tested into brilliant green lactose bile, or formate ricinoleate broth. If gas is formed, continue as with water for definite identification.

374. Bacteriological examination of cream.—Estimations of the bacteriological content of cream samples are made by using same methods as those for milk with the following exceptions:

a. Measuring sample.—Mix sample; weigh 1.0 gm asceptically into a sterile butter boat or directly into a dilution bottle.

b. Dilutions used.—The allowable bacterial content of cream (50,000 to 100,000 colonies per cc) is greater than for milk. Carry dilutions one or two steps farther when making agar plate count.

375. Determination of number of bacteria in plain ice cream.—*a. General.*—(1) In the bacteriological examination of ice cream and of ice cream mix before it is frozen, follow the same general methods as for milk:

(*a*) Use the agar plate method.

(*b*) Make direct microscopic count.

(*c*) Sometimes test for coliform group.

(2) Collect samples of at least 50-cc amounts in unopened cartons or in sterile, wide-mouthed, 125-cc bottles fitted with ground glass stoppers or metal caps. In sampling bulk ice cream, remove top inch of cream with sterile spoon, discard this, and use a second sterile spoon to collect sample. Collect representative samples of ice cream mix at periodic intervals. Send to laboratory immediately for examination; if laboratory is at some distance, keep sample properly refrigerated by packing in dry ice or in water containing cracked ice.

b. Standard plate method.—(1) *Preparation of sample.*—Melt the frozen ice cream by placing the container in a water bath at 45° C. for a period not to exceed 15 minutes.

(2) *Methods of making dilutions.*—(*a*) *Volumetric.*—To reduce the percentage of error when using materials of high viscosity (melted ice cream, ice cream mix, condensed milk, etc.) it is necessary to use large amounts in making the first dilution. To make 1:10 dilution, use 11 cc of sample to 99 cc of sterile water in dilution bottle. Most applicable to plant control work.

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(*b*) *Gravimetric.*—This method is more accurate and should be used for most Army laboratory examinations.

1. Bring the sample to a suitable degree of consistency (about 10° C.) by allowing to stand at room temperature or by heating in water bath at 43° to 46° C.; using either method the total time required must not exceed 15 minutes.
2. Obtain a butter boat or similar piece of apparatus, sterilized in cotton-plugged test tube and a standard dilution bottle containing 99 cc of sterile distilled water and having opening of such size as readily to take the butter boat.
3. Remove the cotton plug from the test tube and slide the the butter boat forward until it projects about ¾ inch beyond the end of test tube. Do not allow boat to touch any contaminated object. Weigh test tube and butter boat to the second decimal place.
4. Pipette 1 gram, or slightly more, of the sample into the butter boat and again weigh to the second decimal place.
5. Allow the butter boat and contents to slide into the dilution bottle.

(3) *Technic of test.*—Make further dilutions, if required, and continue as for examination of milk.

SECTION V

RICKETTSIAE

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376. General.—*a. Description.*—Small pleomorphic diplobacillary micro-organisms; generally appear as diplobacilli in pairs but may occur singly. Their size depends upon various factors, the chief one being the source from which the infectious material is obtained. *Rickettsiae* are rarely found in the animal body except in the tunica vaginalis of infected guinea pigs which show scrotal swelling. In cultures, the various types of *Rickettsiae* vary greatly in size from

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barely visible forms up to long bacillary of filamentous forms. In tissues they stain purple with the Giemsa stain, but in exudate smears (tunica vaginalis) and in tissue cultures they stain best with the Machiavello stain, appearing as red bodies. *Rickettsiae* occupy an intracytoplasmic position in European and murine typhus, while in Rocky Mountain spotted fever they often appear in the nucleus. This is an important morphologic distinction between these two types. *Rickettsiae* grow only in the presence of living susceptible cells and in this respect resemble viruses.

b. Vaccines.—Effective formalized vaccines have been prepared from tissue cultures, from infected animal tissues, and from infected insect vectors.

377. Habitat.—*Rickettsiae* are parasitic on insects (arthropods), animals, and man. The *Rickettsiae* pathogenic for man cause five clearly defined groups of diseases, many of which are primarily harbored by rodents or other animals, being transmitted to man by infected lice, ticks, fleas, or mites. The blood, and hence all the organs of infected man or animal, is infectious, but *Rickettsiae* are found with extreme difficulty in preparations made from these tissues. The common locality in which to find them is in the tunica vaginalis of infected guinea pigs when scrotal swelling exists. In typhus, especially the murine type, the mesothelial cells lining the tunica vaginalis are packed with them. In spotted fever the mesothelial cells in the tunica vaginalis show a sparse infection. The distinctive lesion in this disease is the presence of organisms in the smooth muscle cells as well as the endothelial cells of the arterioles and venules.

a. Typhus fever group.—(1) European or epidemic typhus (*R. prowazeki*).

(2) Murine or endemic typhus (*R. mooseri*).

b. Rocky Mountain spotted fever (*R. rickettsia*) *and related diseases.*—(1) Sao Paulo exanthematic typhus.

(2) Fieure Boutonneuse.

(3) South African tick fever.

c. Trench fever (*R. quintana*).

d. "Q" fever (*R. burneti*).

e. Tsutsugamushi fever group (*R. nipponica*).—Tsutsugamushi fever in Japan.

378. Important species.—*a. R. prowazeki* causes European or epidemic typhus fever, transmitted from man to man by the body louse (*Pediculus humanus* var. *corporis*). The micro-organisms occur in the cells lining the alimentary tract of the infected louse and

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so the disease is transmitted to man via the louse excreta. In from 5 to 7 days after the louse has fed on the blood of an infected man, the excreta of the louse becomes infective and may remain so for 5 days; the louse then dies and therefore is not a reservoir of the disease between epidemics. In man the most characteristic lesion resulting from this infection is in the vascular system, mainly in the skin and brain. This is a filth or war disease which was responsible for about five million deaths in the period of the World War. "Tabardillo" or Mexican typhus belongs to this group.

b. R. mooseri is the cause of endemic or murine typhus. It is normally a pathogen of rats and other rodents and is transmitted from rat to rat and from rat to man by rat fleas (*X. cheopis* and *C. fasciatus*). During epidemic periods it may be also transmitted from man to man by the body louse. In fleas the *Rickettsiae* may exist without causing their deaths.

c. R. rickettsia is the agent of Rocky Mountain spotted fever. This disease in the western part of the United States is transmitted by the tick (*Dermacentor andersoni*), while in the eastern part of the United States, it is transmitted by a different tick (*D. variables*). There is a great variation in the severity of different strains of this disease. The Bitter-root Valley strain is very pathogenic for man and guinea pigs; outside of this strain the disease is equally severe in the East as in the West. The *Rickettsiae* proliferate in the tick without harm to it and are transmitted hereditarily to its descendants. In the tick, intranuclear forms are frequently found. Infected ticks may transmit the disease to man, monkeys, guinea pigs, rabbits, dogs, ground squirrels, and other rodents.

d. R. nipponica is transmitted by the bite of larval form only of a mite (genus *Trombicula*).

e. This disease has occurred in Australia and a few accidental cases have occurred in the United States as laboratory infections. The organism has been isolated from infected ticks in Montana.

f. R. quintana causes trench fever. This is transmitted by the louse. *Rickettsiae* are found in the lumen of the bowel 5 days after the louse has fed on the blood of a patient; its excreta becomes infective and may remain so for at least 4 months. Infection occurs as the result of rubbing the excreta into the skin. This was a common disease in the trench life of the World War.

379. Collection and transmission of specimens for examination.—*a.* Clear, sterile serum of patient for Weil-Felix agglutination test.

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b. Sterile defibrinated or citrated blood for animal inoculation. This material should be inoculated intraperitoneally into guinea pigs as soon as possible.

c. Autopsy (man): Fresh portions of sterile brain or spleen for animal inoculation; tissue fixed in formalin or Zenkers for histopathological examination.

d. From guinea pigs: Slide preparations from scrapings of tunica vaginalis. European typhus is transmitted from guinea pig to guinea pig by intraperitoneal inoculation of an emulsion of infected brain. Murine typhus is transmitted to guinea pigs by an intraperitoneal inoculation of ground-up tunica vaginalis. Rocky Mountain spotted fever is transmitted from guinea pig to guinea pig by an intraperitoneal inoculation of heart blood.

380. Weil-Felix reaction.—*a. General.*—(1) This is a macroscopic tube agglutination test used in diagnosing Rickettsial diseases. The antigen employed is a *Proteus* X strain, originally isolated from the urine of typhus patients. This organism bears no relationship with the *Rickettsiae* which cause the disease (though a common polysaccharide between *Proteus* X and *Rickettsiae* is said to exist). The test is done 2 hours at 56° C. and overnight in ice box. Only the nonmotile or "O" variant, living or heat killed, is used. While the OX_{19} strain is used for typhus (both types) and Rocky Mountain spotted fever, the Kingsbury strain or OXK is used for tsutsugamushi fever. Agglutinins for *Proteus* X appear about the fourth day of the disease and gradually increase in titer from then on, to disappear soon after convalescence. The increase in agglutination titer is of significance.

(2) The OX_{19} agglutination test alone cannot be used in distinguishing the two types of typhus fever from Rocky Mountain spotted fever. A positive agglutination titer of 1:100 is significant and an increase in titer during the course of the disease is of first importance. In typhus a titer of 1:1,000 is frequent and even 1:100,000 may rarely be attained. In Rocky Mountain spotted fever a titer of 1:10,000 has been found.

b. Results.

European or murine typhus OX_{19}+.
Rocky Mountain spotted fever OX_{19}+.
Tsutsugamushi fever OXK+.

381. Reaction in guinea pig.—*a. Transmission.*—Transmission of an unknown infection to the male guinea pig is the most important single process for establishing the diagnosis, for it may induce fever or scrotal reactions or enable one to apply cross-immunity tests with

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known strains of typhus and spotted fever after the guinea pig has reacted. Human blood is commonly infective for guinea pigs only during the first 4 to 6 days of fever. The best method for establishing a strain is to inject large amounts (6 cc) of blood into guinea pigs by the intraperitoneal route. In transferring epidemic typhus from one animal to another, the guinea pig is sacrificed on the third day of fever and a brain suspension is inoculated intraperitoneally. For murine typhus, the ground-up tunica vaginalis is used. In Rocky Mountain spotted fever 1 cc of guinea pig blood is usually sufficient, but in some milder strains 4 cc may be necessary. The diagnosis of the disease in the guinea pig is based on a febrile reaction, presence and type or absence of scrotal lesions, presence of *Rickettsiae* in tunica vaginalis, the transfer of the disease from animal to animal, the development of a specific immunity, the presence of brain lesions, and the sterility of the blood and brain on ordinary culture media.

b. Temperature.—The temperature of the guinea pig may be regarded as normal up to 103.8 or 104; above this, abnormal. In the original guinea pig inoculation the temperature may declare itself at any time between 4 and 24 days. Once the disease has "taken," the course in subsequent transfers remains more or less constant, provided the transfers are made at the same time in the same manner. In murine typhus the incubation period is from 3 to 4 days with slight fever and scrotal swelling. In epidemic typhus the period of incubation is from 8 to 10 days with fever lasting about 6 days. In Rocky Mountain spotted fever the period of incubation is about 6 days and fever is of about 5 days' duration.

c. Scrotal reaction.—This reaction is more frequently found in large male guinea pigs rather than in small ones. The typical reaction is characterized by swelling and redness. The testes are firm and cannot be pushed back into the peritoneal cavity. Scrapings of the tunica vaginalis from guinea pigs infected with murine typhus show many cells filled with *Rickettsiae.* These infected cells are called "Mooser bodies." While scrotal swelling may occur in epidemic typhus, it is not the rule. Here only a few *Rickettsiae* are found. Scrotal swelling only occurs with a virulent strain of Rocky Mountain spotted fever. Here fewer *Rickettsiae* are found and intranuclear forms may be present. With the milder strains no scrotal swelling is present.

382. Relationship between typhus species.—*a. Similarities.*—(1) Guinea pigs and monkeys recovered from one type of typhus are immune to the other.

(2) Weil-Felix reaction with OX_{19} is present in both.

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(3) Brain lesions in experimental animals are indistinguishable.

(4) Clinical course in man is somewhat similar in onset, febrile reaction, rash, and duration (though different in severity and seasonal incidence).

b. Differences.—(1) Murine typhus produces a more rapid rise of temperature in guinea pig and a characteristic scrotal swelling. Scrapings from the tunica vaginalis show large, swollen endothelial cells, filled with *Rickettsiae* (Mooser bodies). European strain does not give obvious scrotal swelling in guinea pigs.

(2) Murine typhus causes a febrile disease in the rat, with *Rickettsiae* in the scrotal sac; European strain causes inapparent infection in this animal.

(3) Murine *Rickettsiae* injected into X-rayed rats will result in heavy diffuse infection; European *Rickettsiae* will not.

(4) European typhus is more severe in man and occurs in winter; murine typhus tends to occur in summer.

383. Cultural examination.—This procedure is not routinely employed. The *Rickettsiae* will not grow on ordinary culture media. The various types of *Rickettsiae* have been grown by the following methods:

a. By classical tissue culture methods.

b. Inoculation into the yolk sac of fertile chick eggs made on the sixth day of incubation (Cox).

c. Serum Tyrode agar medium on which susceptible tissue cells are placed on the surface of the medium (Zinsser, Plotz, and Enders).

d. In insect (arthropod) louse vector (Weigl).

384. Microscopical examination.—Not applicable to the direct diagnosis but only to tissue, animal exudate, and culture materials.

a. Giemsa stain.—Tissue specimens, fixed for ½ hour in alcohol, Zenker's solution, or Regaud's solution, are sectioned and stained for 10 to 24 hours with diluted (1:50) Giemsa solution. Result: *Rickettsiae* appear as purple bodies.

b. Machiavello staining method.—Tissue culture or exudate smears are stained by the following procedure: Stain with basic fuchsin (0.25 percent in distilled water) for 4 minutes; wash in water, then very rapidly with 0.5 percent citric acid solution, then wash rapidly with tap water; stain with methylene blue (1 percent aqueous solution) for 10 seconds; wash and dry.

Result: *Rickettsiae* stain red, cells stain blue.

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TABLE XV.—*Interpretation of laboratory tests for rickettsial diseases*

Disease	Weil-Felix reaction		Results of guinea pig inoculation		
	OX_{19}	OXK	Scrotal swellings	Brain lesions	Immunity
European typhus	+++	—	Rare; few *Rickettsiae* in tunica vaginalis exudate, only intracytoplasmic localization.	++	Immune to itself and murine typhus.
Murine typhus	+++	—	Usual; many *Rickettsiae* in tunica vaginalis (Mooser bodies). Only intracytoplasmic localization.	++	Immune to itself and European typhus.
Rocky Mountain	+++	—	Only with virulent strains (Bitter-root Valley strain). Few *Rickettsiae* in tunica vaginalis. May find intranuclear forms.	++	Immune to itself but not to European or murine typhus.
"Q" fever	------	------	None	—	Immune to itself only.
Trench fever	?	?	None	------	Immune to itself only.
Tsutsugamushi fever.	—	+++	None	—	Immune to itself only.

SECTION VI

FILTERABLE VIRUSES

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Rabies	395

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385. Habitat.—These are strict parasites, responsible for various pathological conditions in man, animals, birds, fish, insects, plants, and bacteria. They are the causative agents of many communicable diseases in man. These diseases are transmitted by contact with a diseased individual or carrier, by infected insects, or by the bite of an infected animal (e. g., rabies by bite of dog). The viruses live and multiply within the living cells of the host and usually infect a particular type of cell or cells from a limited group of tissues.

386. Characteristics.—Uncertainty exists as to the exact nature of viruses; whether living matter, as usually considered, or inanimate substance. The following characteristics are common to most viruses and are used to describe and identify them:

a. Size is from 8 to 275 millimicrons (mμ). Larger ones can be stained and seen with the ordinary microscope (elementary bodies of vaccinia and psittacosis). By use of ultraviolet light, photographs have been made of the smaller ones.

b. They are usually filterable. A large amount of virus is usually lost during filtration.

c. They cannot be cultivated in the absence of living, susceptible cells.

d. Characteristic "inclusion bodies" develop in the cells of the host, infected with many virus diseases.

e. They are resistant to many bactericidal agents.

f. Many viruses have ability to produce protective immunity with specific viricidal and other antibodies.

g. Ability to reproduce rapidly in the tissues of a susceptible host.

h. Ability to adapt themselves to different hosts by repeated passage.

387. Classification.—Due to small size and parasitism for living cells it is impossible to classify viruses by their morphological or cultural characteristics. Viruses may be grouped by the characteristic affinity they have for certain tissues:

a. Those having an affinity for skin are variola, vaccinia, animal pox diseases, herpes, varicella, lymphogranuloma inguinale, foot and mouth disease.

b. Those having an affinity for the nervous system are encephalitis lethargica, St. Louis encephalitis, encephalitis of horses, cattle, and sheep, poliomyelitis, lymphocytic chorio meningitis, and rabies.

c. Those characterized by catarrhal or generalized infection are measles, mumps, psittacosis, yellow fever, dengue fever, distemper, swine fever or hog cholera, rinderpest, infectious anaemia of horses, fowl plague.

d. Those characterized by tumor formation are fowl leukemia, Rous sarcoma of chickens, warts, infectious myxomatosis of rabbits.

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388. Collection of specimens.—While some viruses are resistant, it should be borne in mind that many are fragile. Rabies and poliomyelitis virus may be conserved in 50 percent buffered glycerin, but other viruses should be kept in an ice box containing dry ice, or they may be frozen and dried. The frozen viruses in a liquid state are thawed at 37° rather than at room temperature.

a. Infected tissue specimens collected for laboratory examinations, vary according to the disease suspected. Specimens may include—

(1) Fresh tissue, or tissue fixed in 10 percent formalin, for microscopical examination for inclusion bodies.

(2) Pieces of tissue are conserved in 50 percent buffered glycerin, made as follows:

Stock solution A (Na_2HPO_4) +28.4 grams per 1,000.
Stock solution B (citric acid) +21 grams per 1,000.
Take of solution A, 93.65 cc.
Take of solution B, 6.35 cc.

This makes 100 cc of buffer pH 7.6. Then add 100 cc of glycerin. Divide in specimen jars and sterilize in autoclave.

b. Liquid material (blood, urine, spinal fluid) is conserved by placing material in pyrex Wasserman tubes, stoppering tightly with a cork stopper and covering cork with adhesive tape. This tube is then plunged in dry ice. Liquid virus material may also be kept by freezing and drying.

c. Clear, sterile serum from patient or recovered animal may be tested for specific antibodies.

389. Preparation of virus suspensions.—Preparation of virus suspensions for animal inoculation or for titration: Tissues are ground up in a mortar with sand or alundum. Tyrode solution, Lockes solution, or broth may be used as a diluting fluid. A 10 percent suspension is often used but this depends upon the virus used. Centrifugalize at 1,200 rpm to bring down larger particles, and inject supernatent liquid. If tissue has been left in glycerin, wash several times in diluting fluid before grinding.

390. Filtration.—Virus suspensions may be filtered to remove bacteria or to determine the filterability of the virus. Of the many types of filters available, the Mandler and Berkefeld diatomaceous earth filters are the most suitable for these purposes. These candles come in varying sizes and varying grades of porosity, "V" or coarse, "N" or medium, and "W" or fine for Berkefeld candles and numbers indicating pounds test for Mandler's. Even the largest viruses and many bacteria will pass through the "V" filter, most viruses through an "N," and only the smallest viruses through the "W" candle. Asbestos

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filters of the Seitz type also are excellent; the "E.K." filter pad corresponds closely in porosity to Mandler "W," is used once, and then discarded. (Since these disks are often alkaline, they should be washed in distilled water and dried before use.)

a. Filtering.—(1) Set up filter in filtering flask and sterilize in autoclave.

(2) Attach side arm of flask to suction pump in series with manometer and trap.

(3) Place virus suspension in glass mantle surrounding candle and draw into filter flask using minimum amount of vacuum.

b. Cleaning of filters.—(1) If infectious material has been filtered, sterilize by covering filter as set up with 5 percent phenol or cresol, and allow to stand for a minimum of 1 hour. Rinse off with tap water.

(2) There are several methods for cleaning filters, some of which are destructive, others tedious and time-consuming. One of the best employs 1 percent $KMnO_4$ (potassium permanganate) which is drawn through the filter as set up (50–75 cc for a 1- by ⅜-inch candle). Subsequently, a 5 percent solution of sodium bisulfite ($NaHSO_3$) is drawn through until all color has disappeared from the candle. Distilled water is then drawn through until test of filtrate with $BaCl_2$ (barium chloride) shows no precipitate. Filter should then be dried in incubator overnight before using.

(3) Filters should be handled by the metal parts and should be carefully protected from contact with grease or oil since such substances change the porosity and allow the passage through the filter of objects otherwise retained.

(4) Alternative method: The simplest and easiest method involves the use of Berkchlor and is recommended when this product is available. A 0.5 percent solution of Berkchlor is passed through the filter (50–100 cc for a 1- by ⅜-inch candle). This is followed by distilled water until test with $AgNO_3$ (silver nitrate) shows no trace of chlorine (a white precipitate). In some instances candles may be boiled 15 to 20 minutes in Berkchlor, followed by distilled water as above. Filters should be subjected to brushing only when no other means for freeing them from clogging is successful.

391. Animal inoculations.—Test for the presence of specific virus by inoculating bacteria-free virus-containing material into susceptible animals. If the type virus present is not known, inoculate different animal species with varying dosages and by several different routes. Due to the selectivity of many viruses for certain tissues in specific hosts, it is often necessary to pass a virus in series through several animals before it becomes sufficiently adapted to produce

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recognizable symptoms. The virus specimens most frequently inoculated into animals, animal used, and type reaction to be noted are:

a. Insect-borne diseases (yellow fever and dengue fever).—Blood during first 3 days of fever or, in case of yellow fever, suspensions of liver and spleen may be injected subcutaneously or intraperitoneally into monkey; results in mild infection in monkey after 4 to 10 days.

b. Neurotropic diseases.—Suspensions of brain or cord from the central nervous system are used:

(1) *Poliomyelitis (infantile paralysis).*—Intracerebral or intranasal inoculation of suspension of spinal cord into monkey results in typical disease.

(2) *Encephalitis (lethargica and St. Louis types of sleeping sickness).*—Intracerebral inoculation into monkeys and rabbits or mice results in encephalitis in 5 to 14 days.

(3) *Rabies.*—Intracerebral inoculation of brain suspension or saliva from infected animal into rabbit, mouse, or guinea pig results in rabies within 21 days.

c. Dermotropic diseases.—(1) *Smallpox.*—Inoculation of pus from pox lesion into monkey by scarification of skin produces local lesion.

(2) *Fever blisters (Herpes febrilis).*—Inoculation of vesicular fluid on scarified cornea of rabbit causes a severe keratitis, or subdural inoculation of rabbit produces encephalitis, fatal in 4 to 6 days.

d. Respiratory diseases.—(1) *Influenza.*—Filtrate of nasal secretions or scrapings, inoculated intranasally into ferret may result in pneumonia. After ferret "take," anesthetized mice may be used.

(2) *Psittacosis (parrot fever).*—Blood or filtered sputum may be injected into peritoneum of mouse, guinea pig, or parrot, resulting in typical disease after 7 to 10 days with focal necrosis in liver, the cytoplasm of cells containing tiny coccoid inclusion bodies.

e. Lymphogranuloma inguinale.—Pus from bubo injected intracerebrally into mouse or guinea pig results in encephalitis in 2 to 8 days.

NOTE.—Bacteria-free pus from bubo, diluted 7 to 10 times with saline, or saline suspension of mouse brain after several serial passages, when killed by heat at 60° C. for 90 minutes on first day and for 60 minutes on second day, may be used as a *Frei antigen* after testing on immunes and non-immunes for potency and specificity. Recently elementary bodies from yolk-sac cultures have been used.

392. Inclusion bodies.—Small structures, the exact nature of which has not been determined, found within the cytoplasm or nucleus of infected cells during many virus diseases, are known as "inclusion bodies." Smears or tissue sections may be stained with Giemsa's,

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Mann's or Seller's stain and examined microscopically. The demonstration of inclusion bodies is of great diagnostic value based upon their selective affinity for certain cells, their position within the cell, and their acidophilic or basophilic staining qualities. For example, the diagnosis of rabies is based upon the demonstration of inclusion bodies (see par. 395).

393. Serological tests.—Most virus diseases produce a strong and lasting immunity in their host. The antibodies present in the blood serum are type specific and may be demonstrated by agglutination, precipitin, complement fixation, and neutralization tests.

394. Cultivation of viruses.—They will not grow on ordinary culture media. Any method which permits cells to remain alive or multiply will support virus growth provided living susceptible cells are present. Viruses may be grown "in vitro" in a modified Maitland's medium consisting of Tyrode's solution, with or without addition of fresh serum, and minced susceptible tissue; in proliferating cells in a plasma clot; or on serum-Tyrode-agar medium where cells are placed on the surface of the semisolid medium; or grown "in vivo" on the chorio-allantoic membrane or other tissues of a developing chick embryo.

395. Rabies.—Rabies is the only virus disease usually diagnosed upon the basis of laboratory findings, that is, the demonstration of inclusion bodies, the so-called "Negri bodies," in smears or sections from the central nervous system of the infected animal.

a. Characteristics.—Rabies is an infectious disease, affecting the central nervous system. It is most common in dogs but occurs in man and other animals. Transmission is by bite of infected animal (dog), whereby virus containing saliva is inoculated. The incubation period in dogs is from 2 to 8 weeks, sometimes longer; in man, usually about 40 to 60 days. The mortality rate of rabid animals is 100 percent, death occurring within 5 days.

b. Collection and transmission of specimens for examination.—If a living animal is received, do not kill, but place in quarantine for 2 weeks or until death. If a dog is killed during early stages of disease, "Negri bodies" may not be found. The entire body of a suspected rabid dog that has died or been killed should be sent to the laboratory, if nearby; or send head and several inches of neck packed in ice. If the specimen is to be sent through the mail to a distant laboratory, remove brain and divide longitudinally into two equal parts; place one-half of brain in 10 percent formalin for sectioning; place other half in 50 percent buffered glycerol for use in making touch preparations and for animal inoculations. For local examination, use fresh

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brain, selecting portions of the hippocampus major (Ammon's horn), fissure of Rolando, cerebral cortex, or cerebellum.

c. Examination for Negri bodies.—(1) Make smear of gray matter of brain on slide, or fix small piece of brain (Ammon's horn), with cut surface up, on end of cork stopper and make touch preparations by gently touching three or four times with clean slide.

(2) Fix for 2 minutes with methyl alcohol.

(3) *Seller's stain.*—(*a*) *Reagents.*

1. Methylene blue ..... 15 gm
Methyl alcohol ..... 100 cc
2. Basic fuchsin ..... 32 gm
Methyl alcohol ..... 100 cc

Just before use, mix three parts of solution *1* with one part of solution *2* and five parts of methyl alcohol. The chromatin should stain blue and cytoplasm red. If a clear-cut differentiation of cytoplasm and chromatin is not obtained, additional amounts of either solution *1* or *2* is added until the desired effect is obtained. The stock solutions should be stored in the refrigerator.

(*b*) *Technic for staining.*—"Touch" preparations are flooded with the mixed stain for approximately 10 seconds, washed in water, dried and examined. The methyl alcohol serves as a fixative and no additional fixation is necessary.

(4) *Mann's stain.*—(*a*) *Reagents.*—Keep as stock solutions—

1. Methyl blue (China blue), 1 gm, dissolved in 100 cc of distilled water.
2. Eosin, W. S., 1 gm, dissolved in 100 cc of distilled water.

Prepare fresh stain for use daily. Add 3.5 cc of methyl blue solution to 10 cc of distilled water, and then add approximately 3.5 cc of eosin solution gradually until correct amount is present (drop of stain on filter paper gives blue center surrounded by pink ring). Filter and use.

(*b*) *Technic for staining.*

1. Flood slide with stain for 5 minutes.
2. Rinse with distilled water.
3. Allow to air-dry or dehydrate and mount in balsam.
4. Examine microscopically for deep-pink stained round or oval Negri bodies from 10 to 20 microns in diameter. They are usually found within cytoplasm of blue-stained nerve cells, but may be extracellular. Red blood cells are larger and take an orange-red color.
5. Report—"Microscopic examination demonstrated (failed to demonstrate) Negri bodies."

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(5) Sections of brain fixed in formalin or Zenker's fluid may be made, carried through xylol and alcohols to water, stained with Mann's stain, dehydrated, mounted in balsam, and examined as above.

d. Animal inoculations.—If touch preparations are negative and persons or valuable animals have been bitten, an animal should be inoculated and the final diagnosis of rabies based upon the demonstration or failure to demonstrate Negri bodies.

(1) *Rabbit.*—Make subdural inoculation of 0.15 cc of 10 percent suspension in H_2O of portion of hippocampus. Incubation time is 17 days or more.

(2) *Young white Swiss mice.*—They are the preferred animal for use. Make intracerebral inoculation of 0.03 cc of 10 percent suspension as above. Incubation time is 9 to 11 days.

(3) *Guinea pigs.*—They are not very satisfactory; do not use if mice or rabbits are available.

SECTION VII

FUNGI

Paragraph
General ---------- 396
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Descriptive terms ---------- 398
Materials for examination ---------- 399
Methods of examination ---------- 400

396. General.—The fungi are complex plant organisms, devoid of chlorophyll. The single-cell types, as the common budding yeast (*Saccharomyces cerevisiae*), grow and multiply much as do bacteria, except as to their method of multiplication (by budding, not by fission). Each individual cell combines the functions of nutrition and reproduction. Other fungi, the molds, are made up of many cells, usually cylindrical (hyphae), joined into filaments (mycelia) from which spores (small round cells) develop, the structure built up by the filaments and spores being characteristic for each species.

397. Habitat.—Fungi occur widespread in nature and to a less extent in disease. Saprophytic fungi obtain their food from dead plants or decaying materials. Parasitic fungi obtain their food from living animal or plant life. A few species of fungi are pathogenic and capable of producing minor or major skin infections (dermatomycosis), hair infections (trichophytosis), bronchial infections (bronchomycosis), and certain specific generalized infections (blastomycosis, actinomycosis, and others). Some fungi have commercial

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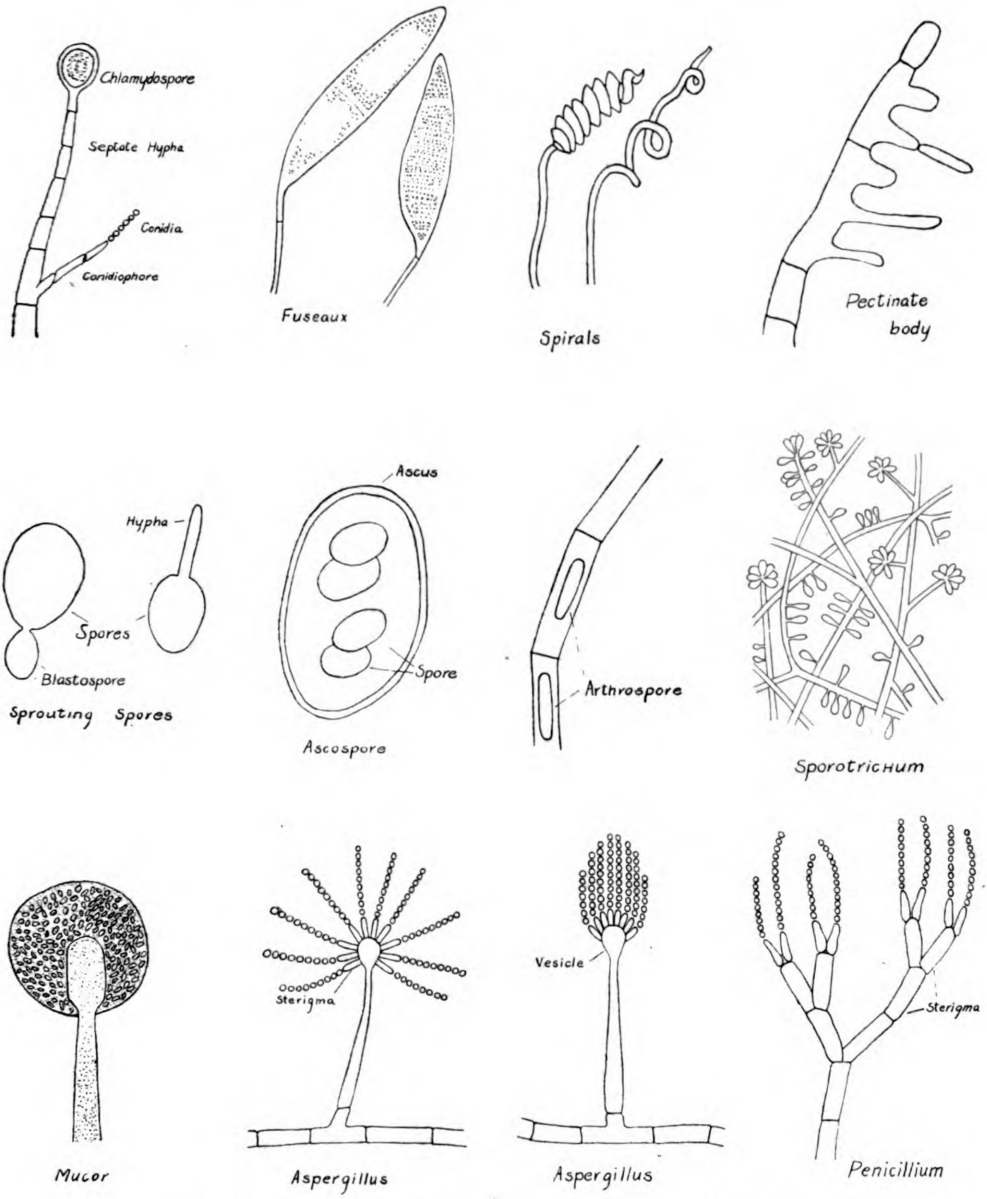


FIGURE 19.—Characteristic morphology of typical fungi.

importance, such as those that give flavor to cheese and cause bread to rise (yeast). Many fungi attain laboratory attention because of their ubiquitousness in dust and their contamination of laboratory media.

398. Descriptive terms.—Fungi occur in many forms, often variable within species under different conditions of growth. Those that are pathogenic tend to grow differently in the tissues than on culture media. Their classification is too complex to be given here. A few terms used in describing and classifying fungi are given below.

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a. Budding fungus.—Yeast-like fungus that grows by budding. In the tissues and in culture they appear as round or oval budding cells and may, but generally do not, develop rudimentary mycelia.

b. Filamentous fungi.—Mold-like fungi that develop long filamentous threads with or without apparent spore formation.

c. Hypha.—The single thread-like portion.

d. Mycelium.—A group or matted mass of branching hyphae.

e. Septa.—Divisions of hypha formed by transverse partitions.

f. Spores.—Cells developed for the propagation or preservation of the species.

g. Thallus.—The actively growing, vegetative organism as distinguished from spores.

h. Ascospores.—Group of spores, four or eight, enclosed in a sac or ascus.

i. Endospore.—A spore formed within an outer envelope.

j. Conidiophore.—Hypha bearing a spore or group of spores.

k. Blastospore.—A spore formed by budding.

l. Arthrospore.—A spore formed of segments of a hypha and released by disarticulation.

m. Chlamydospore.—A large spore with tough and frequently double-contoured wall, undergoing encystment.

n. Sterigma.—A short stalk bearing chains of conidia (as in *Aspergillus*).

o. Sporangium.—A sac containing an indefinite number of spores at the end of a hypha (as in *Mucor*).

p. Fuseaux.—Fusiform septate spores, produced by certain fungi (*Trichophyton*).

q. Spirals.—Terminal coils seen in some species.

r. Pectinate bodies.—Comb-like structures formed by some fungi.

s. Cryptococcus.—A genus of budding fungi devoid of ascospores and mycelia (e. g., *Cryptococcus gilchristi*, the causative organism of one form of blastomycosis).

t. Saccharomyces.—A genus of budding fungi having ascospores but no mycelia (e. g., *Saccharomyces cerevisiae*, brewers yeast, oval or spherical cells, cause alcoholic fermentation).

u. Monilia.—A genus of budding fungi having no ascospores, mycelia of rudimentary type, and capable of fermenting certain sugars with the production of acid and gas (e. g., *Monilia psilosis*, formerly thought to cause sprue).

v. Endomyces.—A genus of budding fungi, having ascospores and segmented mycelia (e. g., *Endomyces albicans*, found in thrush).

w. Madurella.—A genus of filamentous fungi characterized by sep-

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tate, branching hyphae, and chlamydospores. They are contained in black granules of infected tissue (e. g., "Madura foot"; *Madurella mycetomi* in "Madura foot").

x. Nocardia (*Actinomyces*).—A genus of filamentous fungi characterized very fine, nonsegmented mycelial filaments and no spores (e. g., *Nocardia bovis*, the "ray fungus" of "lumpy jaw" of cattle, actinomycosis of man).

y. Sporotrichum.—A genus of filamentous fungi which appear in the tissues as oval spores and develop in cultures as mycelium with characteristic grouped spores or conidia (e. g., *Sporotrichum schenki*, the causative agent of sporotrichosis, appearing in fresh spreads of the pus or tissue as oval or cigar-shaped cells, and when examined by hanging-drop culture appear as fine interlacing septate hyphae with oval or pear-shaped spores attached to the hyphae).

z. Aspergillus.—A genus of filamentous fungi characterized by its spore organ. They are common and troublesome laboratory contaminants, appearing on culture plates as cottony masses dotted with minute colored spots, becoming in older cultures profusely black, yellow, or green, according to the species. Microscopically, the colored spots are seen to be the spore organs, the spores (conidia) borne on aerial hyphae which terminate in a large rounded head with rows of spores projecting in all directions. The main cottony mass is a network of septated mycelial filaments (e.g., *Aspergillus niger*, the black mold).

aa. Penicillium.—This genus differs in its spore organ in that the fertile hyphae show numerous branches, rather than a rounded head, bearing rows of spores, a structure somewhat resembling a broom. The color of the colony varies with the species—green, yellow, etc. A common variety is *Penicillium brevicaule* and its strains, it causes spoilage of cheese and other dairy products.

ab. Phycomycetes.—A group of genera having, in addition to the mycelium, spores contained in a spherical, case-like structure (a sporangium) at the end of a hypha. Species of this group frequently contaminate laboratory media and food products, and occur in soil, dust, and water (e. g., *Mucor mucedo*, the blue-black mold).

ac. Pleomorphism.—This term refers to the great variation in characteristics of morphology and culture which many fungi undergo under different conditions of life. The ringworm group are particularly likely to undergo these degenerative changes, and once a culture has so changed, it cannot easily be restored to its original condition. Prolonged growth on suger-containing media leads to this permanent change, hence the use of "conservation agar" for stock-culture maintenance.

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ad. Ringworm group of fungi.—These filamentous fungi produce superficial skin infections, generally growing as leathery masses of closely interwoven hypae, growing slowly with development of bumps and ridges, and covered by a powdery or velvety "duvet", of aerial hyphae. The next four genera belong in this group. The so-called athlete's foot may be caused by the same group of fungi.

ae. Microsporum.—The small-spored ringworm fungus. In the diseased epidermis they appear as a fine mycelium, 1 to 5 microns in diameter, composed of rectangular elements; they penetrate into the hairs and grow up and down in the hair. When the infected hairs are examined, they are found to be encased with an irregular mosaic of small round spores about 2 microns in diameter (e. g., *Microsporum audouini*, causing the common ringworm of the scalp).

af. Trichophyton.—The large-spored ringworm fungus. The mycelium consists of chains of oval or rectangular spore-like bodies 5 to 8 microns in diameter, in regular alinement. Various species commonly produce ringworm of the scalp, beard, skin, and nails (e. g., *Trichophyton tonsurans*, which is found only within the medulla of the hair).

ag. Epidermophyton.—A genus of skin-invading fungi, appearing as long, interlacing filaments, never invading the hair as do *trichophytons*. May be readily recognized by direct miscroscopic examination of skin scraping (e. g., *Epidermophyton cruris*, causing the common "Dhobie itch" or ringworm of the groin and other areas).

ah. Achorion.—A genus of filamentous fungi of which a species causes favus of scalp (*Achorion schoenleini*).

399. Materials for examination.—*a.* Hairs, skin scales, or scrapings from lesions.

b. Tissue masses or scrapings from internal lesions.

c. Secretions or excretions from infected areas.

d. Incidental cultures (contaminants, etc.).

400. Methods of examination.—*a. Choice of method.*—Method varies with expectancy of findings. Skin scrapings and hair may yield informative data by immediate direct methods only and be less informative on culture or animal inoculation, being difficult to grow, to infect, or to identify exactly. Some species give the desired information only on cultivation or animal inoculation, especially in the case of tissue invaders. A few pathogens may be detected by histopathological examination.

b. Microscopic examination.—(1) *Collection of specimen* (preferably in laboratory by a medical officer).—After cleansing the affected part with alcohol, such materials as hairs, nails, scales, or bits of

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tissue may be scraped into a sterile Petri dish. Moist specimens should be prepared and examined without delay. Biopsy specimens should be divided, one-half for direct examination, the other half for fixation and histological examination. All specimens should be obtained from an active, infected area and not from dried or inactive lesions. Collection of sputum for this examination requires especial care to avoid mouth contaminants by previously rinsing mouth with sterile saline solution, the expectoration then to be placed in a sterile Petri dish and examined within a few hours.

(2) *Fresh preparation for direct study.*—Outline a vaseline circle on a slide, place the material under examination within this circle, add a few drops of 10 percent sodium hydroxide, cover with a cover glass, and examine after a period of digestion—a few minutes to 12 hours. Fungi resist the digestive action of the hydroxide and retain their form, whereas tissue elements disappear. Avoid mistaking artifacts, resembling yeast-like organisms and hyphae.

(3) *Stained spreads.*—Moist materials may be spread on a slide as for bacteriological study, for bacterial stains and for a polychrome (Wright or Giemsa) stain. The former stains will reveal bacterial content, the latter will assist in the study of any fungi present but will also bring out cellular detail and may lead to the discovery of a protozoan infection as leishmaniasis.

c. Cultivation.—(1) Seventy percent alcohol may be used to cleanse scales or hair of contaminating bacteria by allowing the specimens to soak therein for about 1 hour prior to placing them on culture media (control culture to be made of the alcohol to detect contaminating spores in it). Materials, with or without above preparation, should be placed upon media having a pH of 5 to 6, unfavorable for bacterial growth. Inoculate several Sabouraud's maltose agar tubes, planting the inoculum into a slightly broken surface of the slant. Incubate at room temperature (22° C.) and at 37° C. Some pathogenic fungi grow better at 37° C. Many fungi show cultural differences when grown at 22° and at 37° C. Retain the cultures for at least 4 weeks before considering them negative.

(2) Observe the cultures daily, but do not open the tubes unless definite growth is observed, then make a subculture as soon as the tube is opened. Subcultures are made on Sabouraud's maltose agar or on his conservation agar; the former is best for primary isolation, the latter for storage and the study of characteristics. In studying the yeast-like organisms, corn meal agar plates are useful for isolation, purification, and low-power study under the microscope. These are best inoculated by so streaking the plates that the wire produces a slit in the medium.

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(3) Cultures may be studied by observing colony characteristics by naked eye, under low-power magnification, and by slide preparations, fresh or stained, for microscopic examination. Instead of removing, as for bacterial spread examination, a surface loopful of the colony, it is best to remove, with a stiff wire, a fragment of the culture supported intact in a fragment of the culture media; this is placed in a drop of lactophenol (equal parts of lactic acid and phenol) or of water, on a slide, and covered with a cover glass. In studying these slides the microscope light is modified to provide a subdued light (by lowering of substage and control of diaphragm). Study hyphae, branching, budding, sporulation, septation, etc., for descriptive report of the cultured fungus.

(4) Hanging-drop cultures in maltose broth, afford another method of study.

d. Animal inoculation.—Most of the fungi are not pathogenic for laboratory animals. A few species have animal pathogenicity and animals may therefore be used in determining the characteristics of only those few species. The form of the fungi seen in tissues tends to be quite different from the form seen in cultures. Some of the yeast-like fungi, producing as a group, blastomycosis, are pathogenic for animals; diagnostic information is, in these, obtainable by the subcutaneous, intramuscular, intraperitoneal, or intravenous inoculation of the suspected material, or preferably, a pure culture of the isolated fungus. The mouse, rat, guinea pig, or rabbit may be so used, and observed for a prolonged period for local or general evidences of infection.

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CHAPTER 11

SPECIAL SEROLOGICAL METHODS

Paragraphs
SECTION I. Human blood groups 401–405
II. Sero-diagnosis of syphilis 406–414

SECTION I

HUMAN BLOOD GROUPS

Paragraph
Groups and their application 401
Determination of blood groups 402
Cross-typing 403
Universal donor 404
Preparation of typing serum 405

401. Groups and their application.—*a.* According to the classification of Landsteiner, recommended by the National Research Council, human blood is divided into four main groups or types, i. e., "0," "A," "B," and "AB." This grouping depends upon the presence or absence in the blood cells of the two agglutinogens A and B. Both are present in the AB type, while neither is present in the 0 type which should be referred to as "zero."

b. The confusion between the older classifications by Jansky and by Moss is eliminated by this newer grouping. The following table will show the relationship between the systems:

Official	Jansky	Moss	Occurrence (percent)
0	I	IV	43
A	II	II	40
B	III	III	7
AB	IV	I	10

c. The practical application of blood grouping is in its relation to transfusion. These transfusions are given to replace blood losses, to combat various sorts of shock and in the treatment of certain infections. The division into these groups is made necessary because the blood serum of certain of these groups will produce dangerous or even fatal reactions in the blood of certain other groups.

d. If the blood cells of the donor are agglutinated by the serum of the recipient this danger is greatest. Such a donor must not be used. If, however, the cells of the recipient are agglutinated slightly by the serum of the donor, there is less danger. This is because the

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donor's blood is injected into the recipient's circulation so slowly that its serum is generally diluted beyond its agglutinating strength. Such a donor can be used only in the gravest emergency.

e. The donor must belong to the same group as the recipient, except those in group 0. This group is referred to in more detail in paragraph 404.

402. Determination of blood groups.—*a.* The only reagents required for the test are sera from blood groups A and B. The only apparatus required comprises—

(1) Microscopic slides.

(2) Hypodermic syringes fitted with 24-gage needles.

(3) Wood applicators.

b. The test is made as follows:

(1) Divide a slide down the middle with a wax pencil.

(2) Mark the left side *A* and the right *B*.

(3) Withdraw enough A serum for the test into one syringe and enough B serum into another by perforating the rubber stoppers of the ampoules containing the grouping sera.

(4) Place 1 drop of A serum in the center of the *A* side of the slide and a drop of B serum in the center of the *B* side. One drop of each is sufficient for the test.

(5) Puncture the finger or the ear lobe of the person to be tested and transfer a minute drop of blood by means of a clean applicator to the drop of A serum, mixing to a smooth suspension of the cells. With a fresh applicator transfer a like drop to the B serum and mix thoroughly. *Never use the same applicator for both sera.*

(6) Allow to stand for 5 minutes, occasionally rolling or tilting the slide to insure thorough mixing. Any agglutination of the red cells must take place within this time limit. If it is difficult to distinguish between true agglutination and rouleau formation, stir again with an applicator, as rouleaux will be broken up to a smooth suspension thereby, but true agglutination will be unaffected.

(7) The reading of the reaction is best expressed by the following illustration.

(8) Occasionally there will be a doubt as to the agglutination in weakly reacting blood. It will be wise then to use a suspension of the cells in saline. One drop of the blood to be typed in 0.5 cc of saline tested in the same manner as whole blood, will almost always give a reaction which can be read clearly, even if it is necessary to use the low-power magnification of the microscope to determine the presence or absence of agglutination.

403. Cross-typing.—Occasionally the bloods of donor and recipient, even though clearly of the same group, will not match, that

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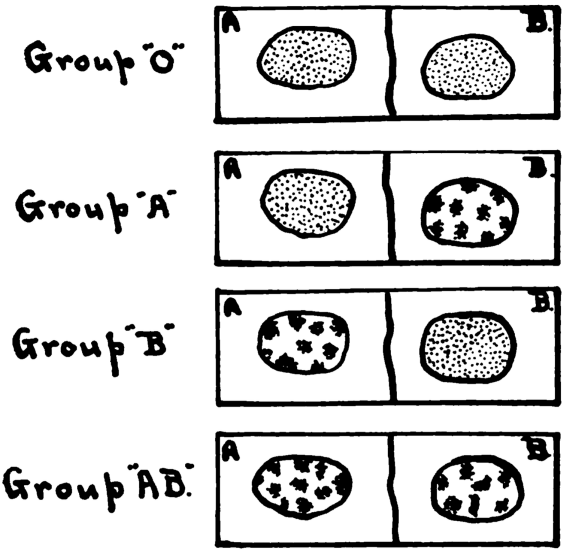


FIGURE 20.—Blood grouping.

is, there will be some agglutination of the donor's cells by the patient's serum or of the patient's cells by the donor's serum. While this may not be fatal, it is potentially a grave danger. It is to be avoided by cross-typing. This is done in the following manner:

a. Take 1 or 2 cc of prospective donor's blood and the same amount of the recipient's blood in separate Wright's capillary ampoules or in small Kahn tubes.

b. Mark the appropriate tubes clearly as DS (donor's serum) and RS (recipient's serum).

c. Set aside until the serum separates. If necessary, centrifuge to hasten separation.

d. Transfer a drop of freshly drawn blood to 1 cc of salt solution from both donor and recipient and mark the appropriate tubes DC (donor's cells) and RC (recipient's cells). Mix to an even suspension.

e. Divide a clean slide as for the standard typing test and mark the left side $\frac{DS}{RC}$ and the right side $\frac{RS}{DC}$.

f. Place 1 drop of donor's serum on the left side and 1 drop of recipient's serum on the right, using fresh capillary pipettes for each transfer.

g. Mix 1 drop of recipient's cells with the donor's serum and 1 drop of donor's cells with recipient's serum, using fresh capillary pipettes for each transfer.

h. The remainder of the test is done in the same manner as for the standard typing. Any agglutination evident within 5 minutes

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should disqualify the donor and another should be tried until one is found giving no trace of agglutination. This is especially true when there is any grade of reaction between donor's cells and recipient's serum.

404. Universal donor.—Any donor belonging to group 0 is considered to be a universal donor, all other things being equal. This is on the assumption that his serum contains no iso-agglutinins for other groups. This may be true but should not be relied upon without cross-typing except in dire emergency. There are by no means infrequent instances of 0 donors whose cells are moderately agglutinated by the particular recipient's cells or vice versa. Unpleasant or dangerous consequences attend their employment. An excellent rule to follow in selecting donors is: Whenever possible use one from the recipient's group and *always cross-type.*

405. Preparation of typing serum.—*a. General.*—Occasionally an emergency may arise when typing serum is badly needed and none is at hand. Strong B serum is difficult to obtain because of the scarcity of B donors in general. It is wise, therefore, always to have an up-to-date list of donors among the local detachment with a note as to the strength of the reaction in each case.

Enough serum can be obtained from 30 to 50 cc of blood to last over almost any emergency and is easy to prepare. Reasonable care as to maintaining sterility while taking the blood and preserving the serum will keep the serum at full strength for many weeks. Choose only donors whose reaction is strong, rapid, and clear-cut. It is wise to take the A and the B blood at different times to avoid all possibility of mixing or mistaking them.

b. Collecting blood.—It has been found best to collect the blood in sterile culture tubes and not more than 15 cc to a tube for ease in handling and to secure the maximum yield of serum. The blood will be collected according to the directions given in paragraph 40*a*.

c. Separating serum.—(1) Remove plugs and flame the mouths of the tubes lightly to burn off wisps of adhering cotton. Do not replug the tubes.

(2) Centrifuge at 1,500 rpm for 5 minutes.

(3) Decant or pipette off the clear serum into fresh, sterile tubes and recentrifuge to get rid of all stray red cells.

(4) Collect in a sterile graduated cylinder to find the exact amount of the serum yield.

d. Preserving type A serum.—(1) Have ready—

(*a*) A 10 percent aqueous solution of sodium citrate.

(*b*) A 1 percent aqueous solution of neutral acriflavin.

(*c*) A 0.5 percent aqueous solution of basic fuchsin.

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(2) To each 9 cc of clear A serum add 1 cc of the sodium citrate solution.

(3) To each 10 cc of citrated A serum add 0.2 cc of the acriflavin solution and 0.2 cc of the basic fuchsin solution.

(4) Mix thoroughly. Store in 2- or 4-cc sterile vials with sealed rubber stoppers. Keep in refrigerator when not in use.

e. Preserving type B serum.—(1) Have ready—

(*a*) A 10 percent sodium citrate solution as above.

(*b*) A 1 percent aqueous solution of brilliant green.

(2) To each 9 cc of clear B serum add 1 cc of the citrate solution, as above.

(3) To each 10 cc of the citrated B serum add 0.2 cc of the brilliant green solution.

(4) Mix and store as for the A serum.

f. The addition of the citrate and stains to the sera serves both to preserve them from bacterial growth and to make them more easily distinguishable from each other. When the stains are not available, the A and B sera may be preserved by simple freezing, provided the laboratory has an electric refrigerator with a freezing compartment. The sera may then be frozen in sterile Kahn tubes each containing 1 cc of serum. The tubes should be plugged with cotton and the plugs sealed with paraffin immediately after freezing. They should be kept in the freezing compartment and thawed out as needed.

SECTION II

SERO-DIAGNOSIS OF SYPHILIS

	Paragraph
General	406
Standard Kahn test	407
Quantitative Kahn test	408
Spinal fluid Kahn test	409
Quantitative Kahn test for spinal fluid	410
Complement fixation test for syphilis (Craig-Wassermann method)	411
Two-tube Kolmer test (sheep system)	412
Spinal fluid complement fixation test	413
Colloidal gold test	414

406. General.—*a.* Three standard tests are here outlined:

Kahn precipitation.
Craig-Wassermann, using the human hemolytic system.
Two-tube Kolmer, using the sheep hemolytic system.

b. The quantitative methods are confined to the Kahn test alone, since it is believed that more clear-cut reactions are achieved by this method. Technic for spinal fluid reactions are given for both Kahn and Kolmer tests.

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407. Standard Kahn test.—*a.* Separate serum from clot and centrifuge until entirely free of cells.

b. Inactivate serum in water bath (56°) for 30 minutes.

c. Mix Kahn antigen by placing 1 cc of antigen in a mixing vial and the required amount of 0.85 percent salt solution (as indicated by titer on antigen bottle) in another vial, then pour salt into antigen and quickly pour from one vial to the other 12 times. Allow to stand for 10 minutes.

d. The standard Kahn test is a three-tube test. The tubes are placed in the rack three deep.

e. After the antigen has stood for 10 minutes it is shaken well to insure an even mixture and is then pipetted into the bottom of the tubes in the following varying amounts:

Back tube, 0.0125 cc
Middle tube, 0.025 cc
Front tube, 0.05 cc

A Kahn antigen pipette (total capacity 0.25 cc) is used to deliver the antigen.

Caution: The antigen suspension is reliable for only one-half hour after dilution. If the number of tests is so great as to take more than a half hour to pipette, the first dilution should be discarded at the end of the half hour and a new dilution made with which to finish the tests. (A skilled technician can pipette antigen for 200 tests in 30 minutes.)

f. The inactivated serum of the patient is then added to the tubes containing the antigen, 0.15 cc to each tube.

g. Three controls are set up at the same time the test is run. The same amounts of antigen (*e* above) are used.

(1) *Control No. 1.*—Add 0.15 cc of 0.85 percent salt solution to antigen in each of the three tubes.

(2) *Control No. 2.*—Add 0.15 cc of known positive serum to antigen in each of the three tubes.

(3) *Control No. 3.*—Add 0.15 cc of known negative serum to antigen in each of the three tubes.

h. All tubes are shaken for 3 minutes.

i. Salt solution is added to all the tubes in the following amounts:

Back tube, 0.5 cc
Middle tube, 0.5 cc
Front tube, 1.0 cc

j. All tubes are shaken enough to mix the salt solution and antigen-serum mixture thoroughly.

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k. Tests are then read. A negative reading shows homogeneous cloudiness with no visible flocs. A four-plus reading shows complete flocculation in a water-clear fluid. One-, two-, and three-plus readings show degrees of flocculation and cloudiness of the fluid varying from the negative to the four-plus.

408. Quantitative Kahn test.—To be done only on sera that show a three- or a four-plus reaction with the standard Kahn test.

a. Separate serum from clot and centrifuge until entirely free of cells.

b. Inactivate serum in water bath (56° C.) for 30 minutes.

c. Mix Kahn antigen by placing 1 cc of antigen in a mixing vial and the required amount of 0.85 percent salt solution (as indicated by titer on antigen bottle) in another vial, then pour salt into antigen and quickly pour from one vial to the other 12 times. Allow to stand for 10 minutes.

d. While antigen is aging for 10 minutes dilutions are made of the serum as follows, using six tubes:

Tube No.	
1	0.6 cc salt solution+0.4 cc serum.
2	0.5 cc salt solution+0.5 cc of mixture from tube 1.
3	0.5 cc salt solution+0.5 cc of mixture from tube 2.
4	0.5 cc salt solution+0.5 cc of mixture from tube 3.
5	0.5 cc salt solution+0.5 cc of mixture from tube 4.
6	0.5 cc salt solution+0.5 cc of mixture from tube 5.

The dilutions will then be as follows:

Tube No.	Dilution
1	1:2.5.
2	1:5.
3	1:10.
4	1:20.
5	1:40.
6	1:80.

The dilutions may be carried higher if the serum potency demands it. One tube may be run with an undiluted specimen of serum if it is apparent that the unit reading should be less than 10.

e. Six Kahn tubes are placed in the Kahn rack in a row, then 0.025 cc of antigen suspension is pipetted to the bottom of each of these tubes.

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f. Then 0.15 cc of each of the serum dilutions is added to the corresponding Kahn tube, beginning with tube No. 6. That is—

0.15 cc from serum dilution tube No. 6 into Kahn tube No. 6;
0.15 cc from serum dilution tube No. 5 into Kahn tube No. 5, etc.

g. All tubes are shaken for 3 minutes.

h. Add 0.5 cc of salt solution to each tube.

i. Shake rack and read results. The highest dilution in which there is a +++ or a ++++ reaction shows the quantitative reading. It may be arbitrarily expressed by multiplying the dilution factor of that tube by four and noting the result as units, for example, if the highest dilution showing a +++ reaction is tube No. 1, the reading in units will be 4+2.5 or 10 units. It may be reported by simply giving the series of readings of the six or more tubes in sequence, as ++++, ++++, +++, +, −, −.

409. Spinal fluid Kahn test.—*a.* Centrifuge spinal fluid until free of cells.

b. Pipette 1.5 cc of clear spinal fluid into centrifuge tube.

c. Add 1.5 cc of saturated ammonium sulfate solution.

d. Place in 56° water bath for 15 minutes. (Globulin can be read at this point.)

e. Centrifuge at high speed for 10 minutes. The precipitated globulin will be packed in the bottom of the centrifuge tube. Pour off the supernatant fluid and drain by inverting the tube on a clean towel for several minutes.

f. Add 0.15 cc of 0.85 percent salt solution and shake gently until the precipitate is dissolved.

g. Mix antigen by placing 1 cc of antigen in a mixing vial and the required amount of 0.85 percent salt solution (as indicated by titer on antigen bottle) in another vial, then pour salt into antigen and quickly pour from one vial to the other 12 times. Allow to stand for 10 minutes.

h. Pipette 0.01 cc of antigen suspension into the bottom of a Kahn tube.

i. Pipette 0.15 cc of the globulin solution from the centrifuge tube into the Kahn tube which contains the 0.01 cc of antigen suspension.

j. Shake for 3 minutes, add 0.5 cc of salt solution, and read results.

410. Quantitative Kahn test for spinal fluid.—*a.* Centrifuge spinal fluid until free of cells.

b. Pipette 3 cc of clear spinal fluid into centrifuge tube.

c. Add 3 cc of saturated ammonium sulfate solution.

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d. Incubate, centrifuge, drain, and dilute antigen as in the standard spinal fluid Kahn procedure.

e. Add 0.3 cc of salt solution and dissolve precipitate. This solution may require centrifugation to free it from undissolved particles.

f. While antigen is aging, dilutions are made of the globulin solution as follows, using five tubes:

Tube No.	
1	This is the 0.3 cc. of globulin solution in the centrifuge tube.
2	0.6 cc salt solution +0.15 cc from tube No. 1.
3	0.4 cc salt solution +0.4 cc from tube No. 2.
4	0.1 cc salt solution +0.2 cc from tube No. 3.
5	0.2 cc salt solution +0.2 cc from tube No. 4.

g. This is a five-tube test. Place five Kahn tubes in the rack in a row.

h. Pipette antigen that has stood for 10 minutes into Kahn tubes, 0.01 cc into the bottom of each tube.

i. Pipette 0.15 cc of dilutions of globulin solution into tubes containing the antigen beginning with the last tube. That is—

0.15 cc from dilution tube No. 5 into Kahn tube No. 5;
0.15 cc from dilution tube No. 4 into Kahn tube No. 4, etc.

j. Shake 3 minutes, add 0.5 cc of salt solution and read results in sequence, for example, ++++, ++++, ++, +, −.

411. Complement fixation test for syphilis (Craig-Wassermann method).—*a. Glassware and apparatus.*

Pipettes: 0.2 cc graduated to 0.01 (for titrations); 1 cc graduated to 0.01 to tip; 10 cc graduated to 0.1 cc.
Test tubes, 100 by 12 mm, heavy wall, without lip.
Test-tube racks carrying 2 rows of 10 tubes each.
Amboceptor cutter and set of 3 ambocepter paper markers (3, 4, and 5 mm).
Centrifuge and graduated centrifuge tubes.
Water baths, one at 37° C. and one at 56° C.
Refrigerator at 6° to 8° C.

All glassware should be chemically clean.

b. Reagents.—(1) *Patient's serum.*—The serum should be separated from the clot and centrifuged until all cells are thrown to the bottom

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of the tube, then it is poured or pipetted from the top and placed into a clean tube. *The serum must be entirely free of cells.* Before the test is run, all sera are inactivated in 56° water bath for 30 minutes.

(2) *Salt solution.*—An 0.85 percent salt solution made with distilled water is used. That is, 8.5 gm of chemically pure sodium chloride in 1,000 cc of distilled water.

(3) *Red-cell suspension.*—A 5 percent suspension of human red cells is used. This is prepared by collecting blood (from a normal person belonging to group "0") in a flask which contains 2 percent citrate in 0.85 percent salt solution, approximately 10 cc of blood to 30 cc of the citrate solution. The red cells are thoroughly washed by centrifuging 4 or 5 times, the supernatant fluid being removed each time and replaced with fresh salt solution. On last washing, centrifuge for 3 minutes at a speed of 1,200 rpm, then read volume of red cells by graduations on the centrifuge tube, remove supernatant fluid, and add salt solution to make a 5 percent suspension. A fresh suspension of red cells is made for each day the tests are run. The speed of the centrifuge and the time of centrifuging are both controlled so that uniform suspensions may be made from day to day.

(4) *Amboceptor paper.*—Serum from rabbits which have been immunized to human red cells (type 0) is soaked up on filter paper and dried in the air. It is kept for 3 months before use.

(5) *Complement.*—The serum used is that of two or more guinea pigs. Serum from a single pig must not be used because of the variability of the complement content in the serum of individual animals. To each 2 cc of fresh serum obtained the day before the test, add 3 cc of salt solution, making a 40-percent dilution. If lyophilized complement is used, regenerate the frozen and dried serum as directed on the ampoule and make the 40-percent dilution from this, as directed above.

(6) *Antigen.*—An alcoholic extract of ether-extracted powdered beef heart, containing 0.4 percent cholesterol.

c. Method employed.—The method employed is a two-tube qualitative test. A cholesterolized alcoholic extract of powdered beef heart is used as antigen and the antihuman hemolytic system (Craig's modification) is utilized.

Assuming that all reagents are new and titrations are unknown, it is best to make a 40-percent dilution of the complement and use 0.1 cc as two units. This is merely an arbitrary dose to be used in the amboceptor titration and the complement must be titrated after the amboceptor unit is found.

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d. Amboceptor titration.—(1) Amboceptor paper is cut in strips 3 mm wide, marked in varying lengths, and tested as follows:

Tube No.	Salt solution (cc)	Complement (cc of 40 percent)	5 percent blood cell suspension	Amboceptor paper (mm)
1	0.9	0.1	0.1	3 by 1
2	.9	.1	.1	3 by 1½
3	.9	.1	.1	3 by 2
4	.9	.1	.1	3 by 2½
5	.9	.1	.1	3 by 3
6	.9	.1	.1	3 by 3½
7	.9	.1	.1	3 by 4
8	.9	.1	.1	3 by 5
9	.9	.1	.1	None
10	.9	None	.1	3 by 10

(2) Incubate in water bath at 37° C. for 1 hour, shaking every 10 minutes, then read the titration. The first tube showing complete hemolysis contains one unit of amboceptor. Use two units, or twice that amount of paper, for the test.

e. Complement titration.—(1) The complement to be used is the 40-percent guinea pig serum, prepared in the manner previously described.

(2) The amboceptor unit is now known and two units are used in the complement titration as follows:

Tube No.	Complement (cc of 40 percent)	Salt solution (cc)	5 percent red cell suspension	Amboceptor units
1	0.02	0.9	0.1	2
2	.03	.9	.1	2
3	.04	.9	.1	2
4	.05	.9	.1	2
5	.06	.9	.1	2
6	.07	.9	.1	2
7	.08	.9	.1	2
8	.09	.9	.1	2
9	.10	.9	.1	2
10	.20	.8	.1	None

(3) Incubate at 37° for 1 hour, shaking the tubes every 10 minutes to liberate the amboceptor from the paper and keep the red cells in suspension. Read the titration.

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(4) The first tube showing complete hemolysis is noted and the amount of complement in that tube is called one unit. Twice this amount, or two units should be used in the test. The control, tube No. 10, should show complete inhibition of hemolysis since it contains no amboceptor.

f. Antigenic strength titration.—(1) A 1 to 10 dilution of the antigen is made by placing 1 cc of antigen in a small flask and adding 9 cc of 0.85 percent salt solution. The salt solution should be added drop by drop while the flask is being shaken constantly. The resultant mixture should present a markedly cloudy appearance.

(2) The amboceptor unit and complement units are now known. The antigen titration is carried out as follows:

Tube No.	Antigen emulsion	Salt solution	Positive human serum	Complement units		5 percent red cell suspension	Amboceptor units
1	0. 02	0. 9	0. 1	2	Incubate at 37° for 30 minutes.	0. 1	2
2	. 03	. 9	. 1	2		. 1	2
3	. 04	. 9	. 1	2		. 1	2
4	. 05	. 9	. 1	2		. 1	2
5	. 06	. 9	. 1	2		. 1	2
6	. 07	. 9	. 1	2		. 1	2
7	. 08	. 9	. 1	2		. 1	2
8	. 09	. 9	. 1	2		. 1	2
9	. 10	. 9	. 1	2		. 1	2
10	none	1. 0	. 1	2		. 1	2
11	none	1. 1	none	2		. 1	2
12	none	1. 2	0. 1	none		. 1	2

(3) The tubes are incubated for 30 minutes in the 37° water bath before addition of the hemolytic system (amboceptor and red cells) and for 1 hour after the addition of the hemolytic system with shaking of the tubes every 10 minutes.

(4) It is noted that tube No. 10 has no antigen emulsion and should therefore show complete hemolysis. Tube No. 11 has neither antigen nor positive human serum and should also show complete hemolysis. Tube No. 12 has neither antigen nor complement and should show no hemolysis. If these control tubes do not comply with these requirements, the titration should be rejected.

(5) In the remaining tubes, the smallest amount of antigen which has completely fixed the complement in the presence of positive serum and therefore shows no hemolysis, is called the antigenic unit. The antigenic dose to be used in the test is twice the antigenic unit.

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g. Procedure for the test.—(1) For each serum to be tested two tubes are necessary, a front and a rear tube. In addition, for each run of tests there must be a control set of two known positive sera and two negative sera. Three single tubes for control of the anti-complementary properties are added.

(2) Each unknown serum and known positive and negative serum has a front and a rear tube:

Front tube	Rear tube
0.9 cc salt solution	0.9 cc salt solution.
0.1 cc serum (patient's or control)	0.1 cc serum (patient's or control).
2 units antigen	
2 units complement	2 units complement.
Incubate at 37° for 30 minutes.	
2 units amboceptor	2 units amboceptor.
0.1 cc red cells	0.1 cc red cells.

Incubate 1 hour at 37° C. with shaking every 10 minutes. Set in ice box for 1 hour.

(3) The three control tubes for the anticomplementary properties of the antigen are set up as follows:

Tube No. 1	Tube No. 2	Tube No. 3
0.8 cc salt solution	0.7 cc salt solution	0.7 cc salt solution.
0.1 cc normal serum	0.1 cc normal serum	———.
6 units antigen	8 units antigen	8 units antigen.
2 units complement	2 units complement	2 units complement.
Incubate at 37° for 30 minutes		
2 units amboceptor	2 units amboceptor	2 units amboceptor.
0.1 cc red cells	0.1 cc red cells	0.1 cc red cells.

Incubate 1 hour at 37° C. with shaking every 10 minutes. Set in ice box for 1 hour.

h. Results.—The positive control tubes should show complete inhibition of hemolysis in the front tube and complete hemolysis in the rear tube, while the negative controls should show complete hemolysis, both front and rear. In the tubes containing the unknown sera, if syphilitic, the front tube should show a complete inhibition of hemolysis or varying degrees of inhibition, according to the

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length of time after infection, the amount and character of treatment, and other factors influencing the strength of the reaction. If syphilis is not present, the front tube containing the patient's serum should show complete hemolysis. All rear tubes should show complete hemolysis. The three antigen control tubes should show complete hemolysis.

412. Two-tube Kolmer test (sheep system).—*a. Glassware and apparatus.*

Pipettes: 0.2 cc, graduated to 0.01 cc; 1.0 cc, graduated to 0.01 cc; 10.0 cc, graduated to 0.1 cc.
Test tubes, 100 by 12 mm, heavy wall, without lip.
Test tube racks, carrying 2 rows of 10 tubes each.
Centrifuge and centrifuge tubes.
Water baths: inactivating, set at 56° C.; incubating, set at 37° C.
Refrigerator, running at 6° to 8° C.

All glassware should be chemically clean and should be used dry or rinsed out with normal saline solution just before using. *Never use any glassware containing the slightest degree of water.*

b. Reagents.—(1) *Patient's serum* (par. 40).—The serum must be inactivated at 56° C. in the water bath for 30 minutes just before using. If the serum to be tested has previously been inactivated, only a 10-minute inactivation is necessary just before running the test.

(2) *Salt solution.*—This is an isotonic solution of sodium chloride. Add 0.85 gram of chemically pure sodium chloride (Merck's blue label) to 100 cc of distilled water.

(3) *Sheep cell suspension* (*indicator antigen*).—Collect the blood by bleeding the sheep from the external jugular vein into 1 to 3 percent sodium citrate solution. The blood may be received into a flask containing a handful of sterile glass beads and shaken well to defibrinate it. Either method prevents clotting. The former method is preferable. Filter a small amount of the blood through cotton into a graduated centrifuge tube, allowing twice as much blood as will be required for the test to be run. Add 2 or 3 volumes of salt solution. Centrifuge at tenth speed for 10 minutes. Repeat this washing five times. On the last washing, centrifuge at the tenth speed for exactly 15 minutes. Do not vary the time or speed, so as to insure the same percent suspension when the cells are finally diluted for use in the test. Read the volume of the cells in the centrifuge tube, carefully remove the supernatant fluid, and prepare a 2-percent suspension by washing the cells into a flask with 49 volumes of salt solution. Always shake well before using to

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secure an even suspension, as the cells rapidly settle to the bottom of the flask on standing.

(4) *Complement.*—See paragraph 411*b*(5).

(5) *Amboceptor.*—Glycerolized amboceptor may be obtained by requisition.

(6) *Antigen.*—While the titration factors are given on all reagents issued by the Army Medical School, it is advisable to keep watch that they retain their potency. Once in 3 weeks is about the proper frequency to check up by titration. The technic is given below.

c. Titration of amboceptor.—(1) Prepare a dilution of 1:100 amboceptor as follows:

Glycerolized amboceptor (50 percent)	2 cc
Salt solution	94 cc
Phenol (5 percent in salt solution)	4 cc

This is to be kept in the refrigerator as a stock solution and is good for several weeks.

(2) Dilute this stock solution for the titration as follows:

Stock amboceptor (1:100)	0.5 cc
Salt solution	4.5 cc

This will be 1:1,000 in strength.

(3) In a series of 10 tubes, prepare higher dilutions as follows:

Tube No.	Amboceptor, 0.5 cc	Plus cc saline
1	1:1, 000	None.
2	1:1, 000	0.5 (1:2,000).
3	1:1, 000	1.0 (1:3,000).
4	1:1, 000	1.5 (1:4,000).
5	1:1, 000	2.0 (1:5,000).
6	1:3, 000	.5 (1:6,000).
7	1:4, 000	.5 (1:8,000).
8	1:5, 000	.5 (1:10,000).
9	1:6, 000	.5 (1:12,000).
10	1:8, 000	.5 (1:16,000).

Mix the contents of each tube thoroughly.

(4) Prepare a 1:30 dilution of the regenerated complement (see par. 411*b*(5)) by diluting 0.2 cc of the regenerated complement with 5.8 cc of salt solution.

(5) Prepare a 2-percent suspension of sheep cells in salt solution.

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(6) In a series of 10 tubes set up the amboceptor titration as shown in the following table:

Tube No.	Amboceptor, 0.5 cc	Complement, 1:30	Saline	Sheep cells, 2 percent
1	1:1, 000	0.3 cc to all tubes	1.7 cc to all tubes	0.5 cc to all tubes.
2	1:2, 000			
3	1:3, 000			
4	1:4, 000			
5	1:5, 000			
6	1:6, 000			
7	1:8, 000			
8	1:10, 000			
9	1:12, 000			
10	1:16, 000			

Mix the contents of each tube thoroughly.

(7) Incubate in the water bath at 38° C. for 1 hour.

(8) Read the amboceptor unit. The unit is the highest dilution of amboceptor that gives complete hemolysis.

(9) Two units of amboceptor are used in the complement and antigen titrations and in the final test. Example: if the unit equals 0.5 cc of the 1:6,000 dilution, then two units will equal 0.5 cc of the 1:3,000 dilution. Dilute just enough of the stock amboceptor for the titrations and the number of tests to be run.

d. Titration of complement.—(1) Prepare a 1:30 dilution of the complement (see *c* (4) above.

(2) Dilute the antigen as indicated by the dilution factor on the antigen bottle, by placing the required amount of salt solution in a small flask and adding the antigen drop by drop, shaking the flask continually until the antigen has all been added. Prepare enough for the complement titration and for the final test.

(3) In a series of 10 tubes, set up the titration as follows:

Tube No.	Complement (1:30)	Antigen dose	Salt solution		Amboceptor 2 units	Sheep cells, 2 percent	
	(cc)	(cc)	(cc)		(cc)	(cc)	
1	0. 1	0. 5	1. 4	Water bath 37° C. for 1 hour	0. 5	0. 5	Water bath 37° C. for 1 hour
2	. 15	. 5	1. 4		. 5	. 5	
3	. 2	. 5	1. 3		. 5	. 5	
4	. 25	. 5	1. 3		. 5	. 5	
5	. 3	. 5	1. 2		. 5	. 5	
6	. 35	. 5	1. 2		. 5	. 5	
7	. 4	. 5	1. 1		. 5	. 5	
8	. 45	. 5	1. 1		. 5	. 5	
9	. 5	. 5	1. 0		. 5	. 5	
10	None	None	2. 5		None	. 5	

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(4) The smallest amount of complement just giving sparkling hemolysis is the exact unit. The next higher tube is the full unit, which contains 0.05 cc more complement. In the antigen titration and in the final test, two full units are used and are so diluted as to be contained in 1.0 cc, as in the following example:

Exact unit	0.3 cc
Full unit	.35 cc
Dose (2 full units)	.7 cc

(5) To calculate the dilution to employ so that 1.0 cc will contain the dose of 2 full units, divide 30 by the dose (0.7). This equals 43, therefore 1.0 cc of a 1:43 dilution will contain the required 2 full units.

e. Titration of antigen.—(1) Prepare a 1:80 dilution of antigen by adding 0.1 cc, drop by drop, with continual shaking, to 7.9 cc of salt solution in a large test tube or a small flask.

(2) Higher dilutions are then prepared as follows:

Quantity of dilution	Salt solution added	Resulting dilution
4 cc of 1:80	4 cc	1:160.
4 cc of 1:160	4 cc	1:320.
4 cc of 1:320	4 cc	1:640.
4 cc of 1:640	4 cc	1:1,280.
4 cc of 1:1,280	4 cc	1:2,560.

(3) Arrange five rows of test tubes with six tubes in each row.

(4) (*a*) In the first tube of each row place 0.5 cc dilution 1:80.

(*b*) In the second tube of each row place 0.5 cc dilution 1:160.

(*c*) In the third tube of each row place 0.5 cc dilution 1:320.

(*d*) In the fourth tube of each row place 0.5 cc dilution 1:640.

(*e*) In the fifth tube of each row place 0.5 cc dilution 1:1,280.

(*f*) In the sixth tube of each row place 0.5 cc dilution 1:2,560.

(5) Heat 3 cc of moderately to strongly positive syphilitic serum in a water bath at 56° C. for 15 to 20 minutes and prepare five dilutions in large test tubes as follows:

Tube No.	Serum (cc)	Saline (cc)	Resulting dilution	Cc of serum in 0.5 cc of dilution
1	1.0	4.0	1:5	0.1
2	.5	4.5	1:10	.05
3	.5	9.5	1:20	.025
4	2.0 (1:20)	2.0	1:40	.0125
5	1.0 (1:20)	4.0	1:100	.005

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(6) To each of the six tubes add dilutions as follows:

0.5 of 1:5 to first row.
0.5 of 1:10 to second row.
0.5 of 1:20 to third row.
0.5 of 1:40 to fourth row.
0.5 of 1:100 to fifth row.

(7) Add 1.0 cc of complement dilution carrying two full units to all 30 tubes.

(8) In a separate rack, set up a serum control carrying 0.5 cc of 1:5 serum and 1.0 cc of complement (2 full units); also a hemolytic system control carrying 1.0 cc of salt solution and 1.0 cc of complement (2 full units).

(9) Shake the tubes gently and place in the refrigerator at 6° to 8° C. for 15 to 18 hours, followed by water bath at 37° C. for 10 minutes.

(10) Add 0.5 cc of amboceptor (2 units) and 0.5 cc of a 2 percent suspension of sheep cells to all 30 tubes and to the control tubes.

(11) Mix thoroughly and place in water bath at 37° C. for 1 hour and make readings. The serum and hemolytic controls should show complete hemolysis.

(12) Chart the results as per the following example observed with a strongly positive serum:

Serum (in 0.5 cc)	Antigen dilutions (in 0.5-cc dose)					
	1:80	1:160	1:320	1:640	1:1,280	1:2,560
0.005	−	−	++	−	−	−
0.0125	−	+	++++	++++	++	+
0.025	+	++++	++++	++++	++++	+
0.05	+++	++++	++++	++++	++++	++
0.1	++++	++++	++++	++++	++++	+++

(13) The dose of antigen to employ in the final test is the largest amount giving a 4 plus reaction with the smallest amount of serum. If three dilutions of antigen give 4 plus reactions with the smallest amount of serum, the dose is midway between the highest and the lowest.

f. Procedure for test.—(1) Having ascertained the exact amounts of the reagents to be used by the above methods, set up the two-tube Kolmer test on the various blood sera for diagnosis as indicated in the following table:

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Tube No.	Patient's serum (cc)	Antigen (cc)		Complement (2 full units) (cc)		Amboceptor (2 units) (cc)	Sheep cells (2 percent) (cc)	
1	0. 2	0. 5	Ten minutes at room temperature.	1. 0	Refrigerator at 6° to 8° C. 15 to 18 hours, then 10 to 15 minutes in water bath at 37° C.	0. 5	0. 5	Water bath at 37° C. for 1 hour, then read.
2	. 2	None (0.5 cc saline)		1. 0		. 5	. 5	

(2) Tube No. 1 is the test tube; No. 2 tube is the serum control tube and should show complete hemolysis. If tube No. 2 shows any residual cells, the serum is anticomplementary and should be so reported.

(3) An antigen, an amboceptor, and a sheep-cell control should be set up with each lot of sera tested, as follows:

Tube No.	Saline (cc)	Antigen (cc)		Complement (2 units) (cc)		Amboceptor (2 units) (cc)	Sheep cells (2 percent) (cc)		
1	0. 5	0. 5	Wait 10 minutes at room temperature.	1. 0	Incubate in refrigerator at 6° to 8° C, for 15 to 18 hours, then 10 to 15 minutes in water bath at 37° C.	0. 5	0. 5	Incubate in water bath at 37° C. for 1 hour.	Antigen control should show complete hemolysis.
2	1. 0	None		1. 0		. 5	. 5		Amboceptor control should show complete hemolysis.
3	2. 5	None		None		None	. 5		Sheep-cell control should show no hemolysis.

(4) A control, consisting of a known positive and a known negative serum should also accompany each lot of sera tested.

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(5) As in the Craig-Wassermann test, the readings are made according to the amount of cells remaining in tube No. 1 for each serum tested. The readings are best made by plus signs, thus:

++++=no hemolysis
+++=25 percent hemolysis } Reported as "Positive."
++=50 percent hemolysis
+=75 percent hemolysis } Reported as "Doubtful."
=100 percent hemolysis—Reported as "Negative."

413. Spinal fluid complement fixation test.—*a.* These are usually tested without any preliminary preparation as they do not contain enough complement to require inactivation by heating at 56° C. If a specimen contains considerable blood which has not had time to settle out, it should be centrifuged.

b. The following table shows the set-up for complement fixation tests on spinal fluids for syphilis:

Tube No.	Spinal fluids	Antigen		Complement (2 units)		Amboceptor (2 units)	Sheep cells (2 percent)	
	cc	cc	Wait 10 minutes room temperature	cc	Incubate in refrigerator at 6° to 8° C. for 15 to 18 hours, then 10 to 15 minutes in water bath at 37° C.	cc	cc	Incubate in water bath at 37° C. for 1 hour.
1	0.5	0.5		1.0		0.5	0.5	
2	.5	none		1.0		.5	.5	

c. Tube No. 2 is the control tube and should show complete hemolysis. The antigen, amboceptor, and sheep-cell control should be run with each lot, the same as for blood serum.

d. A control, consisting of a known positive and a known negative fluid should also accompany each lot of fluids tested.

414. Colloidal gold test.—*a. General.*—This solution, made by the methods in former use, was unreliable in both sensitivity and permanence and was very difficult to make. It could not be made

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successfully in amounts less than 1 liter. The following method is short, simple, reliable, adequately sensitive, very permanent, and needs no titration. It can be made in any amount from 100 cc to 1 liter.

b. Preparation of solution.—(1) *Glassware etc.*

Erlenmeyer flasks, one 2,000 cc and one 250 or 500 cc.
Graduates, one 1,000 cc and one 50 cc.
Pipettes, 10 cc and 1 cc, several of each.
Thermometer, 100° C., one.
Beakers, 250 cc, several.
Four-burner Bunsen, one.

The glassware should be chemically clean and should be rinsed inside and out with double-distilled water before using. Pyrex glass is not essential except in the flask used for boiling. Quicker heating without wire gauze may thus be attained and is important. It is also better to store the completed solution in pyrex but not absolutely necessary.

(2) *Reagents.*—(*a*) Double-distilled water.

(*b*) One percent solution of gold chloride.

NOTE.—Merck's chloride, which should be used, comes in 15-grain ampoules, therefore the contents of one ampoule dissolved in 97.2 cc of double-distilled water makes a 1 percent solution.

Be sure to soak off the label and all paste in warm water and rinse thoroughly in double-distilled water before breaking. Take care that none of the chloride is lost in breaking. Place the halves of the ampoule with their contents in beaker containing 97.2 cc of double-distilled water and stir thoroughly to insure complete and even solution of the chloride.

(*c*) One percent solution of sodium citrate, CP, in double-distilled water. It will be best to make not less than 500 cc to insure accuracy.

(*d*) Hydrogen peroxide, CP (10 volumes, 3 percent). This should be a freshly opened bottle. *Never* use one that has been standing uncorked at room temperature for any length of time.

(3) *Procedure.*—(*a*) Pipette 10 cc of the gold chloride solution into 950 cc of double-distilled water in the 2,000-cc Erlenmeyer flask.

(*b*) Heat as rapidly as possible to between 90° and 95° C. *No higher and no lower.*

(*c*) Remove thermometer from flask as soon as it records 92°.

(*d*) Without removal from the flame, add 50 cc of the citrate solution.

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(*e*) As soon as the solution comes to a boil again add quickly 0.77 cc of hydrogen peroxide (or 10 drops from a standard pipette). When drawing up the peroxide into the pipette take care to exclude the many small bubbles that form in a freshly opened bottle.

(*f*) Remove from flame, cool, and store in a dark place at room temperature. Cork tightly with a tinfoil covered stopper.

(*g*) *Do not shake the flask during procedure.*

(*h*) This method takes about 10 minutes when a hot flame is used.

(*i*) When the solution begins to reheat after the addition of the citrate, a slight bluish tinge begins to appear which gradually changes to the standard color during 3 minutes of boiling. No further change takes place thereafter. This was the original procedure but does not give as sensitive a product as is desirable. The addition of the peroxide, with its immediate color change, gives the required sensitivity. The solution must not be boiled after the peroxide is added. No saline, edestine, or pH titration is necessary if the final color is right.

(*j*) The standard color shows no trace of a bluish tinge. It may be best described as a brownish-yellow shade of burgundy red.

c. Method for making colloidal gold test.—(1) Place 10 chemically clean test tubes in a rack.

(2) In the first tube place 0.9 cc of a 0.4 percent salt solution and in each of the remaining 9 tubes place 0.5-cc amounts of salt solution.

(3) To the first tube add 0.1 cc of the spinal fluid to be tested and mix thoroughly.

(4) Remove 0.5 cc of the mixture from tube No. 1 and place in the second tube, thoroughly mixing as before.

(5) Remove 0.5 cc from tube No. 2 and carry to the third; continue this transfer until the tenth tube is reached; from the latter discard 0.5 cc after mixing.

(6) Add to each tube 2.5 cc of the gold chloride solution.

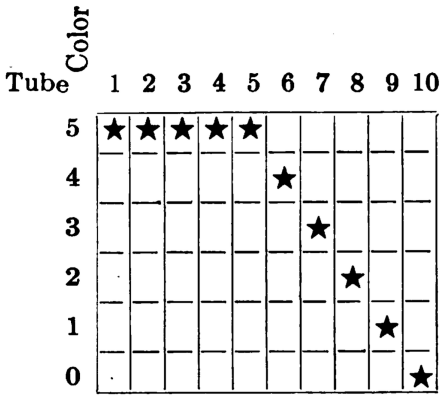
(7) Mix thoroughly by rotation of the tubes and set aside at room temperature for 24 hours. Read results.

d. Readings.—The readings depend upon any change of color which takes place in the tubes after standing 24 hours. For convenience, these color changes are reported by number, thus:

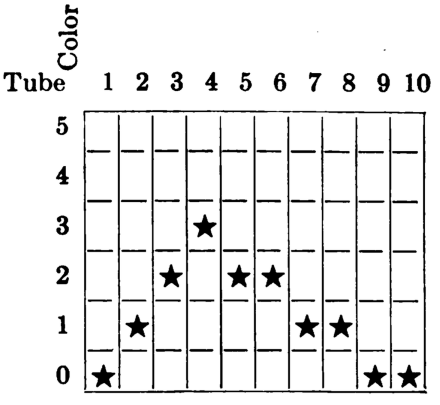
No.
0, no color change.
1, slight violet tinge.
2, bluish red.
3, blue.
4, light, transparent pink or blue (almost decolorized).
5, complete decolorization.

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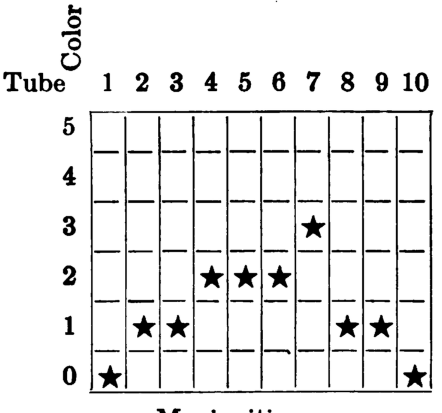
e. Recording.—The recording is made as follows:



Paretic curve.



Luetic curve.



Meningitic curve.

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CHAPTER 12

PROTOZOOLOGICAL METHODS

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SECTION I

PROTOZOA

	Paragraph
General	415

415. General.—*a.* The protozoa are very small animals, consisting of a single cell. Due to their small size they are always studied under a microscope. They are important to the medical soldier because some species of protozoa inhabit various parts of the human body and cause disease. Amoebic dysentery, malaria, and African sleeping sickness are just a few of the diseases caused by these organisms.

b. The protozoa are in general larger than bacteria and their internal structure is more easily seen. This internal structure is usually diagnostic of the species. They are grown only with difficulty on artificial culture media and are usually crowded out by bacteria when these latter forms contaminate the cultures.

c. Although protozoal diseases are more widespread in tropical countries, they are also common in temperate zones. They are of utmost importance to the medical soldier because of their epidemic tendencies, and the fact that a large number of military personnel may have served, or are now serving, in areas where protozoal infections are common. Many of these men may develop chronic cases of disease, and in this "carrier" state serve to infect many others.

SECTION II

INTESTINAL PROTOZOA

	Paragraph
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	Paragraph
Method of staining protozoa in bulk	421
Culture methods	422
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Special methods used in diagnosis of amoebae	424
Confusing objects	425

416. General.—*a. Amoebae.*—Included among the protozoal parasites of man are the amoebae, which resemble each other in moving about by "false feet" (pseudopodia). Of the half dozen species inhabiting the intestinal tract of man, only one, *Endamoeba histolytica*, has been proven to be pathogenic.

b. Endamoeba histolytica.—Although amebiasis (infection with *E. histolytica*) is more commonly found in tropical and near tropical regions, it has reached epidemic proportions in temperate climates (Chicago World's Fair, 1933), and is world-wide in distribution. It is estimated that in the United States alone 6 to 12 millions of persons harbor this parasite, an incidence of 5 to 10 percent.

Accurate diagnosis of *E. histolytica* is absolutely essential, because of the seriousness of the disease. The erroneous diagnosis of one of the harmless amoebae as *E. histolytica* would subject the patient to a needless long and severe treatment. The other intestinal amoeba most commonly confused with *E. histolytica* is *E. coli*. Table XVI and figure 21 will enable the laboratory worker to differentiate the several intestinal species.

c. Other intestinal protozoa.—In addition to the amoebae mentioned above, other intestinal protozoa may occasionally be encountered. Since these forms are either harmless or only rarely found, they are mentioned only in passing. For the most part these organisms are sufficiently different in appearance as not to be confused with *E. histolytica*. For details regarding these forms standard texts are available.

Among the intestinal flagellates are *Chilomastix mesnili*, *Trichomonas hominis*, and *Giardia lamblia*. Some investigators are of the opinion that these species, particularly the latter two, are pathogenic. Until any of these flagellates have been definitely proven to be the cause of disease however, it is better to regard them as harmless.

Balantidium coli is a pathogenic ciliate inhabiting the intestine of man. It produces a dysenteric condition very similar to that caused by *E. histolytica*. However, since human infection with *B. coli* is encountered only very uncommonly, this parasite will not be discussed further here.

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FIGURE 21.—Amoebae (trophozoites, precysts, and cysts). Note.—*A, B, C, D,* trophozoites of *Endamoeba histolytica, Endamoeba coli, Iodamoeba butschlii,* and *Endolimax nana,* respectively; *E, F, G,* precysts of *E. histolytica, E. coli,* and *I. butschlii; H,* trophozoite of *Dientamoeba fragilis,* an amoeba that does not encyst; *I, J. K,* cysts of *E. histolytica, E. coli,* and *I. butschlii; L,* trophozoite of *Endamoeba gingivalis,* an amoeba of the mouth cavity that may confuse the picture by patients expectorating into bedpans.

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TABLE XVI.—*Characteristics of common amoebae of man*

LIVING TROPHOZOITES

	Endamoeba histolytica	*Endamoeba coli*	*Endolimax nana*	*Iodamoeba butschlii*
Motion	Active progression in a definite direction. Form is elongated in motion.	Most strains are not actively progressive but merely change in form.	Some are like *E. histolytica*, but smaller. The majority do not move progressively.	Many strains in culture like that of *E. histolytica*. The majority, however, are like *E. coli*.
Pseudopodia	Finger-like with smooth outline when not in progressive motion. Ectoplasm is clear, glass-like, and easily discernible. When in progressive motion, the ectoplasm may not be clearly differentiated. One-third to one-quarter of the parasite is ectoplasm.	Usually blunt, but it may be like *E. histolytica*. The ectoplasm is usually not clearly differentiated. One-quarter to one-fifth of the parasite is ectoplasm but it is often poorly differentiated from the endoplasm even when the amoeba is in motion.	Like *E. histolytica*. One-half to one-third of the parasite is ectoplasm and is easily differentiated.	Like *E. histolytica* or very broad with coarsely indented outline. One-half to one-third of the parasite is ectoplasm and is easily differentiated.
Color	Faint green	Gray	Gray	Faint green.
Visibility of nucleus (oil-immersion lens).	Usually difficult to visualize except when the nucleus passes into the pseudopodia and is contrasted against the clear ectoplasm.	Quite clear. It is much more readily seen than that of *E. histolytica*.	The karyosome may be defined with ease.	The karyosome may be defined with ease.
Endoplasmic inclusions of diagnostic significance.	Red blood cells are typical and diagnostic. Degenerated and culture forms contain bacteria.	A voracious feeder but usually does not ingest red blood cells. Bacteria, starch grains, and yeast are the principal inclusions.	Bacteria	Bacteria.

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TABLE XVI.—*Characteristics of common amoebae of man*—Continued

STAINED TROPHOZOITES

	Endamoeba histolytica	*Endamoeba coli*	*Endolimax nana*	*Iodamoeba butchlii*
Average size	20 to 25 μ	20 to 30 μ	6 to 10 μ	9 to 13 μ.
Nuclear membrane (stains faintly or not at all).	Lined with minute, fairly even-sized grains of chromatin which stain deeply.	Lined with coarse irregularly sized grains or bars of chromatin which stain deeply.	Chromatin on nuclear membrane in thin line and stains poorly.	A few poorly staining, widely separated, chromatin grains on nuclear membrane.
Karyosome	Short rod or globule of small diameter, centrally suspended within the nucleus. Regular outline. Stains deeply and uniformly.	Short rod or ball or irregular outline, usually eccentric. Diameter greater than that of E. *histolytica*. Stains deeply and uniformly.	Very large, central or eccentric, composed of 1, 2, or more deeply staining masses in a lighter staining matrix. Outline often irregular and oblong.	Similar to that of *E. nana* but larger and more apt to contain a poorly staining central portion. Causes the nucleus to appear like an eye with a widely dilated pupil.
Linin network (stains faintly or not at all).	Contains no chromatin grains between the karyosome and nuclear membrane.	Sometimes contains grains of chromatin. Region just without karyosome halo often appears cloudy after staining.	It is not often discernible. Consists of a few short lines from the karyosome halo to the nuclear membrane. (Karyosome usually the only structure visible in the nucleus.)	Like a web when defined by an excellent stain.

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CYSTIC STAGE

Average size	7 to 15 μ	10 to 30 μ	5 to 15 μ	7 to 15 μ.
Nuclei number	1 to 4, rarely more. Mature cysts contain 4 nuclei.	1 to 8, rarely more. Mature cysts contain 8 nuclei.	1 to 4, rarely more. Mature cysts contain 4 nuclei.	Usually 1. Rarely 2.
Visibility of nuclei in the unstained living state.	Poor but discernible with the oil-immersion lens.	Good	Poor	Good.
Shape	Generally spherical or nearly so.	Generally longer than broad and one side may be less curved than the other.	Irregularity of shape is common. Generally oval.	Great irregularity of shape and outline is common.
Reserve food inclusions. (These disappear in old specimens and are not constant in young cysts.)	Bar-shaped chromatoid bodies in 0 to 90 percent of cysts. Sometimes a small amount of glycogen is present in young cysts. It is diffuse and stains a light brown with iodine.	Acicular chromatoid bodies present in about 10 percent of cysts. A large amount of glycogen may be present and push the nucleus against the cyst wall.	Small granules or masses of volutin and glycogen may be present. Neither is characteristic.	Masses, grains, or rods of volutin may be present but these are not characteristic. The glycogen, almost invariably present, is large in amount, smoothly outlined, and stains a deep brown with iodine. This iodine body is characteristic and diagnostic.

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417. Collection of specimens.—*a.* The specimen should be collected directly in clean, covered receptacles (bedpans, swabs in test tubes containing ½ cc of physiological saline, syringes, bottles, or droppers) preferably sterilized by heat. These receptacles should not be sterilized by chemical disinfectants as protozoa in the vegetative stage are easily killed and quickly undergo autolysis in the presence of only small amounts of such chemicals. If the receptacles are not properly cleaned and sterilized, there is always the possibility of introducing free-living protozoa into the specimen and thus confusing the picture. All specimens should be examined at the earliest possible opportunity after collection. The protozoa degenerate rapidly, and the possibility of an accurate diagnosis diminishes as the time between collection and examination of the specimen increases.

b. If the pathological lesions are in the rectum or sigmoid, specimens may be obtained by the physician by means of the proctoscope or the sigmoidoscope and sent to the laboratory for examination. These are more apt to yield protozoa than the passed feces. However, because of the attending discomfort and perhaps pain to the patient, this method should be used only after it has been demonstrated that the feces are negative.

c. It is practically impossible to find protozoa in a stool after an oil cathartic or following a barium meal. Specimens collected by means of an enema are also unsatisfactory. Therefore, in the event any of the above has been used, examination for protozoa in the feces should be delayed for at least 72 hours.

d. The portions of a stool most likely to contain parasitic protozoa are those showing blood or mucus. In formed stools, small flecks of mucus or mucus and blood can always be found on the surface of the specimen. In semiformed or liquid stools, if the specimen is examined carefully, mucus and blood also can be found.

e. Formed stools usually contain only cysts or precysts of protozoa. Semiformed and liquid stools will ordinarily contain only vegetative forms, and it is these types of stools that best afford an opportunity to diagnose a protozoan infection. If the patient is not already passing such a stool, and it is necessary to rule out the possibility of a parasitic protozoan infection, give a saline cathartic and collect the second or third liquid stool passed for examination.

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f. In patients infected with intestinal protozoa, the number of organisms present on an infected surface at any given time depends upon the type of pathology produced, the bacterial flora present, and the resistance of the host. Because of this multiplicity of governing factors, protozoa usually appear in showers; they may be present in great numbers one day and relatively scarce the next. Therefore, to arrive at a satisfactory diagnosis in questionable cases, repeated examinations should be made on at least 3 consecutive days.

418. Examination of fresh material.—These specimens should be kept in the original receptacle used for collection until examined. The material should be kept at a temperature of 37° C. as all vegetative organisms in this group are quite sensitive to chilling and are also rapidly killed by temperatures of 45° C. or higher. Since drying also affects these organisms the specimen should have its original moisture content when presented for examination.

a. Fecal smear.—The general procedure for examination of dysenteric, diarrheic, or mushy stools, where the vegetative forms (trophozoites) are most likely to be found, follows: Warm a clean slide so that it feels comfortable when touched to the back of the hand. Then secure a small piece of mucus or mucus and blood from the fresh specimen by means of a wire loop or wooden applicator and thoroughly emulsify it in 1 drop of physiological saline on the middle of the slide. Now take a clean No. 1 cover slip between the thumb and forefinger of the right hand, contact the slide with one edge of the cover slip near the drop but not touching it, push the slip along the surface of the slide until its edge contacts the drop, rock it slightly from side to side to allow a portion of the fluid to come under the edge of the slip, and then let the cover slip drop from between the fingers allowing it to fall on the side. The fluid portion of the drop on the slide will then automatically be drawn by capillary attraction under the slip, while the solid particles will be excluded. This method insures a thin, even preparation of not too great a density (when newsprint is viewed through it the words are legible) and insures even apposition of the cover slip to the slide. The preparation is now ready for examination, but if it is to be kept on a warm stage for any period of time it should be ringed with vaseline.

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b. Zinc sulfate centrifugal flotation technic.—In formed or semiformed stools, where cysts are more likely to be found, the zinc sulfate centrifugal flotation technic is very valuable. Ordinary fecal smears should also be made, however, since they give some indication of the intensity of the infection, the zinc sulfate method having its greatest value in enabling the worker to discover light infections.

(1) Prepare a fecal suspension by emulsifying 1 part stool specimen (about the size of a pecan) in 10 parts lukewarm water.

(2) Strain 10 cc of this emulsion through two layers of wet cheesecloth into a Wassermann tube.

(3) Centrifugalize for 45 to 60 seconds at approximately 2,500 rpm, pour off the supernatant fluid, and add 2 to 3 cc of water. Then break up the sediment and repeat the above, centrifugalizing and discarding the supernatant fluid three or four times.

(4) After pouring off the last supernatant fluid add 3 to 4 cc zinc sulfate (33 percent solution), break up the packed sediment, and add enough zinc sulfate solution to fill the tube to about ½ inch from the rim.

(5) Centrifugalize the tube for 45 to 60 seconds at top speed.

(6) Remove several loopfuls of the material floating on the top surface film to a clean slide. Add 1 drop of iodine stain and a cover slip.

c. Iodine stain.—This is a temporary stain used for quick diagnosis on fresh material, and must be made up fresh every 10 days or it will not stain properly. It is prepared by mixing the following:

Iodine	2 gm
Potassium iodide	4 gm
Distilled water	100 cc

After finding trophozoites or cysts and studying them in the fresh, wet, unstained mount, the nuclear details can be clearly visualized and studied microscopically if the cover slip is raised at one edge and a drop of the iodine stain is thoroughly mixed with the contents under the slip.

d. Culture.—Culture specimens are prepared for microscopic examination exactly as fecal smears above except that because of their fluid nature they are not mixed with physiological saline prior to applying the cover slip.

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419. Special staining.—*a.* The specimen should be fresh, and the protozoa should not show evidence of degeneration. If the specimen is old and the protozoa are degenerated, good fixation and staining will be impossible. The best fixative for protozoa in general laboratory use (Schaudinn's solution) is prepared as follows:

Mercuric chloride, saturated solution in physiological saline	65 cc
Ethyl alcohol, 95 percent	35 cc
Glacial acetic acid	5 cc

The glacial acetic acid and the ethyl alcohol should be added to the mercuric chloride solution just before use.

b. The general procedure in fixing protozoan specimens follows: If the material to be fixed is not in the liquid state, mix enough physiological saline with it to form a thin, watery solution. Now rub well onto one-half of the surface of a clean slide 1 small drop of fresh egg white or normal horse serum, then put 1 large drop of the specimen on this surface and spread it evenly. Allow the preparation to dry only until the fluid portion of the specimen will no longer run when the slide is tilted (the film will still be moist), then drop the slide, filmside down, into 50 cc of the above fixing solution contained in a Petri dish. After 10 minutes turn the slide over or place it in a Coplin jar three-fourths full of the same fixative and allow it to remain for 1 to 2 hours.

c. Slide preparations for staining these organisms *amoeba trophozoites* are made in the same way except that as soon as the drop of fecal material is placed on the slide a cover slip is dropped on it. The preparation is allowed to set for 3 minutes and then placed in a Coplin jar. The fixative is *then* carefully added and is allowed to act for 2 hours. After fixation the cover slips are removed from each specimen and the slides are then ready to be carried through the routine of staining. This method of putting the cover slip on the preparation makes it possible to inspect the slide for amoebae prior to fixation and in addition is a great help in keeping the amoebae on the slide during fixation.

After fixation, the preparations should be rinsed in tap water (i. e., water in another Coplin jar, not running water), and then placed in 70 percent ethyl alcohol. They may then either be transferred to 95 percent alcohol for preservation, or stained immediately.

d. The most generally useful staining process for permanent preparations of protozoa is an iron-hematoxylin technic (see par. 420).

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420. Method of staining smears on slides.—*a. Procedure.*—The fixed and washed slide specimens prepared as outlined previously are carried through the successive steps in staining as follows:

(1) Immerse for 10 minutes in 70 percent ethyl alcohol.

(2) Immerse for 10 minutes in 70 percent ethyl alcohol to which has been added sufficient iodine stain to produce a light mahogany color.

(3) Immerse for 10 minutes in 70 percent alcohol.

(4) Mordant in the following solution for 20 minutes:

Iron alum (ferric ammonium sulfate) 4 percent equeous solution	1 part
Ethyl alcohol, 50 percent	10 parts

(5) Immerse for 5 minutes in 70 percent ethyl alcohol.

(6) Stain for 24 hours in Heidenhain's iron hematoxylin:

Hematoxylin	1 gm
Ethyl alcohol, 95 percent	10 cc
Distilled water	90 cc
Thymol	1 crystal

Dissolve the hematoxylin in the alcohol, add the distilled water and thymol, then allow to ripen for 1 month in a clear, glass-stoppered bottle exposed to the sun.

(7) Remove from stain and wash in two changes of tap water.

(8) Destain in the following:

Iron alum, 2 percent aqueous solution	1 part
Ethyl alcohol, 50 percent	10 parts

Differentiate by agitating each slide separately in the above solution until a light gray to blue tinge predominates; control the exact point by observing the staining definition of the organism under the microscope every few minutes.

(9) Rinse in tap water, then wash for 10 minutes each in three separate dishes of 70 percent ethyl alcohol.

(10) Begin dehydration by two changes of 95 percent ethyl alcohol for 5 minutes each.

(11) Complete dehydration by two changes of absolute ethyl alcohol for 10 minutes each.

(12) Replace the absolute alcohol in the specimen by two changes of xylol for 10 minutes each.

(13) Mount in canada balsam.

b. Precautions.—(1) The specimen should not be allowed to dry at any stage in the technic as drying causes the organisms to shrink and become distorted in shape.

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(2) If the destaining agent is not thoroughly washed out of the specimen it will fade the stain.

(3) If the specimen is not properly dehydrated before clearing in xylol, the xylol will become milky and the slide when viewed microscopically after mounting in balsam will appear blurred.

421. Method of staining protozoa in bulk.—In the staining of mucous surface protozoa of man there are almost as many variations in staining technic as there are workers in the field. The chief difficulties encountered in all of the better technics of staining are: first, keeping the organisms on the slide or cover slip during fixation and staining; second, keeping the organisms free from distortion and clear from the debris so that their internal structures are not obscured; third, carrying out proper differentiation of the internal structures of the organisms at the time of destaining; and fourth, having a sufficient number of well-stained organisms on the slide after staining so that a diagnosis will not have to be made on a few more or less atypical organisms. The staining method not only satisfies the above requirements, but also affords a method of concentration, thereby making it possible to secure satisfactory stains from specimens containing very few organisms that could not be stained by other methods. The method of staining differs from other standard hematoxylin staining methods only in that the organisms are not fixed and stained on slides or cover slips, but are fixed and stained in bulk. The organisms are concentrated and then carried through the steps of fixation and staining in 50 cc centrifuge tubes. Very few of the organisms present in the original specimen are lost during fixation, staining, and mounting. The organisms are natural and lifelike and are not distorted by the reagents or manipulations used.

a. Precautions.—(1) There should be no delay in concentration, examination, and fixation of trophozoites after they have been secured from the patient. Degenerated organisms will not take a satisfactory stain.

(2) Destaining must be checked by frequent microscopic observations of the organisms being differentiated. Care should be exercised to carry the destaining to the point that there is sharp differential detail between the nuclear structures and the cytoplasm. The common tendency with this technic is not to carry the destaining far enough. The organisms should be checked for structural detail after the acid destain has been neutralized and if there has been insufficient destaining they should be carried back into acid alcohol and further destained until the desired degree of differentiation has been secured.

(3) The stained material must be completely dehydrated before clearing in xylol.

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(4) Discard the supernatant fluid decanted off after centrifugalizing, in each step of the procedure, or if the alcohols and stains are to be used over again, filter through a Berkefeld N filter as there is danger of carrying organisms in used alcohols and stains to the next case subsequently stained.

(5) The material being stained may be left for 24 to 48 hours additional time in any step of the staining procedure with the exception of the fixing solution and the acid alcohol destaining reagent. This allows the staining procedure to be carried out without interfering with the routine work in a laboratory.

b. Concentration and fixation of fecal specimens.—Thoroughly emulsify 20 cc of feces in 200 cc of warm (37° C.) physiological saline in a settling flask or tall, narrow cylinder; allow to stand for 5 minutes and then decant the supernatant fluid into two 50-cc centrifuge tubes. Centrifugalize the material at approximately 1,850 rpm for 5 minutes, then decant off the supernatant fluid, save the precipitated residue of one tube for fresh examination, and to the tube containing the other precipitate add 25 cc of fresh Schaudinn's fixing solution. Thoroughly mix the precipitate and the fixative and allow the mixture to stand for at least 1 hour, preferably 24 hours.

c. Concentration and fixation of cultures and other liquid specimens.—Pipette the fluid containing the organisms directly into a 50-cc centrifuge tube and then centrifugalize and fix the material as indicated above. (Between each step in the following procedure the material is centrifugalized at 1,850 rpm for 5 minutes, then the supernatant fluid is decanted off and the next solution added to the precipitated residue which is then thoroughly emulsified by rotation or stirring.)

(1) Wash the fixed material two times with distilled water.

(2) Wash 10 minutes with 70 percent ethyl alcohol (containing enough Gram's iodine to give it a light brown color).

(3) Wash 10 minutes with 70 percent ethyl alcohol.

(4) Stain for from 1 to 24 hours by adding Harris' hematoxylin.

Hematoxylin	1 gm
Ethyl alcohol, 95 percent	10 cc
Dissolve the hematoxylin in the alcohol.	
Alum (ammonium or potassium)	20 gm
Distilled water	200 cc

Dissolve the alum in the water by the aid of heat and add the hematoxylin solution. Bring the mixture to a boil as rapidly as possible, and then add one-half gram of yellow oxide of mercury.

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The solution at once assumes a dark purple color. As soon as this occurs, remove the vessel containing the solution from the flame and cool rapidly by plunging into a basin of ice water. As soon as the solution is cool it is ready for use. The addition to the stain of glacial acetic acid to a concentration of 4 percent is supposed to increase the precision of the nuclear staining.

(5) Wash with tap water.

(6) Destain by adding about 20 cc of acid alcohol (1 percent HCl in 70 percent ethyl alcohol) to the stained precipitate in the centrifuge tube. Mix the precipitate and the destaining solution and occasionally stir the mixture with a wooden applicator stick. From time to time check the progress of destaining by taking 1 drop of the mixture, placing it on a slide, applying a cover slip, and then observing the progress of nuclear differentiation of the organisms under the high-dry power of the microscope. The organisms will be fairly easy to find in the average case and destaining should be allowed to go on until the cytoplasm is practically colorless and the nucleus stands out sharp and clear. As soon as the desired definition has been obtained, add sufficient ammonia water (5 drops NH_4OH in 50 cc of distilled water) to neutralize the acid alcohol and turn the solution bright blue.

422. Culture methods.—*a. Media.*—(1) *Boeck-Drbohlav medium.*—This is the medium most generally used for the protozoa found on the mucous surfaces of man. In this medium many of the amoebae and mucous surface flagellates found in man, with the exception of *Giardia lamblia*, will survive and multiply and the individual characteristics are well preserved.

Eggs.

Sterile *Ringer's solution* (autoclaved at 15 pounds pressure for 20 minutes and then allowed to cool) is prepared by mixing:

Sodium chloride	8.0 gm
Potassium chloride	0.2 gm
Calcium chloride	0.2 gm
Distilled water	1,000 cc

Sterile horse or human serum. (Must not contain tricresol or other preservatives.)

Emulsify four whole eggs in 50 cc of the sterile Ringer's solution. Place 4 cc of this mixture in each test tube and sterilize in the autoclave as follows: Close the door and the exhaust valve, open the steam valve, and let the pressure climb to 10 pounds, at which time shut off the steam and (as a caution) allow the pressure to decline

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of its own accord before removing the media. Repeat this procedure on 3 successive days, storing the media at room temperature between sterilizations. Before using add 4 cc of the liquid portion of the medium, consisting of sterile horse serum, 1 part, and Ringer's solution, 8 parts.

(2) *St. John's wheat broth medium.*—This medium is of differential value in the cultivation of amoebae in that only *E. histolytica* can maintain growth in it. However, many strains of *E. histolytica* cannot be cultivated in it. Directions for preparation and use of this medium may be found in Laboratory Methods of the United States Army, 4th edition.

b. Technic.—Protozoa are quite sensitive to changes in the bacterial flora in vitro; they die quickly in the presence of certain bacteria. Therefore, sterile precautions insofar as is possible, should always be used in this technic.

Using an applicator or wire loop, inoculate a portion of the specimen about the size of a pea, consisting of fresh mucus, mucus and blood, or concentrated cysts, into the liquid portion of the medium and thoroughly emulsify it there. Incubate at 37° C. and examine at the end of 24 and 48 hours. Flagellates growing in the medium will be found throughout the liquid portion while amoebae will be found in the very bottom portion of the liquid fraction. The specimen from the culture to be examined for amoebae is obtained by introducing a clean sterile 1-cc pipette, with the index finger held tightly over the upper end, into the culture so that the tip is at the bottom portion of the liquid medium; then gently release the finger pressure allowing only 1/10 cc of the material in contact with the bottom to run into the pipette. Resume the finger pressure on the upper end of the pipette and then withdraw it from the culture. The material in the pipette can then be used to transfer the culture and make fresh, wet, unstained or stained preparations. If it is desired to carry on the positive cultures they should be routinely transferred every 48 hours. However, the survival of these organisms in culture depends a great deal upon the bacterial flora present. A culture in which *Escherichia coli* predominates is usually favorable and one in which spore-bearers, *Pseudomonas aeruginosa* or *Proteus vulgaris*, predominate is usually unfavorable. In the latter case transfers should be made at 24-hour periods.

Cultures for amoebae that are not positive at the end of 48 hours should be transferred and further examined as follows: Allow the

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culture to remain in the incubator for 2 hours, then without unduly disturbing it, remove all but 0.5 cc of the supernatant fluid by means of a sterile pipette equipped with a rubber nipple. Wash the slant with the remaining fluid and then transfer it to new medium. The resulting culture should then be reexamined at 24- and 48-hour intervals before calling it negative.

423. Laboratory animals.—Laboratory animals are of no value as a diagnostic procedure in this group. However, in case of *E. histolytica*, the virulence of this organism can be tested by injecting cultures or freshly isolated trophozoites into the rectum of a young kitten by means of small catheter and syringe. An acute fatal dysentery is usually produced by virulent strains.

424. Special methods used in diagnosis of amoebae.—In the preceding pages technics were described to enable the technician to familiarize himself with laboratory methods of handling intestinal protozoa. Since *E. histolytica* is the intestinal protozoan of most interest to the medical soldier, special methods and aids for confirming diagnosis of infection with this parasite are outlined below.

a. Fresh, unstained, wet mount.—The fresh, unstained, wet mount prepared as outlined under general methods is examined systematically, covering the slide without repeating the microscopic fields. Amoebae in the live state have a particular refractive, translucent, granular character which easily differentiates them from other objects in the specimen. This property can be exaggerated and will greatly aid in picking out the amoebae when the slide is being rapidly examined if the high-power objective of the microscope is focused slightly above the objects in the preparation. If the observer is well trained, amoebae may be recognized under the low-power objective and then a switch made to the high-power objective to determine the detailed characteristics. If the outline below is followed in studying amoebae it will be found to be of great aid in species determination.

(1) Size and color.
(2) Differentiation of ectoplasm and endoplasm.
(3) Granularity of endoplasm and presence of cell inclusions.
(4) Nucleus—visibility, location when in motion, and size.
(5) Motility, type—active, sluggish, progressive or nonprogressive.
(6) Pseudopodia—single or multiple; clear or granular.
(7) Flowing of endoplasm into pseudopod—slow or explosive.
(8) Presence or absence of—red blood cells in endoplasm; bacteria in endoplasm.

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(9) Presence of—contractile vacuoles; food vacuoles.

(10) Presence and characteristics of chromatoid bodies.

(11) Cultural characteristics—growth on R. E. S. and St. John's media on successive transfer.

b. Fresh, iodine-stained, wet mounts.—(1) Nucleus—size, shape, distribution of chromatin and size and uniformity of granules, and location of karyosome.

(2) Presence of glycogen.

(3) Cysts, if present—character of, size, number of nuclei and type, visibility, and glycogen.

c. Smears stained with iron-hematoxylin.—Staining by hematoxylin methods should be made of all amoebae where species cannot be recognized readily in the fresh or iodine-stained preparations.

d. Cultures.—Cultures properly made will increase the number of positive findings and allow further study in determining the true species of an amoeba.

e. Concentration of cysts.—Concentration methods for cysts should be done routinely on all fecal specimens that are examined.

f. Complement-fixation.—At the present time the complement-fixation test is still in the experimental stage in its development. It may become of practical diagnostic significance when a true *E. histolytica* antigen can be developed.

g. Charcot-Leyden crystals.—Charcot-Leyden crystals are indicative of chronic inflammation and are often found in the feces.

h. Differentiation of amoebic and bacillary dysentery.—This can presumptively be done by observing the general character of the fecal exudate. However, one may complicate the other, and amoebic dysentery in the presence of severe bacterial secondary invasion of the ulcers may have many of the characteristics of a bacillary dysentery. Therefore, every effort should be exerted to demonstrate the causative organism in each case. The main differential points are shown in table XVII.

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TABLE XVII.—*Differential diagnosis of fecal exudates in bacillary and amoebic dysentery**

Exudate	Bacillary dysentery	Amoebic dysentery
Blood.	Varying amounts.	Small amounts to actual hemorrhage.
Polymorphoneutrophiles.	About 90 percent of exudate. Many show nuclear degeneration (ringing). Cytoplasm frequently contains fat.	Few. Cytoplasm of some of those present shows degenerative changes and in such the nuclei may appear pyknotic.
Endothelial macrophages.	Present in varying numbers. Actively phagocytic, frequently contain erythrocytes and leucocytes. Undergo toxic degeneration; "ghost cells."	Not seen except in cases also having bacterial dysentery.
Plasma cells.	Present, relatively more abundant early.	Present in small numbers.
Pyknotic bodies.	Proportionately insignificant, but are found.	Constitute about 80 percent of cellular elements.
E. histolytica trophozoites.	Absent unless the two diseases are both present.	Present and must be found to make diagnosis.
Amount of exudate, actual hemorrhage excluded.	Massive; a large part of the stool.	Small.
Bacterial content.	Low.	Very high, usually.

*Callender, G. R., "The Cytological Diagnosis of Dysenteric Conditions and Its Application in the Military Service; the Military Surgeon," June, 1925.

425. Confusing objects.—Tissue cells derived from the host, or ingested as food may at first glance appear as amoebae in the stools, but careful examination of them will easily establish their true nature. Macrophages may be found containing phagocytized red blood cells, but examination reveals their typical nuclear structure and amoeboid movement is not observed. Epithelial cells are pale in color and have nuclear characteristics that easily differentiate them.

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Vegetable cells such as starch granules, pollen granules, yeast cells, or other cells of this type have a certain definiteness of out line and structure that should lead to no confusion. However, yeast cells, such as *Blastocystis hominis*, may be confused with cysts of amoebae. The presence of budding forms and their particular structure should cause no difficulty in differentiating them.

SECTION III

BLOOD AND TISSUE FLAGELLATES

Paragraph
General 426
Leishmanias 427
Trypanosomes 428
Trypanosoma cruzi (Chagas' disease) 429

426. General.—The flagellates found in the blood and tissues of man belong to the genus *Leishmania* and the genus *Trypanosoma*. The organisms in the flagellate group undergo changes in form during certain stages of their life cycles. These stages are generally referred to by the name designated for the form taken by the organism as illustrated by the following chart. Note that the genus *Trypanosoma* may assume any of the forms. The genus *Leishmania* has only two forms.

427. Leishmanias.—There are three generally recognized species of *Leishmania* infecting man, which, although distinct, are identical under the microscope. The results of animal inoculation have been confusing regarding species differentiation. The diseases produced in man, however, are distinct, there being three types of lesions: visceral, cutaneous, and muco-cutaneous.

a. Leishmania donovani (visceral form).—Infection with this parasite is called kala azar, that is "death fever," and is fairly common in the countries bordering the Mediterranean, India, China, and South America. (The Mediterranean type affects children chiefly, but many adults have the infection and it is not uncommon in aged people.) The disease is chronic and is characterized by anemia, loss of weight, and marked enlargement of the spleen. The parasites are found in the endothelial cells of the liver, spleen, lymph nodes, and occasionally in the white blood cells circulating in the blood. It is generally agreed that the parasites are transmitted to man through the bite of an infected fly of the genus *Phlebotomus* (see sec. II, ch. 14), but there is still no definite proof that any insect is the transmitting agent.

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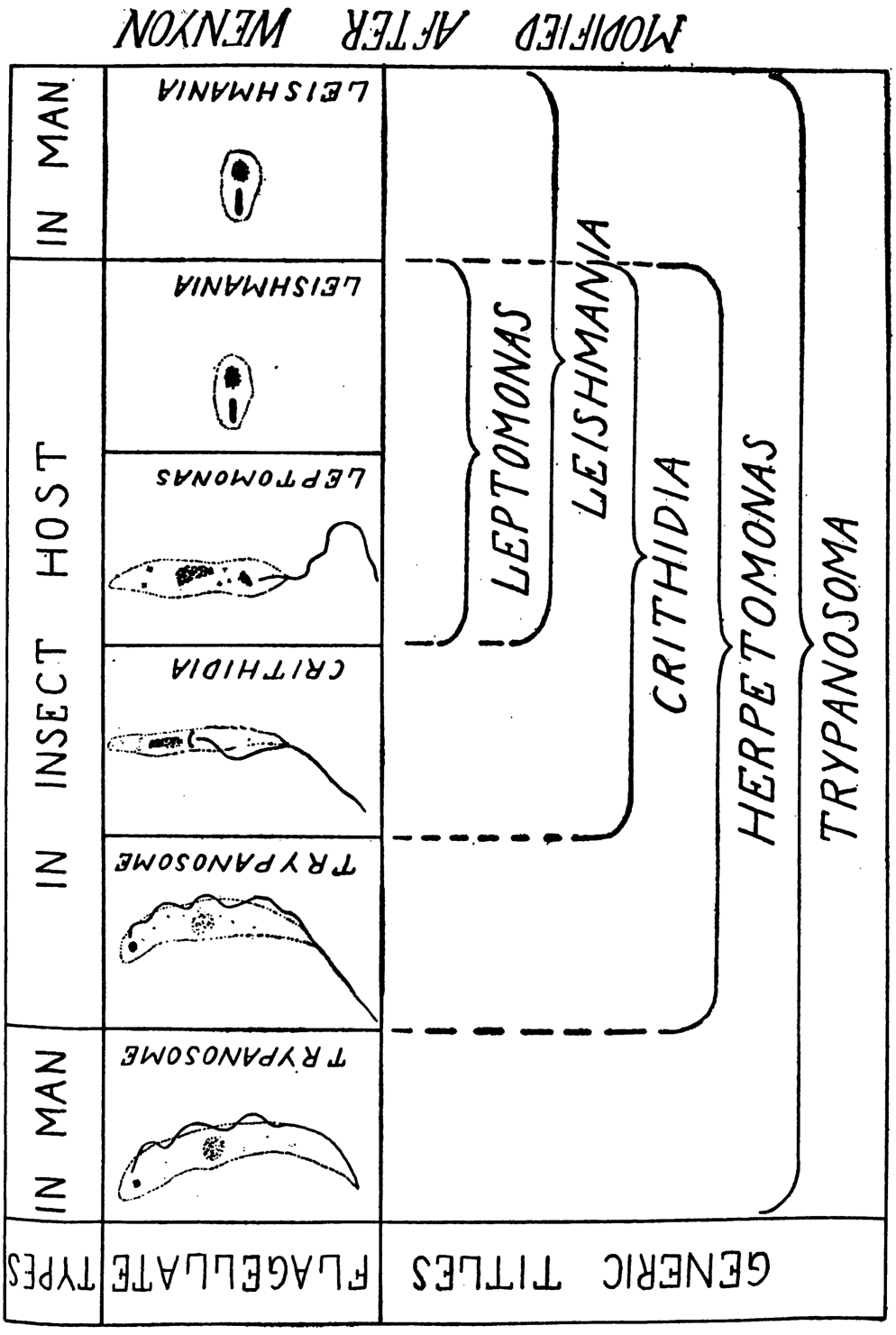


FIGURE 22.—Classification of *Trypanosomes* and *Leishmanias*.

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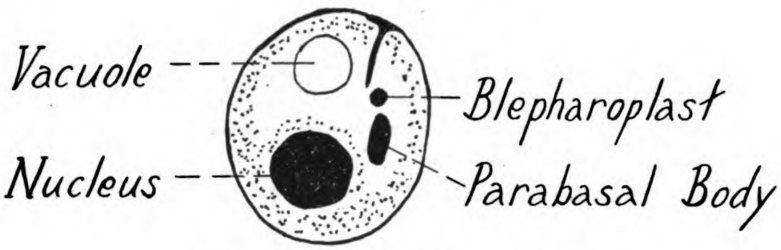


FIGURE 23.—Diagram of a *Leishmania*.

(1) *Morphology.*—In the tissues this species is a very small (1 to 3 microns) round or oval body, with a sharp outline and poorly staining cytoplasm. With Wright's stain the cytoplasm stains a pale blue, the nucleus appearing as several bright red granules. The parabasal body stains deep purple and is the only other definite structure clearly visible in the parasite in man (see fig. 23).

(2) *Diagnostic methods.*—The laboratory methods of diagnosis are, in order of reliability: culture of material obtained from spleen or liver puncture, direct examination of this material, the aldehyde test, and the direct examination of the blood. Complement-fixation and the precipitin tests have been used by certain workers with some degree of success.

(*a*) *Culture.*—The NNN (triple N) medium is ordinarily successful. This medium is made of—

Agar	14 gm
Sodium chloride	6 gm
Distilled water	900 cc

Mix and dissolve by means of heat, then tube in 6-cc amounts and autoclave for 30 minutes under 20 pounds of steam pressure. Remove the tubes and cool to 48° C. Then under aseptic conditions add 2 cc of sterile defibrinated rabbit's blood to each tube, mix well, and slant. Slanted tubes should be placed in the ice box to cool and harden so they will have the maximum amount of water of condensation. When cool, the cotton plugs of each tube should be covered with a rubber cap to prevent evaporation of the water. The tubes should be tested for sterility by incubation for 24 hours before they are used, because trypanosomes will not grow in the presence of bacterial contamination. The material suspected of containing *Leishmania* is planted at the bottom of the slants in the water of condensation and incubated at 22° to 25° C. for 3 to 14 days.

(*b*) *Spleen and liver puncture.*—This is a procedure that should be done only by a competent medical officer due to the danger of

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tearing the organ and causing uncontrollable hemorrhage. The material obtained is spread on a slide in as thin a layer as possible and stained with Wright's or Geimsa's stain. Examination is microscopic.

(*c*) *Aldehyde test.*—To 1 cc of clear serum obtained from the blood of a suspected case add 2 drops of 100 percent formalin (that is, 40-percent formaldehyde solution). If the infection is kala azar the serum will jell within a few seconds to 30 minutes. While this test may also be positive in malaria, tuberculosis, and leprosy, it is nevertheless of value.

(*d*) *Blood.*—*Leishmania* are rarely recovered in the circulating blood. When present they are found in mononuclear and polymorphonuclear leucocytes.

b. Leishmania tropica (*cutaneous form*).—Infection with this parasite, which is common in the Mediterranean area and in Africa, is known as oriental sore, Delhi boil, and bubas. The organisms are found in nodules just under the skin and in the *margins* of ulcers, and may be obtained by puncture and aspiration or by scraping the margins of the ulcers. Material so obtained is spread on a slide and stained and examined as for *L. donovani.* Since the organisms are very scarce, much patience must be used in the search. The parasite may be cultivated in the NNN medium, but usually there will be too many bacteria present.

c. Leishmania brasiliensis (*muco-cutaneous form*).—This organism produces muco-cutaneous leishmaniasis or espundia of Central and South America. Its morphology is the same as the other species described above. The above-mentioned NNN medium is again used in culturing this parasite. The method of transmission is unknown although it is believed to be by the sandfly *Phlebotomus intermedius* which is common in regions where the disease is found.

428. Trypanosomes.—*a. General.*—Trypanosomes are protozoa of a somewhat elongated shape, and tapering at both ends. Along the convex margin is an undulating membrane, to which is attached the flagellum. The flagellum arises from a small granule, the blepharoplast near the back end of the organism. It then passes along the free margin of the undulating membrane and may emerge at the anterior end of the organism as a free flagellum of variable length. Just posterior to the blepharoplast there is usually a dark, shining, round or rod-like body known as the parabasal body. The nucleus of the organism is round or oval and centrally located. The cytoplasm is clear but may contain granules which stain red with Giemsa's stain. Figure 24 shows the internal structure of a trypanosome.

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TABLE XVIII.—*Trypanosomes of medical importance*

Species	Length	Definitive host	Intermediate host	Geographical distribution	Susceptible animals	Culture	Disease
*T. gambiense*___	15μ to 30μ	Man, domestic animals	Tsetse fly, *glossina palpalis.*	Tropical Africa, but mostly western and central.	All laboratory animals except monkey.	NNN plus glucose.	Gambian sleeping sickness.
*T. rhodesiense*__	12μ to 35μ	Man, big game animals, antelope.	Tsetse fly, *G. morsitans.*	Eastern Africa, eastern Rhodesia, etc.	All laboratory animals except monkey.	NNN plus glucose.	Rhodesian sleeping sickness.
*T. cruzi*_____	20μ	Man, cat, armadillo, opossum, wood rats, bats.	Kissing bug, *Panstrongylus megistus.*	South and Central America. Organism found in reservoir hosts in United States.	Guinea pigs, white rats, monkey.	NNN_____	Chagas' disease.

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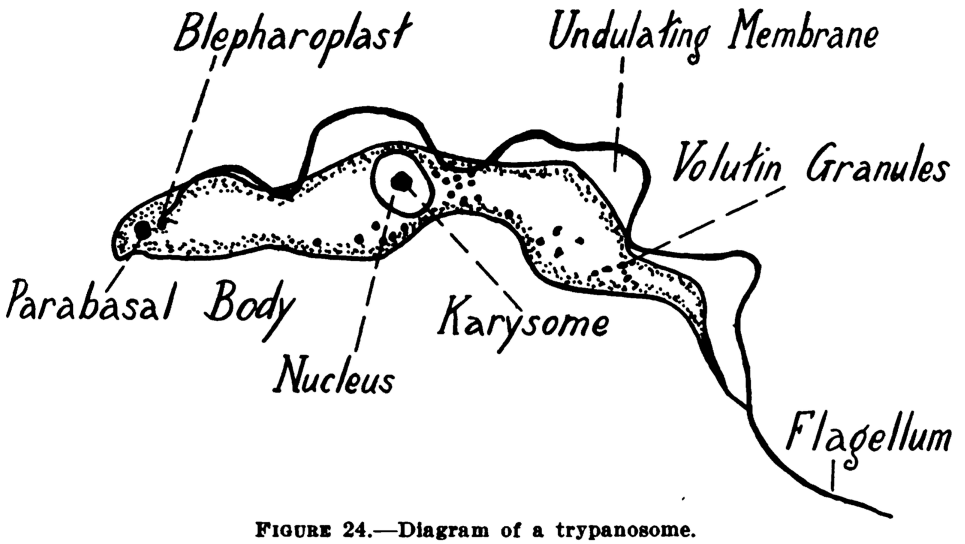


FIGURE 24.—Diagram of a trypanosome.

b. Species of medical interest.—The trypanosomes of medical interest are:

(1) *Trypanosoma gambiense* and *T. rhodesiense*, the causative organisms of African sleeping sickness. For discussion of these the interested worker is referred to standard texts.

(2) *Trypanosoma cruzi*, the causative organism of Chagas' disease. This parasite is discussed in detail in paragraph 429.

(3) *Trypanosoma lewisi*, a trypanosome of rats, which though not important medically, is of interest since it may be confused with *T. cruzi*. Rats are used in the laboratory in animal inoculation methods of diagnosis of Chagas' disease, and since *T. lewisi* and *T. cruzi* are very similar in appearance, the rats used in diagnosis must be free from *T. lewisi*.

429. Trypanosoma cruzi (Chagas' disease).—This is a fairly common trypanosome infecting man in tropical and subtropical South and Central America. Although this is primarily a childhood disease, it is sometimes found in adults.

a. Life cycle.—In man *T. cruzi* as seen in the peripheral blood is a trypanosome about 20 microns in length. Its body tends to be shaped like the letter "C" and the posterior end is sharply pointed. The parabasal body is oval in shape and very conspicuous. There is a free flagellum. The nucleus is oval in shape and centrally located. There may be some variations in the width of the body, as the young forms are narrower than the old. After a variable period in the blood stream, the mature trypanosome invades the muscle fibers or other tissue cells, assumes the leishmania form and multiplies by splitting lengthwise. This multiplication of the leish-

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mania stage continues until the cell ruptures from so many parasites. Each then develops a short flagellum, the body lengthens, the parabasal body moves posteriorly, and the young trypanosome now returns to the blood stream or invades other tissue cells. There is considerable local tissue damage and destruction due to the invasion of tissue cells by this parasite. In the triatomid bug (see sec. II, ch. 14) about 6 hours after the bug has fed upon an infected individual the ingested trypanosomes round up and assume the leishmania form. These forms multiply rapidly as they pass along the intestinal tract. In the hind-gut they again develop flagella and in about 25 to 30 hours become crithidial forms (see fig. 22). These forms multiply in the hind-gut and are found in greatest numbers near its junction with the kidney tubules. The crithidial forms now develop into trypanosome forms which are infective for man. When the bug feeds again, it deposits a small drop of fluid from the rectum upon the skin of the individual. This fluid excrement may contain great numbers of trypanosomes which are capable of passing through intact mucous membrane or small skin abrasions into the blood stream, thus infecting the individual. Persons may inoculate themselves by accidentally rubbing the deposited feces into the feeding site.

b. Diagnostic methods.—(1) *Collection of specimens.*—The most favorable time to find these organisms in the suspected infection is during the periods of fever. In most cases of trypanosomiasis there are usually very few organisms present in the blood stream. It is necessary, therefore, to use concentration methods as well as thick blood films. A suitable concentration method follows: Using sterile technic, 10 cc of blood are withdrawn from a vein and immediately mixed with 40 cc of sterile, warm (37° C.) distilled water in a 50-cc sterile centrifuge tube. The tube is shaken and as soon as hemolysis is complete it is centrifuged at approximately 2,500 rpm for 5 minutes. The supernatant fluid is decanted and the trypanosomes present will be found in the residue, which can then be examined microscopically. If the preparation has been kept sterile it may be used to inoculate culture media. Lymph-gland puncture is the method most apt to give positive results. One of the enlarged lymph glands is punctured aseptically with a Luer syringe equipped with a 19-gage needle and some of the gland juice is aspirated. This aspirated material may then be examined under the microscope, cultured, or inoculated into a susceptible animal.

(2) *Slide preparations.*—Fresh, unstained, wet mounts and thin

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and thick stained films can be prepared as outlined in the section on malaria. Parasites are not apt to be numerous.

(3) *Culture.*—Concentrated, laked blood; aspirated contents of lymph glands; spinal fluid; or biopsy tissue from a suspected case of trypanosomiasis, when inoculated into a suitable medium may yield cultures positive for trypanosomes.

One of the best media upon which to culture *T. cruzi* is the NNN medium described in paragraph 427*a*(2)(*a*).

(4) *Animal inoculation.*—In diagnosis by animal inoculation either the blood or an emulsified portion of an infected lymph node may be injected intraperitoneally into a guinea pig, white rat, or monkey—preferably guinea pig.

SECTION IV

MALARIAL PARASITES

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430. General.—The malarial parasites are single-celled, pigmented, amoeboid organisms, living within the red blood corpuscles of man or some other vertebrate. The asexual cycle is confined entirely to the red blood cells of man or animals, whereas the sexual cycle is found in the mosquito. Figure 25 shows diagrammatically the life cycle of the malaria parasite in man and the mosquito.

431. Plasmodium vivax.—*a. Life cycle in man.*—(1) The sporozoites, which constitute the infective stage for man, are injected into the body during the bite of the infected female anopheline mosquito. After reaching the blood stream these elongated forms (sporozoites) parasitize red blood cells, round up, and assume the typical signet-ring form. These ring forms are about one-third to one-fourth the diameter of the infected red cell and when stained by Wright's or Giemsa's method, the chromatin stains red and the cytoplasm blue. In about 6 hours, the parasite increases in size by one-third and shows marked amoeboid movement within the infected cell. The

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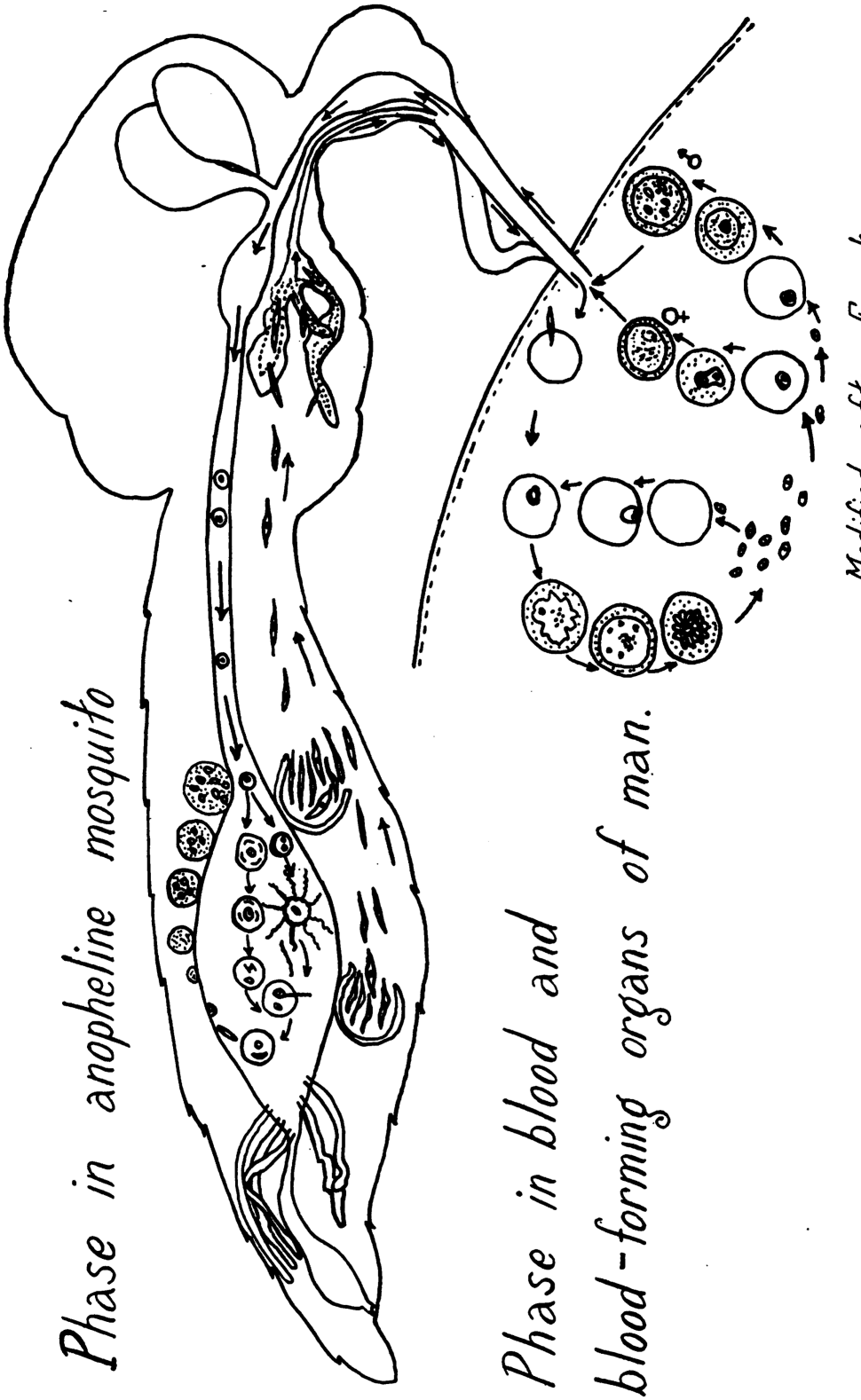


FIGURE 25.—Life cycle of malaria parasite.

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infected red cell has now enlarged until its diameter is from 10 to 12 microns and its color is paler than the uninfected red cells. If the infected red cells are properly stained, fine pink dots or stippling (Schüffner's dots) are usually present in the cytoplasm, and minute granules of light-brown pigment are seen near the chromatin of the parasite. The growth of the parasite is fairly rapid and by the end of 36 hours it fills two-thirds of the enlarged stippled, parasitized red cell. After about 40 hours, the parasite fills the cell and its chromatin usually divides into 12 to 16 segments. Small yellowish-brown granules of pigment are now scattered throughout the body of the parasite. The pigment then clumps toward the center of the parasite whose cytoplasm then divides into equal sections, one about each segment of the chromatin. The small bodies resulting from the division of the mature parasite are called merozoites. At the end of 48 hours the merozoites rupture the red cell membrane (at which time the patient's chill begins), and then each liberated merozoite seeks out and parasitizes a new red blood cell, to repeat the cycle. The pigment is liberated into the blood stream where it is destroyed. At any given time, practically all of the stages in the life cycle can be demonstrated in the blood stream, although one stage always predominates. The probable reason for this is that infections in man are due to the bite of more than one infected mosquito, or that in a single bite, some of the sporozoites instead of being inoculated intravenously are inoculated subcutaneously, thus delaying the start of their life cycle. *P. vivax* requires 48 hours for completion of its asexual cycle.

(2) The gametocytes (which will become sexual forms when taken up by the mosquito) develop from certain undifferentiated merozoites. The stimulus for their formation is unknown, but it is probably a response to developing protection on the part of the host. Four days are required for the development of the ring forms into mature gametocytes, and these must be from 7 to 10 days old before they are infectious for the mosquito. When fully grown the gametocytes are rounded in form and have fairly uniform cytoplasm. They appear in the blood cells within about 7 days after the initial fever. Their life in the blood stream is about 10 to 20 days. They are incapable of reproducing themselves or of starting the asexual cycle in man without first undergoing changes in the female anopheline mosquito.

(*a*) The male gametocyte (microgametocyte) is 7 to 8 microns in diameter and occupies an enlarged red cell without completely filling

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it. Schüffner's dots may be present in the margin of the red cell. The cytoplasm of the parasite stains faintly blue-gray and contains numerous granules of brown pigment. The chromatin stains red and is scattered over a fairly wide area, sometimes in the shape of a band, near the center or edge of the parasite.

(*b*) The female gametocyte (macrogametocyte) is larger, 8 to 10 microns, fills the parasitized cell more completely, and its cytoplasm stains a darker blue than the male gametocyte. The chromatin of the female gametocyte is in one compact clump, usually near the edge of the parasite, and the pigment is in coarse, brown, compact granules. There are usually from three to six female gametocytes for every male gametocyte in the peripheral blood.

b. Life cycle in female anopheline mosquito.—The gametocytes are drawn into the mosquito's stomach along with its blood meal. Within 20 minutes the male gametocyte develops 8 to 10 flagellar-like processes which soon exhibit violent lashing motion. These processes soon separate from the original cell body and are then called microgametes. While this process has been going on in the male gametocyte, the female gametocyte extrudes two bodies, each containing one-half of the chromatin. These are now ready for fertilization and are called macrogametes. The microgamete penetrates the cell wall of the macrogamete, the chromatin of each fuses, and the parasite now becomes a motile organism called an ookinete. The ookinete now penetrates through the wall of the mosquito's stomach, rounds up, becomes immotile, and is then called an oocyst. The chromatin of the oocyst undergoes multiple divisions until there may be as many as 10,000 granules which are grouped together in small clumps. The oocyst now contains from 10 to 20 groups of these chromatin granules and is known as a sporoblast. Each chromatin granule now develops a cigar-shaped cytoplasmic process and becomes a sporozoite. The sac containing the sporozoites is called a sporocyst. The sporocyst ruptures into the body cavity, liberating the motile sporozoites, which soon get into the mosquito's salivary glands. This process in the female anopheline mosquito requires about 7 to 12 days for its completion, if the temperature is about 20° C. and the air is about 70 percent saturated with moisture. Then the mosquito is ready to infect a new person. The infection apparently does not harm the mosquito.

432. Plasmodium malariae.—This parasite causes quartan malarial fever and it is the rarest of the three common species in man. Except for infected travelers, it is probably confined to the tropical

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or subtropical portions of the world. In subtropical regions, the greatest number of new cases of this infection occur during the fall months. The symptoms of this infection are much more severe than would be expected from the number of parasites infecting the red cells. In this infection, all stages are usually present in the blood stream at one time, although one stage always predominates.

a. Life cycle in man.—(1) The human phase of *P. malariae* is similar to that of *P. vivax* with the following exceptions:

(*a*) *P. malariae* requires 72 hours to complete its asexual cycle.

(*b*) During the first 6 hours of its growth it has largest ring form of all the types of malaria, and when it is 24 to 48 hours old, it develops typical band forms.

(*c*) Its segmenting forms develop only 8 to 12 merozoites.

(*d*) The infected red cells are not enlarged.

(*e*) Schüffner's dots are *not* present.

(*f*) The pigment present is coarse and stains dark brown or black.

(2) The gametocytes in *P. malariae* are similar to those in *P. vivax*, except that the organisms are smaller and the red cells are not enlarged and do not contain Schüffner's dots. The time required for its gametocytes to develop is approximately 7 days.

b. Life cycle in female anopheline mosquito.—The life cycle in the female anopheline mosquito is the same as for *P. vivax*, except that it takes about three times as long to complete the cycle.

433. Plasmodium falciparum.—This is the organism of aestivo-autumnal, subtertian, malignant tertian, or pernicious malaria. Its distribution is similar to that of *P. malariae*, but it is second only to *P. vivax* in the frequency of its occurrence. It is the most severe of all the malarial fevers of man and requires early diagnosis and prompt treatment.

a. Life cycle in man.—(1) The asexual cycle is similar to that of *P. vivax* with the following exceptions:

(*a*) The ring forms are smaller when they first parasitize a red blood cell.

(*b*) There are more apt to be multiple infections of a red cell. Ring forms may show two dots of chromatin.

(*c*) The rings may persist for about 24 hours in the peripheral circulation during which time they double or triple in size.

(*d*) Ring forms, and mature gametocytes which are crescent-shaped, ordinarily are the only forms found in the peripheral blood. In severe, overwhelming infections by this parasite peculiar thread-like forms may occasionally be present in the peripheral blood.

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(*e*) Developing amoeboid forms and immature gametocytes are usually found only in the capillaries of the internal organs (spleen, liver, etc.). These organisms have a marked tendency to clump together and stick to the endothelial cells, thus bringing about capillary blockage.

(*f*) Segmenting forms of *P. falciparum* develop 12 to 32 merozoites.

(*g*) The parasitized red blood cells are not enlarged.

(*h*) Schüffner's dots are *not* present in the parasitized cells, but large granules staining purplish red, called "Maurer's dots" or malignant stippling are present.

(2) The gametocytes are crescent-shaped with rounded ends and they are greater in length than the diameter of the red cells which they have parasitized. The male gametocyte stains bluish gray and has a central nucleus with diffusely scattered chromatin. The pigment present is in coarse grains and stains brownish black. It is scattered between the grains of chromatin. The tips of the male crescent are usually more rounded than those of the female crescent. The female gametocyte (crescent) stains sky blue and its nucleus is made up of a compact mass of chromatin and pigment.

b. Life cycle in female anopheline mosquito.—The life cycle of *P. falciparum* in the female anopheline mosquito is essentially the same as that of *P. vivax*, except that one-third to one-half again as much time is needed to complete the cycle.

434. Plasmodium ovale.—This organism resembles *P. malariae* except that in stained preparations the ring forms show Schüffner's dots and the infected red blood cells are oval-shaped. The red blood cells are larger than normal, but not so large as cells infected with *P. vivax*. This malarial infection is rare in the Americas but some cases have been reported recently. The following chart shows graphically the differential diagnostic points in the three genera commonly affecting man.

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TABLE XIX.—*Differential table of the malarial parasites of man*

Species		Parasitized red cells				Schizogony									Gametogony				Sporogony	Seasonal prevalence in subtropical regions where all 3 types of malaria are present
		Size	Color	Stippling	Presence of multiple infection	Size		Motility	Length of life cycle	Number of merozoites formed	Stages present in peripheral blood at any given time	Pigment			Differential characters of schizonts	Size		Shape	Length of life cycle	Time required to complete cycle at 20° C. relative humidity 70	
						Ring forms first hour	Mature forms in parasitized red blood cell					Presence and amount	Type	Color		♂	♀				
P. vivax (tertian or benign tertian)	Unstained	Enlarged to 10μ or 12μ	Pale yellowish red	Fine granules	Uncommon	1.5μ to 1.75μ	Fills cell	Active ameboid	48 hours	12 to 16	All stages, but one predominates	Yes, large amounts	Fine grains	Light brown	See illustrations	7μ to 8μ	9μ to 10μ	♂ ♀ round ♀ ♂ oval	4 days. Infective for mosquitoes in 7 days	7 to 12 days	Spring and early summer
	Stained		Pale brownish red	Pink granules of Schüffner																	
P. malaria (quartan)	Unstained	Not enlarged	Dull green	Not present	Uncommon	2μ to 2.5μ	Fills cell	Sluggish ameboid	72 hours	8 to 12	All stages, but one predominates	Yes, large amounts	Large amount coarse grains	Dark brown or black	Band forms. See illustrations	5.5μ to 6.5μ	6μ to 7.5μ	♂ ♀ round ♀ ♂ oval	7 days. Infective for mosquitoes in 10 days	20 to 24 days	Late fall
	Stained		Greenish red or normal	A few basophilic granules																	

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TABLE XIX.—*Differential table of the malarial parasites of man*—Continued

Species	Parasitized red cells				Schizogony					Pigment			Gametogony			Sporogony	
	Size	Color	Stippling	Presence of multiple infection	Size		Motility	Length of life cycle	Number of merozoites formed	Stages present in peripheral blood at any given time	Presence and amount	Type	Color	Differential characters of schizonts	Size	Shape	Length of life cycle	Time required to complete cycle at 20° C. relative humidity 70	Seasonal prevalence in subtropical regions where all 3 types of malaria are present
					Ring forms first hour	Mature forms in parasitized red blood cell									♂ ♀				
P. falciparum (aestivo-autumnal, malignant tertian, subtertian, or pernicious tertian)	Unstained																		
	Stained	Brassy copper	Few coarse grains	Fairly common	0.5μ to 1.5μ	Fills only ⅔ cell	Little or none	48 hours	12 to 32	Ring form and gametocytes only. Other forms in capillaries of internal organs except in severe cases	Yes, small amount	Coarse grains	Dark brown or black	See illustrations	12μ × 3μ	12μ × 2.5μ	Crescent shaped with the male having ends more rounded	4 days. Infective for mosquitoes in 7 days	16 to 20 days	Late summer and early fall
	Not enlarged	Purplish red or normal	Malignant stippling of Maurer stains deep purple																

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435. Laboratory diagnosis of malaria.—*a.* The most favorable time to find malarial parasites in the blood in a clinical case of malaria is the period beginning 12 hours after the chill up to 1 hour before the next chill. Do not take specimens of blood for examination during a chill.

b. Suspected positive findings should be confirmed by the laboratory officer before being reported.

c. Quinine or other anti-malarial drugs used in treatment within 4 days before taking the sample make it very difficult to demonstrate the parasites except by the thick film method.

d. Repeat the examination as many times as necessary to prove or disprove the diagnosis.

e. Use only glass slides that are chemically clean and free of scratches, grease, or fogging.

436. Thin blood films.—Thin films for staining are prepared by securing blood from the patient, and then making thin smears on a clean slide as if for a differential white blood cell count. The smear should be so thin that the cells are in a single layer and do not override one another. When the smear is too thick it is usually due to the use of too large a drop of the patient's blood or to the streaking slide being held at an angle of less than 35° when the smear was made. The smear should not be blotted, but allowed to air-dry rapidly without the use of heat. Do not blow the breath upon the smear to hasten drying, because it will lake the red blood cells. If smears are stained before they are dried, the cells will not be properly fixed, the stain will be precipitated, and the result will be unsatisfactory.

a. Flood the thoroughly dried blood smear with Wright's stain for ½ to 1½ minutes.

b. Dilute the stain on the slide with an equal volume of buffered distilled water pH 6.8 or enough of the water so that there is produced a metallic scum on top of the mixture. Allow this mixture to stand for 3 to 5 minutes.

c. Thoroughly wash the stained blood film with neutral distilled water until it is light pink in color, then blot and allow to air-dry.

437. Thick blood films.—Thick films for staining are made by securing a large drop of the patient's blood on a clean glass slide, then spreading the drop with the corner of another slide so that the blood covers an area about the size of a 10-cent coin. The film is then allowed to air-dry thoroughly at room temperature or in 37° C. incubator. If the film is thoroughly dried before it is stained,

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the blood cells will stay on the slide. Thick smears are best stained by the Giemsa method (see combined thick and thin film method, par. 438). However, if this stain is not available, they may be stained with Wright's stain.

a. Immerse the thoroughly dried thick smear for 10 minutes in a solution made of—

Formalin	5 cc
Acetic acid	1 cc
Distilled water q. s. ad	100 cc

This solution fixes the parasites and white blood cells, but dissolves the red cells.

b. Remove the smear and wash thoroughly in tap water in a Coplin jar following with distilled water.

c. Allow slide to thoroughly dry and then stain by Wright's method as for a thin smear.

438. Combined thick and thin blood films.—The combined thick and thin stained smears are prepared and stained by Giemsa's or Wright's method as outlined below. They are methods of choice in searching for malarial parasites in carriers, clinical cases that have very few parasites in the peripheral blood, and in malarial surveys. The appearance of the parasites in a stained thick preparation after the red cells have been laked out is different from that in stained thin preparations. They are quite typical, but the body of the parasite may be distorted in shape due to the destruction of the red blood cell. Therefore, where possible, the novice should not use this method until he has become thoroughly familiar with the malarial parasites and those confusing objects that are found in the thick stained smear. A convenient way of handling and staining large numbers of thick smears in a malarial survey is the one outlined by Barber and Komp of the United States Public Health Service. "In handling large numbers of thick smears it is convenient to carry out the technique in groups of 25 slides. With this in mind, the thick film is placed about one inch from one end of the slide and the other end is used for labeling. The slides are assembled in groups, a cardboard $\frac{1}{16}$ to $\frac{1}{8}$ inch thick and $1\frac{1}{2}$ inches long, is inserted between the slides at the labeled ends and the whole fastened together by means of a stout rubber band. The entire block may now be stained and dried as a single unit.

The combined thick and thin smears for staining are prepared by making a thick smear on one end of the slide and a thin smear starting $\frac{1}{2}$ inch from the thick smear, and then streaking it toward

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the opposite end of the slide. Draw a line with a wax pencil between the two smears and they are now ready for staining. Proceed as for a thick smear but *be careful to immerse only the thick smear in the acidulated formaldehyde solution.* If the thin smear comes in contact with this solution, the red cells will be dissolved out and the smear will be useless. Failure to stain by Wright's method is usually due to insufficient lapse of time after diluting the stain with distilled water, or to contamination of the stain, or other reagents, or material, with acid. The precipitation of granules of stain on the blood film is either due to improper drying of blood films before starting the stain, introduction of water into the stock stain, or too much evaporation of the alcoholic stain before dilution. Red cells stained blue, except for the occasional cells showing polychromatophilia, are either overstained (too much time allowed after diluting the stain) or have been insufficiently washed during the last stage of the staining process."

439. Examination of specimens.—The laboratory diagnosis of malaria infection in man consists of finding and recognizing the malarial parasites and their type when present in the blood preparations made from suspected malarial fever patients. In examining preparations made for malaria the oil immersion objective should be used. Malarial parasites are best seen when the light coming through the substage is slightly reduced. The proper amount of light may be obtained by moving the slide until a blood platelet is centered in the field, then adjusting the substage so that the maximum definition of its morphological detail is obtained. In searching for the parasites the slide should be covered in an orderly manner, moving back and forth over the smear so as not to repeat any field previously examined. *Never make a diagnosis on the first parasite found;* cover enough of the slide so that if two species of malarial parasites are present you will find them. If in doubt about any single abnormal parasite found, remember that where there is one malarial parasite there are bound to be more, and careful search will usually reveal an easily recognizable form. When the technician has determined the presence of malarial parasites he should submit the slide to the laboratory officer for confirmation. No positive reports for malarial parasites should leave the laboratory except those slips signed by a responsible officer. If no officer is available and you are sure there are malaria parasites present, report as a suspected positive. Save the slide for a final confirmation at a later date.

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Early diagnosis and treatment are of utmost importance if a favorable prognosis is to be expected in *P. falciparum* infections, owing to its tendency to produce early and unexpected cerebral complications. Therefore, if *P. falciparum* infections have been found in the laboratory in which you are working and you have a blood smear in which you are certain there are malarial parasites but you are unable to determine the type, report the case as positive for malaria so that treatment may be started. Then make additional smears and study carefully to determine the type.

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CHAPTER 13

HELMINTHOLOGICAL METHODS

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SECTION I

CLASSIFICATION

	Paragraph
General	440
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440. General.—*a.* The helminths of importance to the medical soldier may be classified in three main groups: flukes (trematodes), tapeworms (cestodes), and roundworms (nematodes).

(1) Adult flukes and tapeworms are usually flat, and are grouped, along with some nonparasitic forms, in the phylum *Platyhelminthes* (platy=flat+helminth=worm). Adult flukes resemble leeches in appearance although they are in no way related to this group. On the other hand adult tapeworms are somewhat ribbon-like, with a long series of segments set behind a very small head.

(2) The roundworms (also called threadworms) are grouped in the phylum *Nemathelminthes* (nema=thread+helminth). Many of the smaller forms resemble bits of thread, but in the larger forms the worm-like character is more evident. These larger roundworms are the ones to which reference is usually made when a child, a puppy, or a kitten is said to be "passing worms."

b. The life cycles of many parasitic helminths are very complicated since they may involve several hosts. Some of the immature stages are recognizable only to experts. Lack of space prohibits the mention of more than a single life cycle from each of the above groups. For details of these life cycles, and those of other parasitic forms, the medical soldier is referred to standard works in this field.

441. Life cycles.—*a. Fluke* (large intestinal fluke, *Fasciolopsis buski*).—Adult flukes living in the small intestine lay eggs which are passed out in the feces of the host. The larva (called a miracidium) develops in the egg, breaks out, swims around in the water, finds a snail, penetrates the soft parts, migrates through the snail's tissue,

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and undergoes changes in structure. The larva is now called a sporocyst. Many smaller individuals (rediae) develop in each sporocyst, increase in size, and rupture the sporocyst (still within the snail, however). Another set of rediae then develop in each redia (singular of rediae). They may be considered as daughter rediae. Other larvae (cercariae) develop within the daughter rediae, many cercariae developing in each daughter redia. The cercariae escape from the daughter rediae, erupt from the snail's tissue, and swim to plants on which the snails feed. Cercariae encyst on these plants and remain there until ingested by a suitable host. They are called metacercariae after encystment. In the intestinal tract of the host, the immature fluke escapes from the cyst and attaches itself to the wall of the intestine where it develops to the adult stage.

b. Tapeworm (beef tapeworm, *Taenia saginata*).—The adult tapeworm in the small intestine either extrudes ripe eggs, or the terminal segments containing ripe eggs break off from the worm. In either event eggs are evacuated in the feces of the host. Eggs ingested by oxen hatch in the intestinal tract. The embryos burrow through the intestinal wall into the lymphatics or the blood stream. They are carried to various parts of the host's body and are filtered out in tissues, usually muscles, where the larvae develop into mature cysts (cysticerci). When insufficiently cooked beef containing the cysticerci is eaten by man, the immature worms are set free and attach themselves to the wall of the small intestine where they develop into adults.

c. Roundworm (American hookworm, *Necator americanus*).—Adult females in the small intestine extrude eggs which are evacuated in the feces of the host. These eggs embryonate and hatch; the larvae (rhabditoid larvae) feed and in a few days moult, then resume feeding and growing. These larvae then transform into another type (filariform larvae), moult, but remain within the "sheaths." After a quiescent period, the filariform larvae shed these sheaths and become active. This is the infective stage for man. On contact with human skin they penetrate it and enter the blood stream, whence they are carried through the heart to the lungs where they "break out" into the alveoli. They are then transported up the air passages to the epiglottis, then down the alimentary tract to the small intestine where they develop into adults. During this passage another moult occurs either in the trachea or in the small intestine. Still another moult takes place in the intestine.

442. General considerations for laboratory diagnosis.—It may be seen from these life cycles that worms living in various parts

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of the body may cause pathological changes with the appearance of certain clinical symptoms. Many cases of helminthic infection may be diagnosed by clinical symptoms alone, but it is always more reliable if the parasite itself (in any stage—egg, larva, or adult), or a part of the parasite, can be demonstrated. In many cases identity of the adult helminths is difficult to determine, and some of the larval forms are even more difficult. The eggs of the various species, however, are usually sufficiently distinctive, that, when eggs can be recovered, they serve as an excellent means of diagnosis in the routine laboratory.

Where diagnostic material (that is, eggs, larvae, adults, or pieces of parasite) will be found depends upon the life cycle of the parasite. The adult large intestinal flukes live in the small intestine and discharge their eggs directly into the lumen. Therefore, an examination of the host's feces for eggs will reveal the presence or absence of these flukes. Beef tapeworms usually do not extrude eggs; the terminal segments of the worm, which contain ripe eggs, break off from the worm and are passed out in the feces. Here diagnosis is based on recovery of part of the adult. Again fecal examination is useful in the case of hookworms, whose eggs are extruded directly into the lumen of the small intestine. It will be seen that the presence of a large number of forms can be diagnosed by an examination of the feces, even though some of the adults may not inhabit the intestine. However, since the life cycles of the various helminths differ, the diagnostic material of some species may be found in other media. In the case of some of the filarial worms (nematodes) the eggs or larvae may be found in the blood where they have been deposited by the adult females. In trichinosis (infection with *Trichinella spiralis*) the larvae may be found in the muscle tissue, recovered by biopsying small bits of muscle. The presence of other forms may be revealed by an examination of the urine or the sputum. Once again, the life cycle of the worm determines where diagnostic material is most likely to be found; the clinical examination furnishes clues as to the offending parasite; and the laboratory examination confirms diagnosis.

SECTION II

IMPORTANT PATHOGENIC SPECIES

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443. General.—The principal helminths causing disease in man are grouped below according to the medium in which diagnostic material is usually found. Species marked with an asterisk (*) are common parasites of man.

444. Helminths in feces.—*a. Manson's blood fluke** (*Schistosoma mansoni*, fig. 26, *R*).—Infection is acquired by swimming or wading in water containing the infective larval stage. These larvae (cercariae) penetrate the skin and enter the blood stream. The adults usually inhabit the mesenteric vessels draining the large bowel. Diagnosis is based on recovery of eggs in the feces of the host. Distributed in parts of Africa, particularly the Nile delta and a very large equatorial section extending east and west to both coasts. Also distributed in the northeastern section of South America, several of the Lesser Antilles, and Puerto Rico.

*b. Oriental blood fluke** (*Schistosoma japonicum*, fig. 26, *Q*).—Infection is acquired in a similar manner to Manson's blood fluke. Adults of this species also inhabit the mesenteric vessels draining the large bowel, and diagnosis is again based on recovery of eggs in the host's feces. Distribution is limited to the Orient.

c. Sheep liver fluke (*Fasciola hepatica*, fig. 26, *T*).—This parasite is rarely found in man. Infection is acquired by accidental ingestion of encysted larval forms (metacercariae). Adults live in the bile passages of the liver. Diagnosis is based on recovery of eggs in the feces of the host. World-wide in distribution.

*d. Large intestinal fluke** (*Fasciolopsis buski*, fig. 26, *T*).—Infection is acquired by accidental ingestion of infective larval cysts (metacercariae), usually while eating pods, stems, roots, or bulbs of water plants, or in peeling water chestnuts with the teeth. The adults inhabit the duodenal region of the small intestine and diagnosis is based on recovery of eggs in the host's feces. The eggs of this species cannot be differentiated from those of the sheep liver fluke in routine laboratory diagnosis. Distributed in the Orient.

*e. Chinese liver fluke** (*Clonorchis sinensis*, fig. 26, *E*).—Infection is acquired by ingestion of encysted larvae (metacercariae) during consumption of infected fresh-water fish. Adults live in the bile passages of the liver. The eggs carried into the intestine by the bile flow and evacuated in the host's feces constitute the basis for diagnosis. Distributed in Sino-Japanese areas of the Orient.

*f. Broad fish tapeworm** (*Diphyllobothrium latum*, fig. 26, *C*).—This tapeworm is acquired by eating raw, salted, pickled, or insufficiently cooked fresh-water fish infected with the larval form (sparganum). The adults inhabit the small intestine, where they

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lay eggs. Diagnosis is based on recovery of the eggs in the host's feces. The infection is common in the north and central regions of Europe and in the Great Lakes region of the United States.

*g. Beef tapeworm** (*Taenia saginata*, fig. 26, *L* and *N*).—This is the most common human tapeworm. It is acquired by eating insufficiently cooked beef that contains the larval form (cysticercus). The adults usually inhabit the small intestine, and specific diagnosis is based on recovering segments of the adult worms in the host's feces. Eggs may also be recovered from the feces, but are not sufficiently distinguishable from those of the pork tapeworm for practical diagnosis. Since segments of the pork tapeworm may also be recovered from the feces, it is necessary to differentiate the segments of these two worms. In mature segments of the beef tapeworm the uterus contains more than 15 lateral branches (fig. 26, *N*), whereas the uterus of the pork tapeworm has fewer than 13 lateral branches (fig. 26, *M*). Infection with the beef tapeworm is world-wide.

*h. Pork tapeworm** (*Taenia solium*, fig. 26, *L* and *M*).—Two types of human infection with this helminth may occur. The most common is infection with the adult, acquired by accidently ingesting infective larval forms (cysticerci) with insufficiently cooked infected pork. The adults usually inhabit the small intestine. Diagnosis of infection with the adult is accomplished by recovering segments of the adult worm, or eggs, in the host's feces. It is necessary to differentiate these segments from those of the beef tapeworm (see *g* above). The less common but more serious infection is acquired by ingesting the eggs. The larval form (cysticercus) develops from the eggs and may encyst in vital centers with serious results. The ocular region and the brain are chosen as sites of encystment more commonly than any other parts of the body in human cysticercosis. It is entirely possible for a person infected with the adult worm, to accidently ingest eggs from this parasitizing adult and acquire the cysticercus infection. Diagnosis of cysticerci is difficult, and is usually not attempted in the routine laboratory unless the cysts are located in superficial tissues, whence they may be excised and examined. X-rays are helpful in diagnosing calcified cysts located in vital centers. A history of infection with the adult worm may be of aid in confirming diagnosis. Both types of infection are world-wide in distribution.

*i. Dwarf tapeworm** (*Hymenolepis nana*, fig. 26, *D*).—This is the most common tapeworm in the southern United States. Infection is acquired by accidental ingestion of eggs. The adults usually inhabit the small intestine. No intermediate host is necessary in this infection, and the person harboring the infection can readily reinfect

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himself by accidently ingesting eggs evacuated in his own feces. Diagnosis is based on recovery of eggs in the feces of the host. The infection is world-wide in distribution.

*j. Large intestinal roundworm** (*Ascaris lumbricoides*, fig. 26, *F*, *G*, and *H*).—This very common intestinal roundworm is acquired by ingestion of embryonated eggs. Adults usually inhabit the small intestine and eggs evacuated in the host's feces serve as a basis for diagnosis. The laboratory worker should be able to recognize fertilized, unfertilized, and decorticated *Ascaris* eggs. Decorticated eggs should not be confused with hookworm eggs (fig. 26, *H* and *I*). The parasite is world-wide in distribution.

*k. Human whipworm** (*Trichocephalus trichiurus*, synonym *Trichuris trichiura*, (fig. 26, *A*).—This is another very common intestinal roundworm acquired by ingestion of embryonated eggs. Adults usually live in the cecum, but may be found in other parts of the large bowel and even in the appendix. Diagnosis is based on recovery of the eggs in the host's feces. World-wide in distribution.

*l. Strongyloides stercoralis** (fig. 26, *K*).—The infection is acquired by invasion of special larval forms (filariform larvae) through the skin, or by accidental ingestion of these larvae. In either event the larvae enter the blood stream and reach the alimentary tract indirectly. The adult males are not tissue parasites and are voided in the feces, but the females usually inhabit the walls of the duodenum and upper jejunum. The eggs are laid and hatch in the tissues, the larvae (rhabditoid larvae) migrating into the intestinal lumen and passing out in the host's feces. Sometimes these larvae, which are normally not infective, may transform into the infective type (the filariform larvae) during their transit in the intestine. These infective larvae may invade the tissues of the lower bowel producing reinfection. Diagnosis is based on recovering larvae in the host's feces. Distributed in warm, moist regions throughout the world.

*m. Hookworm** (*Necator americanus* or *Ancylostoma duodenale*, fig. 26, *I*, *J*, and *O*).—Infection is acquired by invasion of filariform larvae through the skin. The adults usually live attached to the mucosa of the small intestine. Diagnosis is based on recovering eggs in the feces of the host. Distributed in warm, moist regions throughout the world. For the most part, however, the American hookworm (*Necator americanus*) is found in the Western Hemisphere and in the middle and southern two-thirds of Africa; whereas, the Old-World hookworm (*Ancylostoma duodenale*) is usually found in

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Europe and in the northern third of Africa. In Asia, the East Indies, and Australia both species of hookworm are encountered.

*n. Human pinworm or seatworm** (*Enterobius vermicularis*, synonym *Oxyuris vermicularis*, fig. 26, *B*).—Infection is acquired by ingestion of embryonated eggs. Eggs are usually embryonated when deposited by the female. The adults live in the cecum, appendix, and adjacent parts of the large and small intestines. The gravid females usually pass out through the anus and deposit eggs on the perianal and perineal folds. Eggs are not commonly deposited in large numbers in the bowel, and in examining for evidence of pinworm infection, ordinary fecal examination will not suffice. A special technic with an anal swab is recommended (par. 449*c*, NIH anal swab). Of course, eggs or adults found in the feces also serve as a basis for diagnosis. This parasite is world-wide in distribution and is probably much more common than surveys (which usually do not include anal swabs) have indicated.

445. Helminths in blood.—*a. Bancroft's filaria** (*Wuchereria bancrofti*).—Infection is acquired during the bites of mosquitoes infected with special larval forms. Adults normally inhabit the lymphatic vessels and the lymph glands, microfilariae probably being deposited in these places and carried into the blood stream. Diagnosis is based on recovery of the microfilariae in thick blood films. X-rays are helpful in chronic cases where the microfilariae may not be found. Distributed throughout the world in tropical and temperate regions.

*b. Persistent filaria** (*Acanthocheilonema perstans*).—Infection is acquired during the bite of the "punkie" (*Culicoides*) infected with special larval forms. Adults live in the body cavities and associated tissues, including mesentery, pleural cavity, etc. Microfilariae recovered in the blood stream serve as a basis for diagnosis. Distributed in Africa, South America, and the East Indies.

*c. Eye worm** (*Loa loa*).—Infection is acquired during the bite of the mango fly (*Chrysops*) infected with special larval forms. The adults move about in the subcutaneous and deeper cutaneous tissues, microfilariae being discharged into the passages produced during these migrations. Diagnosis is based on recovery of adults from the migratory tunnels, or upon the presence of microfilariae in blood films. This parasite is widely distributed in central West Africa.

For routine laboratory examination it is probably not necessary to differentiate the microfilariae of the various species inhabiting the blood, because there is no known treatment for any of these species. Technic for examination of blood is given in chapter 12.

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446. Helminths in tissue.—*a. Sparganum**.—Under the term "sparganosis" is grouped infection with the larval forms (sparganum stages) of certain helminths, probably tapeworms. In most cases the adults are unknown, but the most common sparganum of man (*Sparganum mansoni*) is in the adult stage a tapeworm of dogs and cats. The spargana are found in the subcutaneous and ocular tissue of man where, particularly in the latter region, they may produce intense pain and tissue reaction. Infection is usually acquired by poulticing an inflamed surface of the body with the infected flesh of a cold-blooded vertebrate (e. g., frogs). Diagnosis is based on the excision of the sparganа from the site of infection. The great majority of cases of human sparganosis have been reported from the Orient, but sporadic cases have appeared in other parts of the world, including the United States.

*b. Hydatid worm** (*Echinococcus granulosus*).—Hydatid disease is infection with the larval stage of a dog tapeworm (*Echinococcus granulosus*). Human infection is acquired by accidental ingestion of the eggs. The larval form (hydatid) develops from the egg and may encyst in various organs, particularly the liver and the lungs. The most accurate diagnosis of hydatid cysts is based on an intradermal reaction (Casoni test). Hydatid infection is distributed chiefly in the sheep- and cattle-raising regions of the world, and has been reported from the United States.

*c. Trichina worm** (*Trichinella spiralis*).—Infection is acquired by eating insufficiently cooked pork which is infected with the encysted larvae. The females deposit larvae in the lymphatics of the duodenum and upper jejunum, probably also in the mesenteric veins. Larvae are carried to all parts of the body and migrate out and encyst in striated muscles. Diagnosis is usually based on an intradermal test or upon recovery of larvae in excised muscle. Distributed chiefly in the United States and central Europe. Rarely reported from parts of Africa and South America.

*d. Ancylostoma braziliense**.—This species, *Ancylostoma braziliense*, is a tropical hookworm of dogs and cats. On contact with human skin, the larvae enter; but since they cannot penetrate the blood vessels, they migrate in the skin layers producing a "creeping eruption." Many human cases of creeping eruption have been reported from the southern United States, but cases are likely to be encountered wherever animals harbor the adult hookworm.

*e. Convoluted filaria** (*Onchocerca volvulus*.—Infection is acquired during bloodmeal of the black fly (*Simulium*) infected with certain larval forms. Adults usually live in tumors in subcutaneous or con-

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nective tissues and deposit larvae inside of these nodules. Diagnosis is based on demonstration of adults or microfilariae from excised nodules; or when no palpable nodules are present, microfilariae from the skin or conjunctiva. Distributed in Africa, Central America (particularly western Guatemala), and southern Mexico.

*f. Guinea worm** (*Dracunculus medinensis*).—Infection is acquired by ingestion of infected small crustaceans (*Cyclops*) in raw drinking water. Adults develop in the viscera or in the subcutaneous tissue, and when mature migrate to a position just under the skin. A blister develops on the skin just above the head of the worm; after a few days this blister bursts, and on contact with fresh water, the worm deposits larvae into the water through the ruptured blister. Diagnosis is based on finding the worm under the skin when local lesions have developed. Distributed in Africa, southern Asia, the West Indies, the Guianas, and Brazil. Sporadic cases have appeared in other localities.

447. Helminthsin urine.—The Vesical blood fluke* (*Schistosoma hematobium*, fig. 26, *S*). Infection is acquired in a similar manner to Manson's blood fluke. Adults of this species inhabit the portal blood and vesical plexuses. Eggs found in the urine, rarely in feces, constitute the basis for diagnosis. Distributed extensively in Africa.

448. Helminthsin sputum.—The Oriental lung fluke* (*Paragonimus westermani*, fig. 26, *P*). Infection is acquired by consumption of raw crabs and crayfish harboring infective larval cysts. The adults usually inhabit the bronchioles of the lung although they may be found in other sites. Diagnosis is usually based on recovery of the eggs in blood-flecked sputum, but eggs may also be recovered in the feces, and rarely in cutaneous lesions. For the most part this fluke is oriental in distribution.

Section III

METHODS OF EXAMINATION

	Paragraph
Feces	449
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449. Feces.—*a. Ordinary smear.*—In many cases of helminthic infection an ordinary fecal smear examined under the microscope will show eggs or larvae of the offending parasite. For details of this technic see section on protozoology (sec. II, ch. 12).

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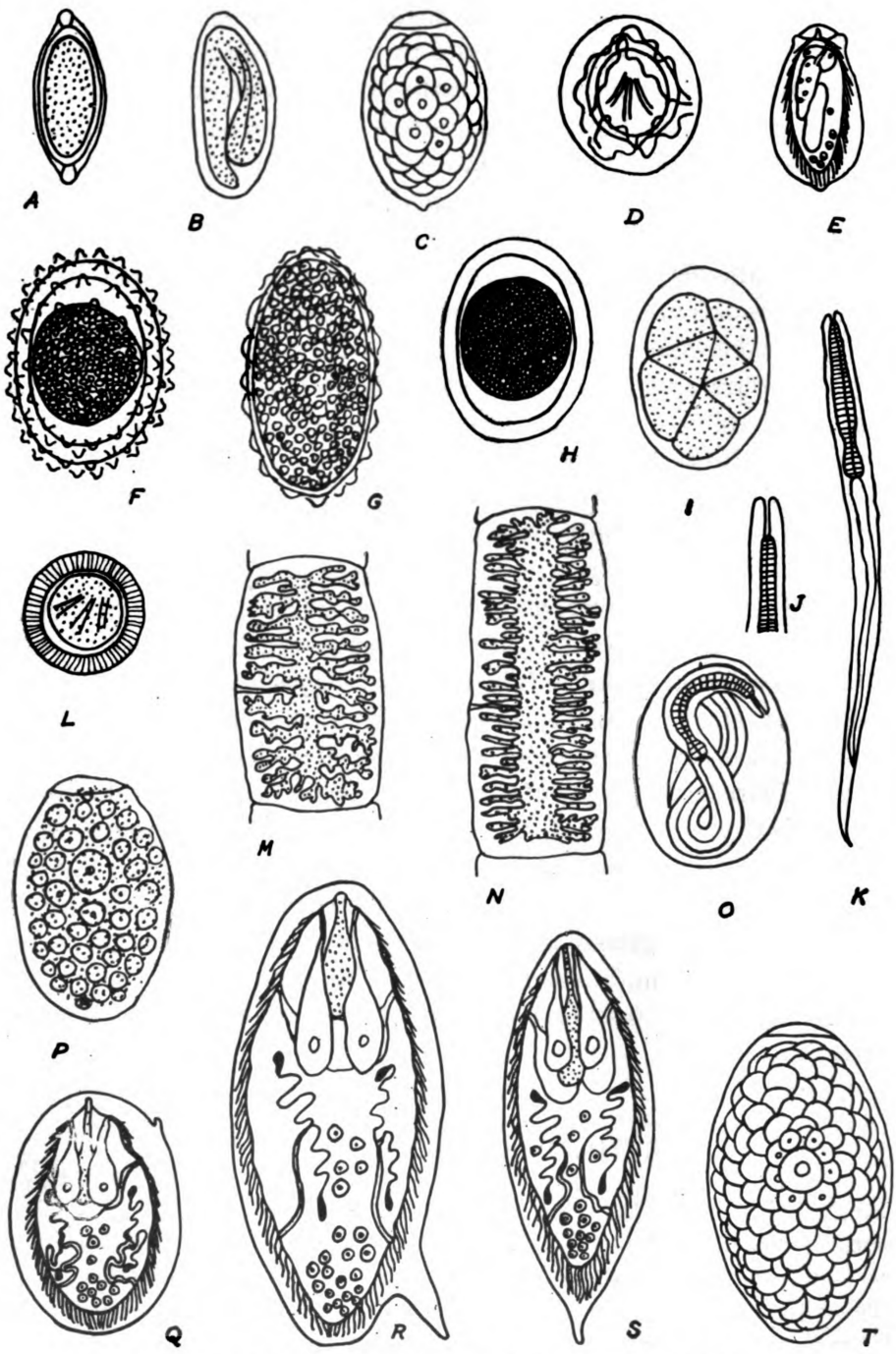


FIGURE 26.—Helminths (diagram of diagnostic material).

NOTE.—*A*, egg of the whipworm (*Trichocephalus trichiurus*); *B*, egg of the pinworm (*Enterobius vermicularis*); *C*, egg of the broad fish tapeworm (*Diphyllobothrium latum*); *D*, egg of the dwarf tapeworm (*Hymenolepis nana*); *E*, egg of the Chinese liver fluke (*Clonorchis sinensis*); *F*, *G*, and *H*, fertilized, unfertilized, and decorticated eggs, respectively, of the large intestinal roundworm (*Ascaris lumbricoides*); *I*, egg of hookworm

(*Note continued on page 371*)

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b. Zinc sulfate centrifugal flotation technic.—Many cases of helminthic infection are sufficiently light that no eggs or larvae will be recovered in an ordinary fecal smear. It is then necessary to employ the zinc sulfate centrifugal flotation technic. It is a good policy to examine each stool by this technic, because the presence of a helminthic infection by an ordinary smear is no indication that other parasites are absent.

c. NIH anal swab.—(1) In pinworm infection eggs are more commonly deposited on the perianal folds than in the feces. Therefore, swabbing and mild scraping of these parts yield eggs even when the feces is negative. The NIH anal swab is the most efficient anal swab and scraper yet devised. Its preparation follows:

(*a*) Insert a glass rod through a rubber stopper so that approximately 1 inch protrudes from the larger end of the stopper.

(*b*) Using a rubber band, secure a cellophane square to the other end of the rod as shown in figure 27. (The rubber bands should be about 2 mm wide and may be made from rubber tubing having a 3-mm bore and walls 2 mm thick.)

(*c*) Fit the swab into the test-tube housing as shown in the diagram.

(2) Before describing the actual swabbing procedure, a few suggestions regarding the use and examination of the swab may be in order.

(*a*) The swabbing should be done in the morning immediately after the patient arises, before he has bathed or defecated.

(*b*) The swab is to be used dry.

(*c*) Certain defects in cellophane resemble pinworm eggs in outline and the worker should guard against this possible source of error in diagnosis.

(3) Swab and examination is as follows:

(*a*) Stroke the cellophane-covered tip firmly, with an outward motion, over the perianal folds and across the anal opening.

(*b*) Release the cellophane square by sliding the rubber band towards the rubber stopper.

(Note continued from page 370)

(*Necator americanus* or *Ancylostoma duodenale*); *J*, fore-part of rhabditoid larva of hookworm showing long buccal cavity. Compare with short buccal cavity of rhabditoid larva of *Strongyloides stercoralis*, *K*; *L*, egg of either of the pork tapeworm (*Taenia solium*) or the beef tapeworm (*Taenia saginata*); *M* and *N*, gravid segments of the pork and beef tapeworms, respectively, showing difference in the number of uterine branches (fewer than 15 in pork tapeworm, more than 15 in beef tapeworm); *O*, egg of hookworm showing fully developed embryo (commonly found in constipated stools of hookworm patients); *P*, egg of the oriental lung fluke (*Paragonimus westermani*); *Q*, egg of the oriental blood fluke (*Schistosoma japonicum*); *R*, egg of Manson's blood fluke (*Schistosoma mansoni*); *S*, egg of the vesical blood fluke (*Schistosoma hematobium*); *T*, egg of the sheep liver-fluke (*Fasciola hepatica*) or the large intestinal fluke (*Fasciolopsis buski*). (Approximate magnifications, *E* X600; *M* and *N*, X3; all others X300.)

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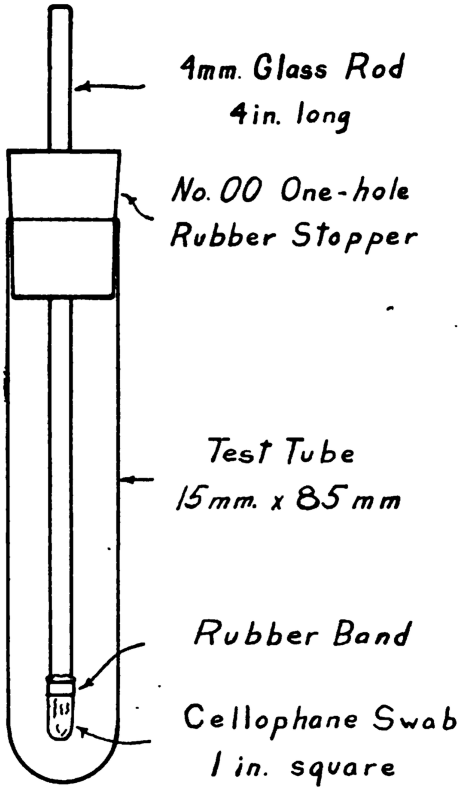


FIGURE 27.—NIH anal swab.

(*c*) Mount the cellophane square in water on a microscopic slide and examine under a microscope.

450. Tapeworm segments.—It is necessary to differentiate segments of beef or pork tapeworms passed in the feces. This is done as follows:

a. Clean and relax the segments by shaking in physiological saline.

b. Place the specimen between two glass slides and press it flat.

c. Hold it up to a strong light (so that the light shines through it), and count the number of lateral branches of the uterus. (Since these lateral branches subdivide, they are to be counted where they arise from the main part of the uterus.)

451. Blood.—Thick films are very useful in demonstrating microfilariae. For details of this technic see paragraph 437.

452. Tissue.—There are several specialized technics employed in the diagnosis of tissue parasites, most of which are not entirely satisfactory. Need for these technics is not very common, but when necessary, the technic is probably best performed by the medical officer in charge. Details of these technics are available in standard texts.

453. Urine.—*a.* In cases of heavy infection with the vesical blood fluke, eggs may be found in the urine, particularly in the last portion passed. The technic follows:

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(1) Have the patient pass urine into a urinalysis glass. (This should especially include the last portion of urine voided.)

(2) Let the material settle for 15 to 20 minutes.

(3) Take up a small portion of the sediment in a pipette and place it on a microscopic slide.

(4) Examine under the microscope.

b. In cases of light infection this procedure may yield negative results. It is then necessary to centrifuge a representative portion of the urine for 1 to 2 minutes and examine some of the sediment under a microscope.

454. Sputum.—In many cases of suspected helminthic infection of the respiratory passage, examination of the sputum is necessary. The technic follows:

a. Rinse the mouth thoroughly with diluted hydrogen peroxide.

b. Pass sputum into a jar.

c. Transfer small bits of sputum, particularly blood-flecked portions, to a microscopic slide.

d. Examine under a microscope.

455. Transmission of specimens.—*a.* From time to time it is necessary to send specimens to other laboratories, sometimes for identification, sometimes for study purposes. In all such cases complete notes should accompany the material and should include such data as locality, host, date, collector's name, number of specimens obtained, condition of the specimens, tissue, organ or medium from which recovered, and any other pertinent information. Material should be treated for shipment as indicated below.

(1) *Eggs.*—Feces containing eggs may be diluted with water and agitated until an even mixture is obtained. To this mixture is added an equal volume of steaming (80° C.) 10 percent formalin. This will also fix any nematode larvae present.

(2) *Larvae and adults.*—Larvae and adult helminths should first be shaken in physiological saline. This cleans and relaxes the specimens. They may now be fixed by adding an equal volume of steaming (80° C.) 5 percent formalin to the saline containing the worms.

(3) *Pathological tissues.*—Pathological tissues may be fixed in 10 percent formalin, or if it is available, Zenker's fluid.

(4) *Intermediate hosts.*—Intermediate hosts may be fixed and preserved in 70 percent alcohol.

b. Specimens should always be carefully packed to avoid breaking or spilling of the contents. Containers should be filled with the preserving liquid to avoid breaking of the specimens if the package is handled roughly. Jars and glass vials packed separately in a box with excelsior, shredded paper or cotton, and marked "Fragile" will usually survive.

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CHAPTER 14

ENTOMOLOGICAL METHODS

		Paragraphs
SECTION I.	General	456-457
II.	Arthropods of medical importance	458-477
III.	Handling and shipment of specimens	478-479

SECTION I

GENERAL

	Paragraph
General	456
Classification	457

456. General.—*a.* Medical entomology is the study of insects and insect-like animals (arthropods) and their relation to human disease and discomfort. These arthropods may be associated with a number of diseases of importance to troops in garrison, camp, or campaign. The principal disease relationship of these forms is that of transmission: malaria and yellow fever by certain mosquitoes, plague by fleas, and typhus by lice. Also of importance are the arthropods that cause disease directly, they themselves serving as pathogenic organisms. The itch mite of man, for example, invades the human skin and produces a severe irritation; this disease (scabies: the itch) caused thousands of men to be admitted to hospitals during the World War. Many arthropods, as nuisances, cause discomfort to troops, such as the annoyance provoked by the common house flies and mosquitoes, especially when they occur in large numbers.

b. Due to the widespread distribution of arthropods, and to their close association with troops at all stations, it is necessary that Army personnel be prepared to determine whether the species present in a locality are likely to be of medical importance. This may entail collection of representative forms, making a tentative identification, and if certain specimens are likely to be important, forwarding them to large central laboratories where positive identification may be accomplished. The medical technician is not expected to know all of the various species by their scientific names, but if called upon to send in a representative sampling, for example, of mosquitoes in the vicinity, he should be able to send mosquitoes, and not a various assemblage of small beetles, flies, midges, fleas, moths, etc. It is the purpose of this section to familiarize the medical technician with the

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medical importance of various arthropods and to aid him in roughly identifying the forms.

457. Classification.—Classification of arthropods is based upon a system of organization. As an army is divided into corps, which are further divided into divisions, then brigades, regiments, battalions, companies, etc., down to the individual men, similarly animals are grouped into phyla, which are subdivided into classes, orders, families, genera, and species. The phylum *Arthropoda* may be divided into several classes of which only four will be here considered, namely, *Insecta* (insects), *Arachnida* (ticks, mites, spiders, scorpions, etc.), *Myriapoda* (centipedes and millipedes), and *Crustacea* (crayfish, shrimp, etc.). These classes can be further divided again and again until the individual species are reached; the yellow fever mosquito may be classified as follows:

Phylum—Arthropoda. Order—Diptera. Genus—*Aedes.*
Class—Insecta. Family—Culicidae. Species—*Aegypti.*

The scientific name of an animal is a combination of the names of the genus and the species: the scientific name of the yellow fever mosquito is *Aedes aegypti.*

SECTION II

ARTHROPODS OF MEDICAL IMPORTANCE

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Centipedes	462
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Anoplura (lice)	470
Diptera (flies, mosquitoes, etc.)	471
Mosquitoes	472
Mosquito-like insects	473
Stout-bodied biting flies	474
Nonbiting flies (filth flies and myiasis-producing flies)	475
Heteroptera (bedbugs, kissing bugs, cicadas, etc.)	476
Siphonaptera (fleas)	477

458. Procedure in identification of specimens.—*a.* Since all members of any of the above classes possess certain common

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characteristics that differentiate them from the other classes of arthropods, use can be made of these characteristics in making "classification keys." These keys enable the worker to identify specimens. A working example of such a key follows:

1. If the specimen has three or four pairs of legs 2
   If it has numerous pairs of legs 3
2. With three pairs of legs (fig. 34) ..... *Insecta* (par. 469)
   With four pairs of legs (fig. 31) ..... *Arachnida* (par. 463)
3. Lives in water (fig. 28) ..... *Crustacea* (par. 459)
   Lives on land (fig. 29) ..... *Myriapoda* (par. 460)

b. An examination of the above key will reveal that identification of specimens is not too difficult a matter. It is simply necessary to "run it through the key." If by running the specimen through the key it should prove to be an arachnid, it is necessary only to turn to the section on *Arachnida* where a key to this class will be found. By subjecting the specimen to several such keys, identification can be made.

c. The key is only an aid, not a final proof of identification. Since in the insect class alone there are thousands of species, it is obvious that the keys and outlines in this manual will not serve to identify all specimens. They will, however, enable the worker to identify many forms, even though the identification may not be entirely accurate in all cases. For more satisfactory identification this manual should be supplemented by standard texts, and for absolute identification specimens may be transmitted to entomological centers.

459. Crustacea of medical importance.—Crustaceans (crayfish, shrimp, etc.) are of little importance to the medical soldier, but are worthy of mention because a few species are associated with human disease. Several species of microscopic forms serve as intermediate hosts in the transmission of certain intestinal worms. A typical (but medically unimportant) crustacean is illustrated in figure 28.

460. Myriapoda of medical importance.—Myriapods include the centipedes (*Chilopoda*) (fig. 29①) and the millipeds (*Diplopoda*) (fig. 29②). They may be differentiated by the number of legs on each body segment, centipedes possessing one pair of legs per segment, whereas millipedes have two pairs on each segment.

461. Millipedes.—The millipedes may for practical purposes be omitted from the list of offenders. They have no fangs and are, therefore, harmless so far as venomous species are concerned.

462. Centipedes.—Certain centipedes, on the other hand, are very important. Although nearly all of the species possess fangs, they are for the most part unable to penetrate the human skin. While

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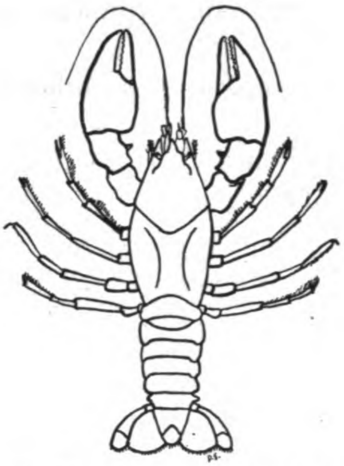
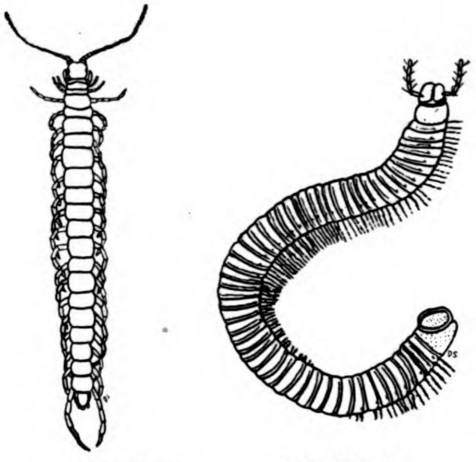


FIGURE 28.—Crayfish (a crustacean).



① Centipede. ② Millipede.

FIGURE 29.—Myriapods.

no deaths have been recorded from centipede bite, painful injury can be accomplished. Species of *Scolopendra*, *Geophilus*, and *Lithobius* are capable of injury.

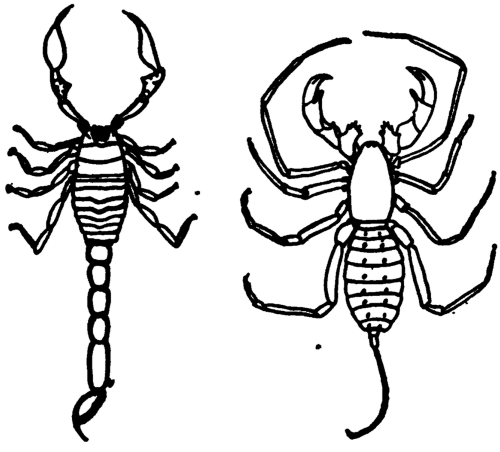
463. Arachnida of medical importance.—The class *Arachnida* is very important from a medical standpoint, containing many species that serve as transmitters of disease, as well as species that cause disease directly. The following key will serve to separate the more important orders:

1. With abdomen divided into segments 2
   Abdomen not divided into segments 4
2. Body divided by constriction into two main parts (fig. 31) Spiders (par. 466)
   Body not divided by such a constriction 3

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3. Minute species (smaller than a pinhead in size) (fig. 32)
Mites (par. 467)
Medium-sized species (larger than a pinhead in size) (fig. 33)
Ticks (par. 468)
4. With spine at tip of tail (fig. 30①)----------Scorpions (par. 464)
Without spine at tip of tail (fig. 30②)---Whip scorpions (par. 465)

464. Scorpions.—Scorpions (fig. 30①) are offensive to man because of their sting, which is accomplished by a spine at the tip of the tail (abdomen). Although many of the smaller species are harmless because they are not able to penetrate the human skin, some species, particularly of the genus *Centruroides* are very important. In the city of Durango in northern Mexico one species, *C. suffusus*, causes on the average of 50 deaths per year. The 20 or more common species



① Scorpion. ② Whip scorpion.

FIGURE 30.—Comparison of scorpion and whip scorpion.

of the southern United States are generally capable of producing only a painful sting. Of interest to soldiers is the fact that some scorpions have a tendency to crawl into shoes during the night.

465. Whip scorpions.—Whip scorpions (fig. 30②) are very ferocious in appearance but are entirely unimportant from a medical standpoint. Their only interest to soldiers is the tendency to confuse them with scorpions.

466. Spiders.—Although all spiders (fig. 31) produce venom, only a few possess fangs sufficiently powerful to penetrate the human skin. Of most importance to the soldier is the "black widow" spider, *Latrodectus mactans*. This is a small black spider, which can be distinguished by the reddish hour-glass marking on the underside of its abdomen. It may be found in grass, shrubs, old outhouses, and privies. Its bite produces severe symptoms and in some cases death.

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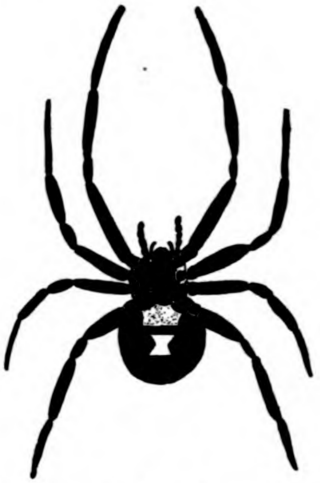


FIGURE 31.—Black widow spider (an arachnid).

Tarantulas present a ferocious appearance, but compared to the black widow their bite is mild. Their hairy bodies serve as a convenient resting place for many bacteria and secondary infection of the wound is common.

467. Mites.—All species of mites (fig. 32) are very small, many being barely visible to the naked eye. In general, only three forms are of medical importance.

a. The common itch mite of man, *Sarcoptes scabiei*, tends to be found where many people may be forced to live together under unhygienic conditions. In times of national emergency where many soldiers come in close contact with each other, and especially where bathing facilities are meager, cases of scabies may appear. When the mites attack, they usually invade the skin between the fingers, and spread to other parts of the body from these foci. The females bur-

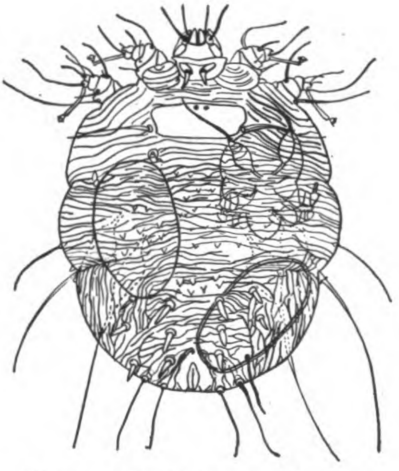


FIGURE 32.—Itch mite of man.

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row into the skin and lay their eggs in the tunnels made during their migrations. The intense itching which this skin invasion produces results in scratching, with subsequent secondary infection.

b. The genus *Trombicula* contains two very important species. The most important is the Japanese chigger, *T. akamushi*, which transmits Japanese River Fever. This disease is very similar to Rocky Mountain Spotted Fever and is as fatal to man. These mites generally occur on small rodents, especially field mice, which serve as reservoir hosts of this disease. These mites are widely distributed throughout certain parts of Japan.

c. The other species of medical interest is the American chigger or redbug. These tiny mites, hardly larger than a pinpoint, are larval forms of *Trombicula irritans*. Since these larvae are very numerous in the fields during the late spring and early summer, troops working in high grass and weeds during warm weather are subjected to their attacks. The larvae, which have only three pairs of legs instead of four pairs, attach themselves to the exposed parts of the body where their bites produce an intense itching that may last several days. This species is widely distributed in the United States, and is a common cause of annoyance to soldiers.

468. Ticks.—*a.* The ticks are important chiefly as transmitters of harmful organisms and incidentally as direct cause of disease. They have a widespread occurrence, particularly in the tropics and subtropics. For purposes of general classification ticks can be divided into two groups: The soft-bodied ticks (family *Argasidae*) and the hard-bodied ticks (*Ixodidae*).

(1) "Head" concealed beneath front margin of body, shield on upper surface of body absent (fig. 33①), soft-bodied ticks (*b*(1) below).

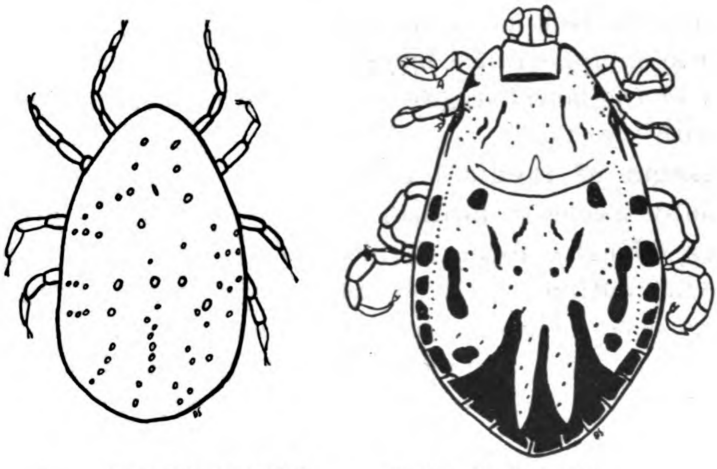
(2) "Head" not concealed beneath front margin of body, shield on upper surface of body present (fig. 33②), hard-bodied ticks (*b*(2) below).

b. (1) *Soft-bodied ticks.*—The members of this group (*Argasidae*) (fig. 33①) are not fixed parasites as are the hard-bodied ticks (*Ixodidae*). Their habits are similar to bedbugs, visiting the host for a blood meal, then returning to the cracks and crevices in which they live. Of the two genera that belong to this family, the genus *Ornithodorus* contains nearly all the species of medical importance The members of this genus are very similar in appearance to those of the genus *Argas*, but may be differentiated since the edges of the body are rounded, whereas these edges are sharp-angled in *Argas* species. Eyes are frequently present in *Ornithodorus* and absent in *Argas*.

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Soft-bodied ticks may transmit relapsing fever from animals, human cases, or carriers to other animals or to man. The tick acquires the spirochetes (*Borrelia duttoni*) when it takes a blood meal, harbors them for prolonged periods, and later infects the person (or animal) when it takes another blood meal. Hereditary transmission from adult tick to its offspring, through the egg stage, may occur. Opossums and armadillos may serve as reservoirs from which the ticks acquire their infections. The more important species are *O. moubata* in Africa, *O. talaje* in South and Central America and Mexico, and *O. turicata* in the southern United States.

(2) *Hard-bodied ticks.*—The members of this family (fig. 33②) differ in feeding habits from soft-bodied ticks in attaching themselves



① Soft-bodied tick. ② Hard-bodied tick.

FIGURE 33.—Ticks.

to their hosts and feeding for long periods of time. They are by far the more widely distributed group, being very well represented in tropical, subtropical, and temperate regions. Of the eight genera belonging to this family, the genus *Dermacentor* is by far the most important. Others of importance are *Haemaphysalis* and *Ixodes*. In general, differentiation of the hard-bodied ticks is rather difficult and for such identification the worker is referred to standard texts.

Hard-bodied ticks are found associated with various human diseases, the most important of which are Rocky Mountain Spotted Fever, tularaemia, and tick paralysis. Rocky Mountain Spotted Fever, an acute infectious disease of man having a high mortality, is caused by a rickettsial organism (*Dermacentroxenus rickettsi*). The tick of most importance in the transmission of this micro-organism to man is the Rocky Mountain wood tick, *Dermacentor andersoni*. The

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North American rabbit tick, *Haemaphysalis leporis-palustris*, is important in transmitting the disease from rodent to rodent. Tularaemia (rabbit fever), caused by *Pasturella tularensis*, may be transmitted to man by the bites of the wood tick *D. andersoni*. Again, the rabbit tick, *H. leporis-palustris*, is important in transmitting the disease among rodents (rabbits).

c. In addition to transmitting disease, many ticks are able to inflict injury by the bites alone. Certain species, particularly the wood tick, *D. andersoni*, and various species of *Ixodes* are offensive in this respect. The bites of these species rarely cause a form of paralysis, and in a few cases, deaths have resulted. The exact mechanism by which the paralysis is effected is not known, but it is probable that the tick secretes some sort of neurotoxin into the wound.

d. At times the technician may be presented with small forms that resemble ticks in appearance, but have only three pairs of legs. These forms may be the larval stages (called seed ticks), and should not be confused with insects.

469. Insecta of medical importance.—The class *Insecta* also contains many species of medical importance. In this group are included such offensive forms as morquitoes, lice, fleas, bedbugs, etc. The following outline will serve as a guide in classifying the more important orders. Those orders of medical importance are marked with an asterisk (*). For more complete information and keys the reader is referred to standard entomological texts.

Insect order	Common names of forms	Figure
**Anoplura*	Lice	35
Coleoptera	Beetles, weevils	34 (2)
**Diptera*	Flies, mosquitoes, midges, etc	40
**Heteroptera*	Bedbugs, kissing bugs, cicadas, etc	43 and 44
Hymenoptera	Ants, bees, wasps, etc	34 (4)
Lepidoptera	Butterflies, moths, skippers	34 (3)
Orthoptera	Roaches, crickets, grasshoppers	34 (1)
**Siphonaptera*	Fleas	45

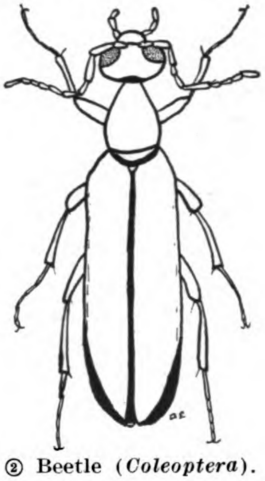
Only the above insect groups indicated as of medical importance will be discussed below. The other groups have been included in order that the technician may observe examples of those groups and thus avoid confusion with important species. He should bear in mind, however, that this list is far from complete.

470. Anoplura (lice).—There are two species of lice that infest man, namely, the head louse and body louse (*Pediculus humanus*)

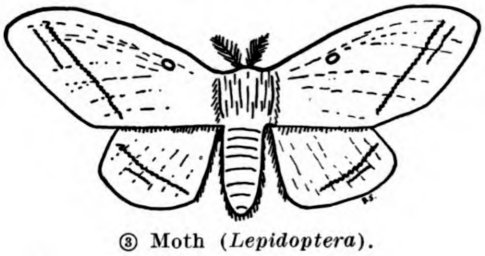
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① Cockroach (*Orthoptera*).



② Beetle (*Coleoptera*).



③ Moth (*Lepidoptera*).



④ Ant (*Hymenoptera*).

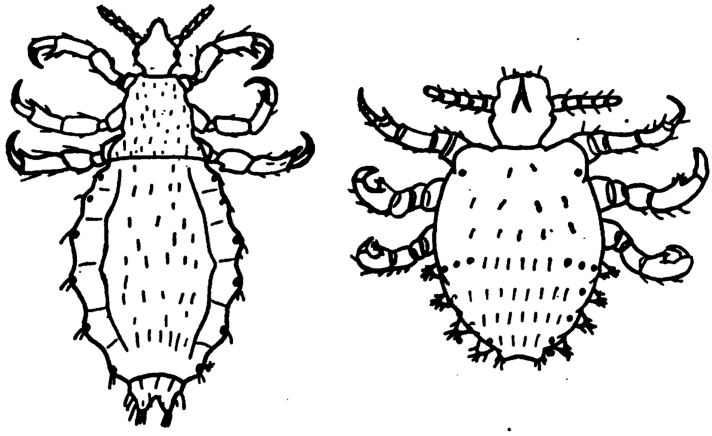
FIGURE 34.—Insects.

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and the crab louse (*Phthirus pubis*). The head louse and body louse are two varieties of the same species, and are called *P. humanus* var. *capitis* and *P. humanus* var. *corporis*, respectively. The head and body lice and the crab lice are easily differentiated by the length of the body in proportion to its width. Both varieties of *P. humanus* are about three times as long as they are broad, whereas crab lice are as broad as they are long (see fig. 35).

a. The head louse lives among the hairs of the head of its host and attaches its eggs near the base of the hairs by means of a glue formed in a special gland. The hairs around the ears and back of the head are most frequently used as sites for depositing the eggs.

b. The body louse usually infests the clothing along the seams, where it attaches its eggs to the fibers of the cloth. Woolen cloth-



① Body louse. ② Crab louse.

FIGURE 35.—Lice (*Anoplura*).

ing seems to be the material of choice of the body louse, because the eggs may be easily attached to the wool fibers.

c. Man is affected by the head and body louse in two ways: by the direct, mechanical effect of the bites, and by their transmission of pathogenic organisms. The bites produce minute hemorrhagic spots which are accompanied by irritation, often with intense itching, leading to scratching and secondary infection.

d. Among the infectious diseases transmitted by lice are typhus, trench fever, relapsing fever, and plague. European or epidemic typhus which resulted in many deaths among soldiers in the European armies in the World War, is caused by a rickettsial organism, *Rickettsia prowazeki*, transmitted by the head and body lice (especially the latter). Transmission may be accomplished in three ways: by deposition of the louse's feces on the injured skin, by

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crushing the insect against the skin, and by its bite. Trench fever is caused by *Rickettsia quintana* and transmitted by the bite of the body louse.

e. A relatively mild type of relapsing fever caused by the spirochete, *Borrelia recurrentis*, is transmitted by lice when infective lice are crushed on the skin. The disease is not transmitted by the bite.

f. It has been shown recently that lice on marmots in western Montana were naturally infected with the organism of plague, *Pasturella pestis.*

g. The crab louse generally frequents the pubic hairs, but it has been found also on other hairy parts of the body, the legs, armpits, beard, eyebrows, and eyelashes. As in the case of the head louse, the crab louse lays its eggs attached to the hairs of the host. This insect provokes some local irritation, but has not yet been incriminated in the transmission of any disease.



① Larva of house fly.



② Pupa of house fly.

FIGURE 36.—Flies.

471. Diptera (flies, mosquitoes, etc.).—The *Diptera* is another arthropod order which contains species that may serve both to transmit disease and to cause it directly. The importance of some mosquitoes and tsetse flies in malaria and sleeping sickness is common knowledge, but there are many other mosquitoes and biting flies that carry disease. There are many species living and breeding in close contact with Army personnel that may cause disease directly. It is well known that in their life cycles flies pass through egg, larval, pupal, and adult stages (fig. 36). The larvae (maggots) of some species may gain entrance to the human body and invade the tissue, such an invasion of tissue with fly larvae being spoken of as myiasis.

Since the order Diptera contains so many species with variance of form and habit, it is impossible to present a simple key to all the major groups. The following key-outline will, however, serve as a convenient guide in separating the forms. It should be remem-

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bered that this outline is one of convenience, and for a more accurate classification standard texts should be consulted. Since the mosquitoes constitute the most important dipterous group, their classification is treated in more detail, and the technician should be able to make tentative examination of kinds of mosquitoes even though the other forms may be regarded more lightly. Separate any specimens collected as follows:

1. Fragile (mosquito-like) (fig. 40①)---------- 2
   Stout-bodied (housefly and horsefly-like) (fig. 40②)---------- 3
2. Scales on wings (fig. 38)----------Mosquitoes (par. 472)
   No scales on wings (fig. 40)----------Mosquito-like flies (par. 473)
3. Biting flies (fig. 41①)----------Stout-bodied biting flies (par. 474)
   Nonbiting (fig. 41②)----------Filth and myiasis flies (par. 475)

472. Mosquitoes.—*a.* The family *Culicidae* to which the mosquitoes belong may be divided into three subfamilies. Of these latter groups the mosquitoes are the only ones that suck blood and are of medical interest. They may be easily distinguished from other common mosquito-like insects by the presence of scales on the veins and margins of the wings (fig. 38).

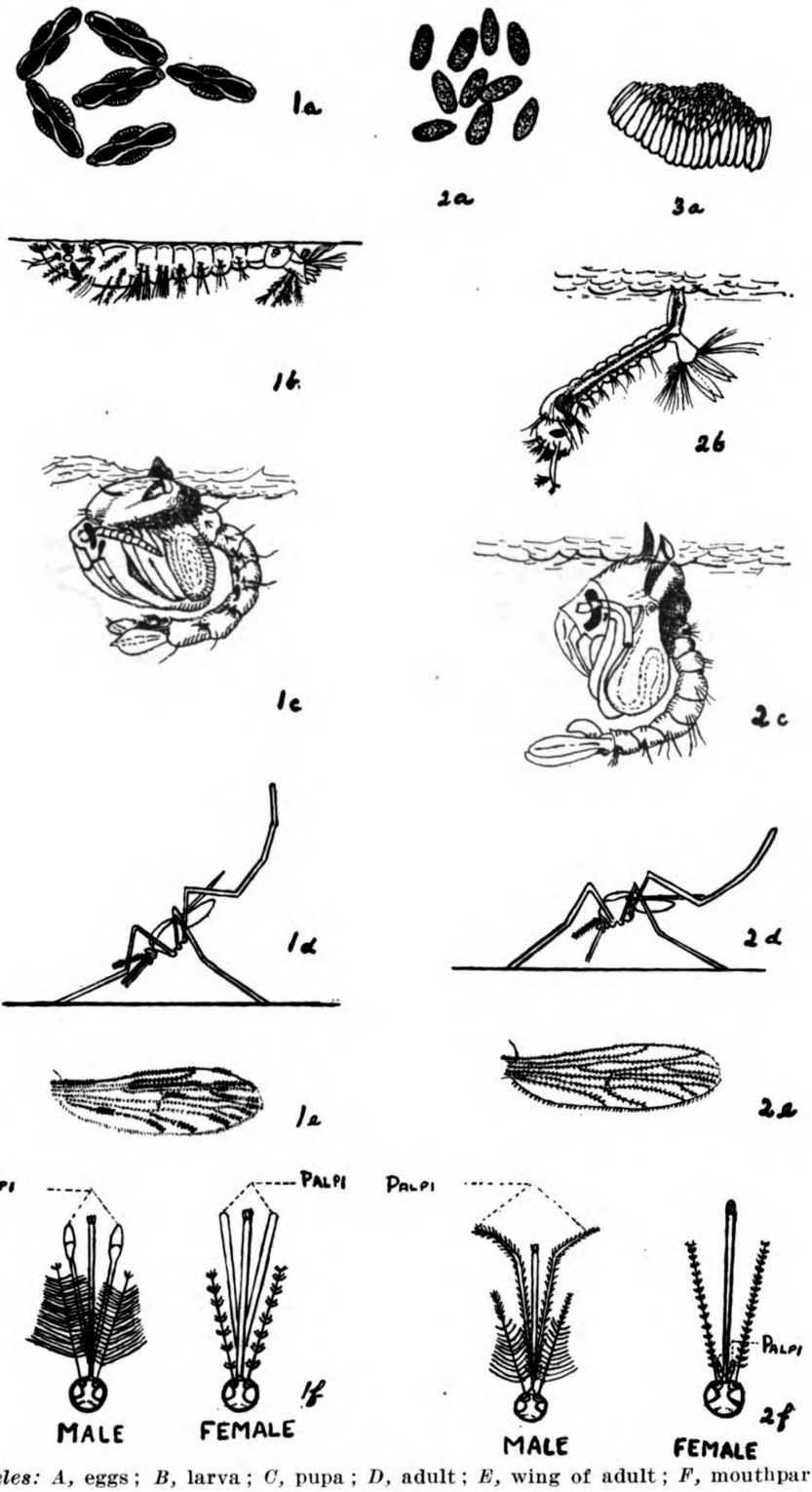
b. Mosquitoes are classified into several genera, most of which will be omitted since they are unimportant as disease transmitters. The forms mentioned in this manual are the malaria mosquitoes (genus *Anopheles*), the yellow-fever mosquitoes (genus *Aedes*), and the common house mosquitoes (genus *Culex*, etc.).

c. The adult anophelines (genus *Anopheles*) can generally be differentiated from the adult culicines (genera *Aedes*, *Culex*, etc.) by the presence of spots on the wings (fig. 38), these spots being absent in the latter groups. Other characters that serve to differentiate anophelines and culicines are the position of the body when at rest (fig. 37) and the structure of the mouth parts, especially the palpi (fig. 37). Differences between eggs, larvae, and pupae of anophelines and culicines are illustrated in figure 37.

d. To a certain extent members of the genus *Aedes* can be differentiated from the other culicines by the presence of white bands on the legs, this character being generally absent in the others. The character is, however, not a valid one, and is given here merely as a suggestion in tentative classification. For more accurate identification the worker is referred to standard texts.

e. Malaria, a common, sometimes fatal, infection caused by various protozoan parasites (*Plasmodium*) and discussed elsewhere in this manual, is transmitted by mosquitoes of the genus *Anopheles*. Among the more important species are *A. quadrimaculatus* and

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1, Anopheles: A, eggs; *B,* larva; *C,* pupa; *D,* adult; *E,* wing of adult; *F,* mouthparts of adult male and female.

2, Aedes: A, eggs; *B,* larva; *C,* pupa; *D,* adult; *E,* wing of adult; *F,* mouthparts of adult male and female.

3, Culex: A, typical raft of eggs.

FIGURE 37.—Mosquitoes. (Comparison of various stages of anopheline and culicine mosquitoes.)

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① *A. crucians.*



② *A. punctipennis.*



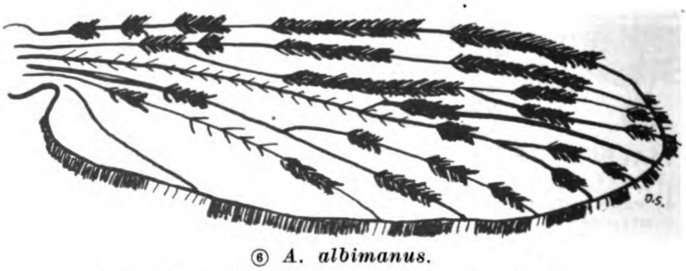
③ *A. maculipennis.*



④ *A. quadrimaculatus.*



⑤ *A. pseudopunctipennis.*



⑥ *A. albimanus.*

FIGURE 38.—Wings of *Anopheles* mosquitoes.

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A. maculipennis in the United States, and *A. albimanus* and *A. pseudopunctipennis* in Mexico and Central America. In the West Indies, Panama, and northern South America, *A. albimanus*, *A. pseudopunctipennis*, and *A. punctimacula* are the principal malaria-carrying species. Most important in the Philippines is *A. minimus*. The wings of several common species are illustrated in figure 38. Larval characters are shown in figure 39.

f. Yellow fever is a very fatal virus disease which at times may reach epidemic proportions. It is transmitted by *Aedes aegypti*. Although many other species of *Aedes* have been experimentally incriminated in the transmission of yellow fever, *A. aegypti* is the only one proven to transmit the disease naturally.

g. Dengue (breakbone fever) is a virus disease transmitted by mosquitoes. Although not a very fatal disease, during epidemics it causes a great deal of disability. Among the more important transmitters of this disease are *Aedes aegypti* and *A. albopictus*.

h. Filarial worm infections are also transmitted by mosquitoes. In these diseases the mosquitoes serve as intermediate hosts, and when feeding on a person the filarial larvae are transferred to the human host. Although the common house mosquito of the Southern states (*Culex quinquefasciatus*) is usually said to be the principal vector in filarial infections, many other species included in several genera have been incriminated.

473. Mosquito-like insects.—These are very small, hairy flies that may be confused with mosquitoes. They are differentiated from that group, however, by the absence of scales on the wings.

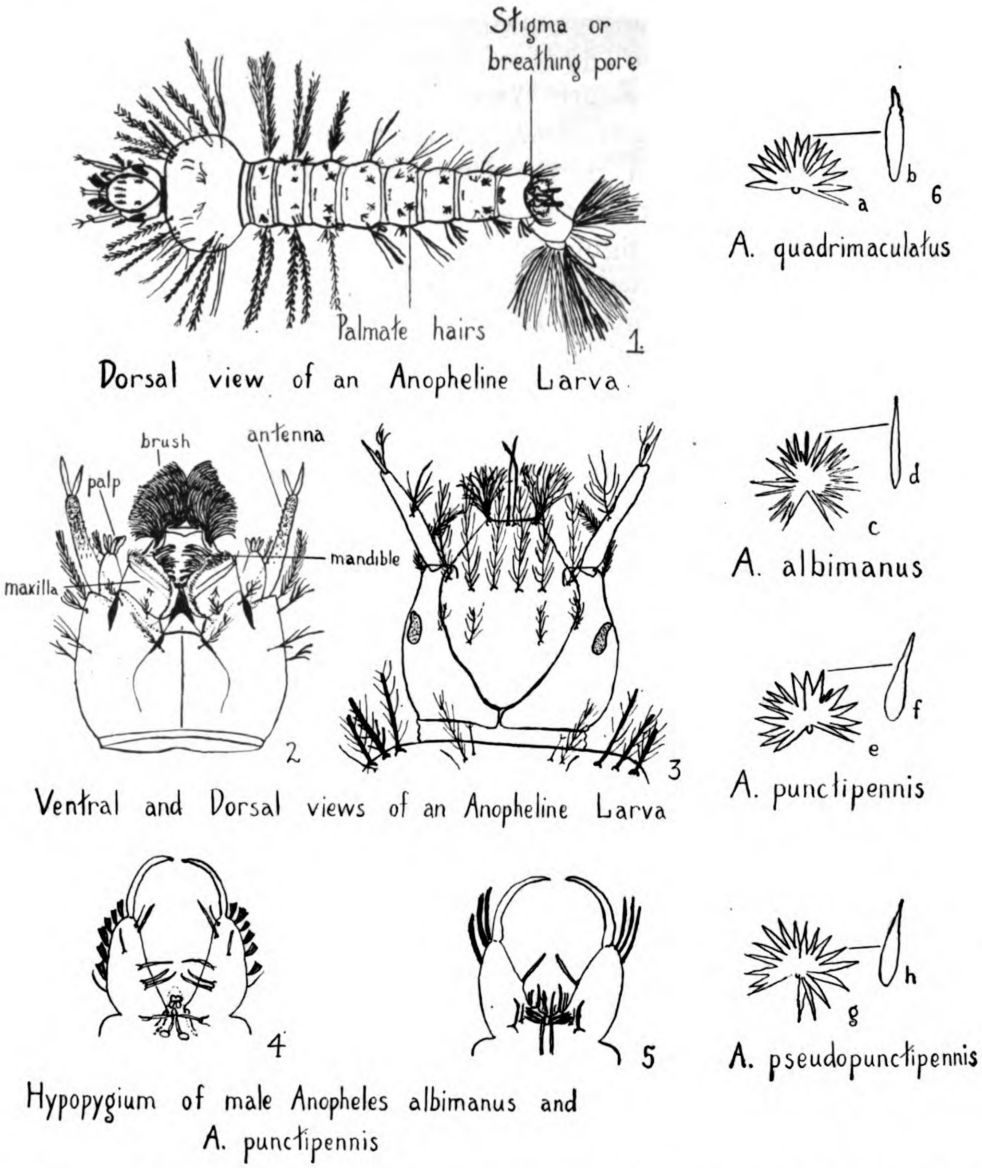
a. The sand flies (family *Psychodidae*) include several species of the genus *Phlebotomus*, some of which transmit "Pappataci" fever, kala azar, tropical sore, and some other diseases.

b. The gnats (family *Simuliidae*) include one group of medical interest, the genus *Simulium*. Certain species are instrumental in the transmission of onchocerciasis in Africa, Guatemala, and southern Mexico.

c. The midges (family *Chironomidae*) are most liable to be confused with mosquitoes. The larvae of this family are the familiar "blood worms" found so often in stagnant water. The medically important genus is *Culicoides*, since many of the species are very annoying biters. Of even more importance is the fact that species of *Culicoides* transmit a filarial worm from man to man throughout tropical Africa.

d. The crane flies (family *Tipulidae*) are of no medical importance but are mentioned in passing (fig. 40). These large, mosquito-like forms are commonly encountered in warm weather and are almost

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NOTE.—*1.* Dorsal view of an Anopheline larva. *2.* Ventral view of the head of an Anopheline larva. *3.* Dorsal view of the head of an Anopheline larva. *4.* Hypopygium (male sex organ) of an adult *A. albimanus.* *5.* Hypopygium of adult male *A. punctipennis.* *6.* Palmate hairs (hair tufts) of various Anopheline larvae; *a, c, e,* and *g* are the hair tufts; *b, d, f,* and *h* are the individual hairs.

FIGURE 39.—Structural characteristics of *Anopheles* mosquito larvae and hypopygium of adult males.

always thought by the layman to be large mosquitoes. They may be differentiated from mosquitoes quite easily, since they have no scales on the wings.

474. Stout-bodied biting flies.—This group includes the horse-flies, deer flies, stableflies, and tsetse flies. The species of this group

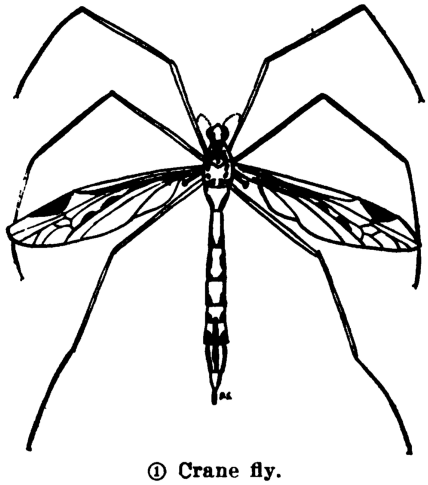
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are of interest due to disease transmission as well as annoyance from their bites.

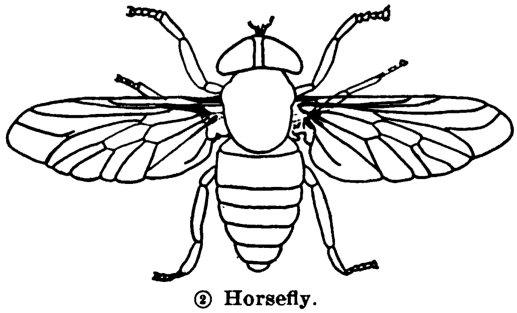
a. The horseflies (*Tabanus*) and deer flies (*Chrysops*) (family *Tabanidae*) (fig. 40) are voracious blood-feeders. In addition to this annoying habit, which is limited to the females, species of *Chrysops* serve as transmitters of a filarial worm infection and of tularaemia. Species of *Tabanus* have been recorded as transmitting anthrax to man and tularaemia to guinea pigs.

b. The stablefly, *Stomoxys calcitrans* (family *Muscidae*), is a biting fly that resembles the common housefly very much in appearance. Occasionally before a rain it will be noticed that the houseflies are very annoying, and may even bite. When this occurs, it is not the housefly as is commonly believed, but the stablefly. They may be differentiated from houseflies by their biting mouth parts (fig. 41). It has been suggested, but not proven, that the stablefly is a vector of poliomyelitis, anthrax, and tetanus. Both males and females feed on human beings.

c. The tsetse flies (family *Muscidae*) are of extreme importance in Africa, certain species of the genus *Glossina* being known to transmit



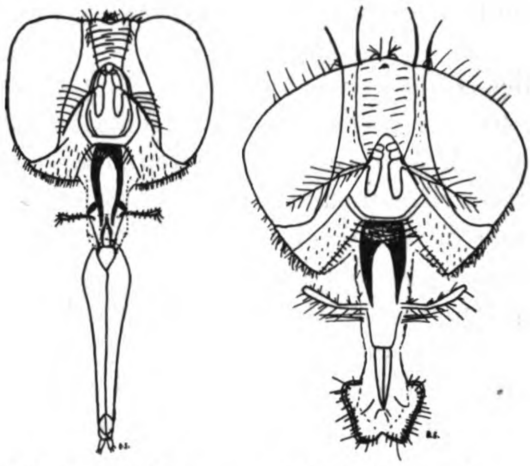
① Crane fly.



② Horsefly.

FIGURE 40.—Flies.

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① Head of stablefly showing biting mouth parts.

② Head of housefly showing nonbiting mouth parts.

FIGURE 41.—Fly mouth parts.

Gambian and Rhodesian sleeping sickness. Both males and females take blood meals. The trypanosomes causing sleeping sickness may be transmitted directly through mere mechanical action, or after undergoing a developmental cycle in the tsetse fly. These flies are unique in depositing larvae rather than laying eggs.

475. Nonbiting flies (filth flies and myiasis-producing flies).—This group includes the houseflies, flesh flies, blue and green metallic-colored flies, and other species usually associated with decaying filth and garbage. These flies are of interest because they produce disease in two ways, by mechanical transmission of harmful organisms or by direct invasion of the human body. The mechanical transmission of disease is easily accomplished by the flies' habits of frequenting filth, garbage, excreta, etc. The housefly is important in the direct transmission of many human diseases, particularly typhoid fever, and including dysentery, tuberculosis, cholera, and anthrax. In addition to transmitting disease by passing from infectious material to food, it lays eggs in the infective filth and the maggots and adults that follow are infected with the pathogenic organisms.

The housefly (*Musca domestica*) is the most important member of this group. Others are the flesh fly (*Sarcophaga*), the nonbiting stablefly (*Muscina stabulans*), the lesser housefly (*Fannia canicularis*) and the blue and green metallic-colored flies (*Cochliomyia*, *Lucilia*, and *Calliphora*).

In addition to transmitting human disease, some of the above, along with other species, serve to infect man directly. This is accomplished

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by the gravid females depositing eggs or larvae on the skin or mucous membranes. The eggs hatch and the larvae penetrate the tissues, causing myiasis.

In many species laying of eggs on the human host is purely accidental, or the females may have been attracted to a sore or open wound, but in other forms definite attempts are made to deposit eggs on certain parts of the body that the larvae may hatch and invade the tissue. Another not uncommon method of infection is for a person to eat food upon which a fly has deposited eggs. The larvae hatch, and if present in considerable numbers, may produce severe irritation of the intestinal mucosa. These larvae may be found in feces sent to the laboratory for examination and should not be confused with parasitic worms. Some species lay eggs in the nostrils, and the maggots developing in the nasal passages have been known to cause extensive tissue damage and even death. Although in many cases the sites of invasion of these maggots serve as sites for the entrance of harmful bacteria, some fly larvae are known to keep the

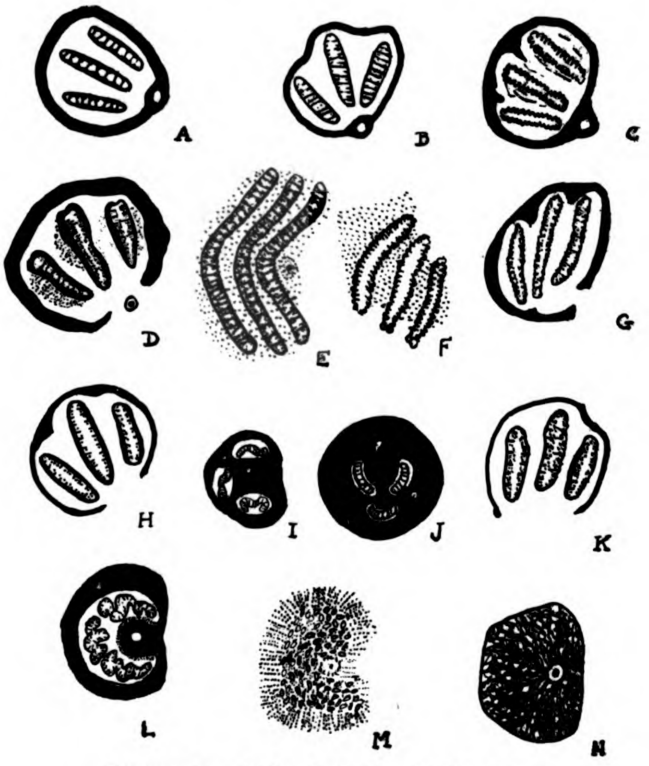


FIGURE 42.—Stigmal plates of fly larvae.

NOTE.—*A*, Blowfly (*Calliphora*); *B*, Green-bottle fly (*Lucilia*); *C*, Blue-bottle fly (*Cynomyia*); *D*, Screw-worm fly (*Cochliomyia*); *E*, Botfly (*Gasterophilus*); *F*, Warble fly (*Dermatobia*); *G*, Flesh fly (*Sarcophaga*); *H*, Black blowfly (*Phormia*); *I*, Biting stablefly (*Stomoxys*); *J*, Nonbiting stablefly (*Muscina*); *K*, Flesh fly (*Wohlfahrtia*); *L*, Housefly (*Musca*); *M*, Cattle botfly (*Hypoderma*); *N*, Sheep botfly (*Oestris*).

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wounds very clean. Maggots living in the wounds of soldiers injured in battle have been noted to remove the debris and bone fragments and thereby promote rapid healing, the basis of the maggot treatment of certain types of wounds.

Among the more important myiasis-producing flies are the flesh flies (*Sarcophaga* and *Wohlfahrtia*), screw-worm fly (*Cochliomyia*), bluebottle or blowfly (*Calliphora*), green-bottle fly (*Lucilia*), housefly (*Musca*), warble-fly (*Dermatobia*), botfly (*Gasterophilus*), lesser housefly (*Fannia*), cattle bot (*Hypoderma*) and sheep bot (*Oestris*).

Identification of the adult flies is rather difficult and will not be attempted herein, but reference should be made to standard texts. Maggots (fig. 36①) are likely to be recovered in stool examinations, and may be presented for identification from breeding areas around Army camps. These larvae, especially in the stage before pupation, can be identified by the pattern of the stigmal plates. These plates are a pair of tiny chitinous structures located at the hind end of the maggot. They may be removed from the maggot and by using the microscope compared with those of the species illustrated in figure 42. In this figure only the left stigmal plate is illustrated. For descriptive details consult standard texts.

476. Heteroptera (bedbugs, kissing bugs, cicadas, etc.).— Although the term "bug" is applied to all members of the class *Insecta*, strictly speaking only the members of the order *Heteroptera* are true bugs. The true bugs are very common, many living in water, while some abound on plants and feed on the juices. Some are blood suckers and are very troublesome or even dangerous to man.

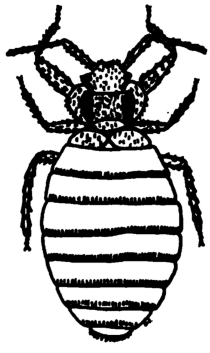


FIGURE 43.—Bedbug (*Heteroptera*).

a. The common bedbug (*Cimex lectularius*) (fig. 43) is world-wide in distribution and is a temporary parasite of man, feeding on his blood and living and breeding in the cracks and crevices of beds and other furniture, and in the walls and floors of his home. In the absence

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of the human host, bedbugs will feed on lower animals. Although the bedbug has been charged with the transmission of human disease, notably European relapsing fever, kala azar, tularaemia, and plague, there has been no definite proof, and its only interest to the medical soldier is the local irritation that some persons suffer from the bites.

b. Certain assassin bugs (fig. 44) are of very definite medical interest in some localities. These bugs, called kissing bugs because they usually take their blood meals from the lips, are important in the transmission of Chagas' disease. This disease, caused by a trypanosome

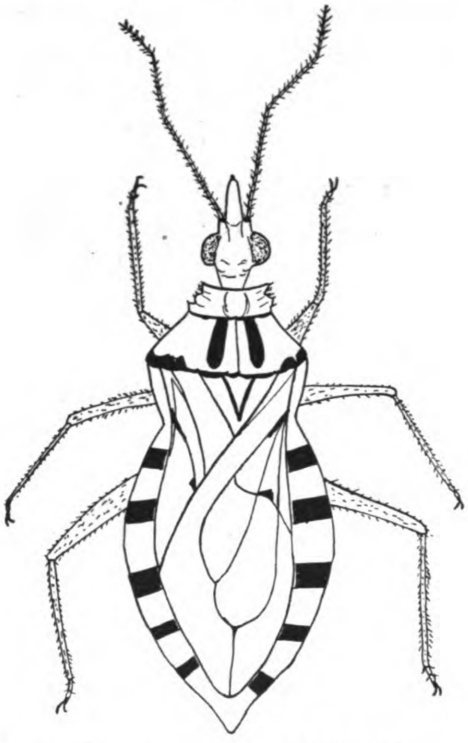


FIGURE 44.—Kissing bug (*Heteroptera*).

(*Trypanosoma cruzi*), is chiefly a childhood disease, but it not infrequently occurs in adults and is very fatal. It occurs in South America, especially Brazil. The South American kissing bug, *Triatoma megista* (synonyms *Conorhinus*, *Panstrongylus*), is particularly important to preventive medicine since it serves as the principal vector of this disease. Closely related species occur in the southern United States, but no human cases of Chagas' disease have been reported from this country.

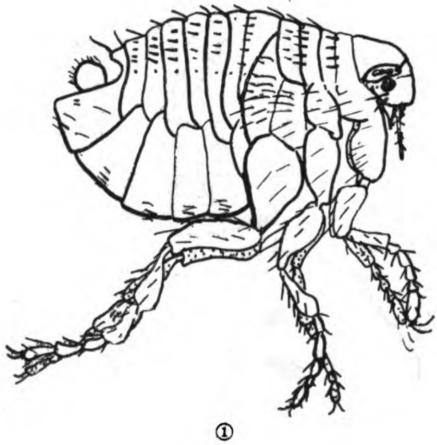
477. Siphonaptera (fleas).—There are seven species of fleas of interest to the medical soldier: four because they are associated with human disease; three because they are commonly encountered and

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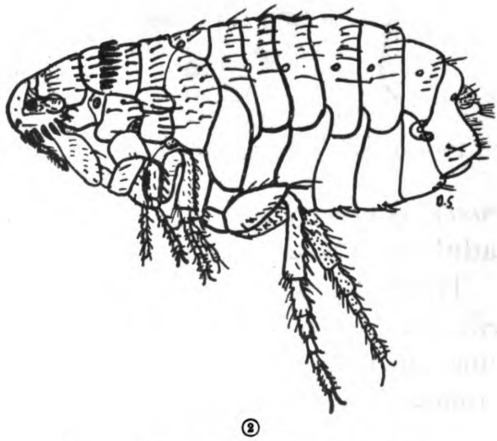
apt to be confused with those of medical importance. In this group also are to be found insects that both transmit disease and cause it directly.

a. The human flea (*Pulex irritans*) (fig. 45①) is found wherever man lives, but is widely distributed throughout the Western States, especially California. Like the bedbug, this flea lives in the cracks and crevices of the home, in the floors, rugs, and bedding, emerging at night to attack the hosts. The human flea feeds readily on dogs, squirrels, and other animals as well as on man.

b. The chigoe (*Tunga penetrans*) is the smallest flea known, and passes its life cycle as a fixed parasite of man and animals. The skin between the toes is most frequently attacked, with irritation and swelling. This results when the female fixes her mouth parts in the



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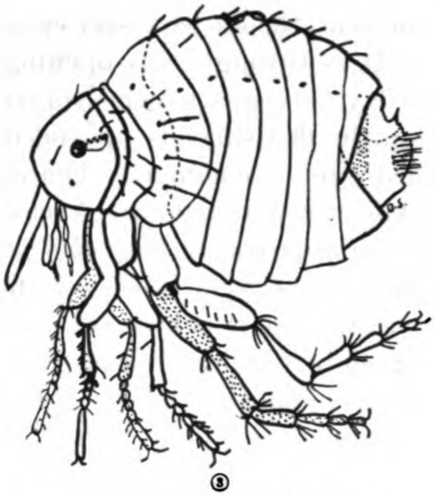


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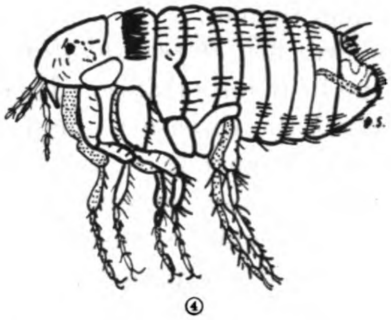
FIGURE 45.—Fleas (*Siphonaptera*).

④ Human flea (*Pulex irritans*). ② Dog flea (*Ctenocephalus canis*). ③ Chicken flea (*Echnidnophaga gallinacea*). ④ Temperature zone rat flea (*Ceratophyllus fasciatus*). ⑤ Tropical rat flea (*Xenopsylla cheopis*). ⑥ Heads of human flea (left) and tropical rat flea (right) showing arrangement of stout bristles in relation to eyes. (Note that in human flea a stout bristle is directly below the eye, whereas in the tropical rat flea it is in front of the eye.)

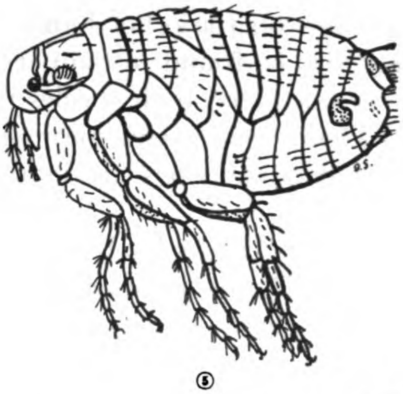
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FIGURE 45.—Fleas (*Siphonaptera*)—Continued.

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skin. The swelling encircles the entire insect except for a small opening at the hind end. It is through this opening that the chigoe is able to get air, and to lay her eggs which drop to the ground. Following this egg laying, the flea shrivels up and dies. Secondary infections of the attacked sites is common. The chigoe is widely distributed in tropical America and tropical Africa. It resembles the chicken flea very much in appearance (fig. 45③).

c. The tropical rat flea (*Xenopsylla cheopis*) (fig. 45⑤) is the most important vector of disease, particularly of bubonic plague. This flea is widely distributed in tropical regions throughout the world, and as a rule is not found in colder climates. It is distributed in the western and southern parts of the United States. Although primarily a parasite of the rat, during an epidemic it transmits plague from rat to rat, rat to man, and man to man.

d. The temperate zone rat flea (*Ceratophyllus fasciatus*) (fig. 45④) is another species of medical importance. It, too, maintains plague among rats and transmits the disease to man. It is world-wide in distribution, but for the most part is confined to the temperate zones.

e. The dog and cat fleas (*Ctenocephalus canis* and *Ctenocephalus felis*) (fig. 45②) are world-wide in distribution. The dog flea is widely distributed throughout the temperate climates of the United States and is the dominant ectoparasite of domestic pets, especially dogs and cats. The cat flea on the other hand, is more prevalent in warm climates, but may also be found in temperate regions. The cat flea has a wider range of hosts, but both species may infest man, rats, and other mammals. These two species may be found in enormous numbers in homes where cats and dogs are kept as pets and allowed to sleep in the house.

f. The mouse flea (*Leptosylla segnis*) is a common ectoparasite of mice and rats in the Eastern Hemisphere but is also widely distributed in the Americas. This species is important in transmitting plague from rat to rat.

g. The chicken flea (*Echnidnophaga gallinacea*) (fig. 45③) is of interest because it is commonly encountered and may be confused with more important species. It is very similar in appearance to the chigoe.

h. The most important human disease transmitted by fleas is plague, that age-old destroyer of mankind, caused by a bacillus (*Pasturella pestis*). Of all the fleas known to transmit this disease to man the tropical rat flea is by far the most important. The temperate zone rat flea is able to transmit this disease but does not have much opportunity to do so since plague is somewhat limited to tropical countries. The human flea is considered important in transmitting plague among

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small animals and in times of epidemic may transmit the disease to man. Although other fleas may serve in this respect during epidemics, they are of very minor importance.

i. Murine or endemic typhus, a mild infection with *Rickettsia mooseri*, is also transmitted by fleas. This infection, although not very common, is found along the South Atlantic and Gulf Coasts. Although this disease may be transmitted from man to man by human body lice, it is commonly transmitted from rat to man by the tropical rat flea and the temperate zone rat flea.

j. Some fleas, particularly the dog fleas, cat fleas, and human fleas, serve as intermediate hosts in certain helminth infections.

SECTION III

HANDLING AND SHIPMENT OF SPECIMENS

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Preparation, identification, and shipment 479

478. Collection of specimens.—*a.* Collect lice and ticks by picking them with forceps from the person or animal infested. Before picking ticks, a few drops of chloroform or ether should be placed on the specimens. This kills the tick and permits easy withdrawal of the mouth parts from the skin. If this procedure is not followed, the heads frequently remain in the puncture sites resulting in secondary infection.

b. Collect fleas from infested persons by picking. They may be collected from rats by killing the rat and combing out the fleas with a fine-toothed comb.

c. Adult mosquitoes may be collected by trapping them in a wide-mouthed bottle while resting or feeding. "Killing bottles" used for this procedure may be made by moistening cotton with chloroform or ether and stuffing it in the bottom of the bottle, then covering cotton with a circle of blotting or filter paper. This killing bottle may also be used to kill other arthropods.

d. Mosquito larvae may be collected by dipping from water in which they breed.

e. Mites may be collected by scraping infected areas of skin.

479. Preparation, identification, and shipment.—*a. Preparation.*—(1) Fleas, lice, and mites may be mounted in balsam on microscopic slides employing the usual technical methods of dehydrating and clearing.

(2) Spiders and ticks may be studied unmounted.

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(3) Larger insects may be mounted by pinning through the thorax.

(4) Arthropod larvae may be killed by dropping into very hot water. They may be preserved in 70 percent alcohol.

b. Identification.—(1) Using this manual as a guide, make a tentative identification of the specimen.

(2) Check the identification with figures and descriptions in any standard texts available, especially those given in the appendix.

(3) If the specimen appears to be of medical importance, or if it belongs to a medically important group, forward it to the Army Medical Museum, Washington, D. C., for accurate identification.

c. Shipment.—(1) Pack the specimen loosely in a small box between strips or sheets of lens paper or soft toilet tissue.

(2) Do *not* pack the specimen in cotton, because this material clings to the specimen and makes manipulation difficult.

(3) Send all data relating to specimen, including source, date, collector, conditions under which collected, and any other pertinent remarks.

(4) Mark the package "Fragile."

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CHAPTER 15

PATHOLOGICAL METHODS

Paragraphs
SECTION I. Gross pathology methods 480-486
II. Histologic technic 487-493

SECTION I

GROSS PATHOLOGY METHODS

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Restoration of body 481
Embalming 482
Fixation of tissue 483
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Gross specimens 485
Preparation 486

480. The morgue.—*a. General.*—The morgue should have light, ventilation, good artificial illumination, running water, and gas. A floor drain and an overhead water tap to which a short hose is attached are desirable. A cabinet should be provided for the instruments and these should be sterilized in a creosote solution and then thoroughly washed, completely dried, and carefully placed in the cabinet after each autopsy. If an autopsy table is not supplied one can be improvised by covering a wooden table with galvanized iron or sheet lead, allowing a gentle slope to the foot of the table and a drain with a pipe fixture to open over the floor drain. The table should be thoroughly scrubbed with a creosote solution after each autopsy. Rubber gloves should be used by all engaged in performing autopsies or handling the organs. These should be washed while on the hand with soap and water, then reversed as they are removed and the insides similarly cleaned. Both surfaces should be thoroughly dried and covered with talcum. They need not be sterilized but should be thoroughly cleaned after each use.

b. Collection of specimens.—Specimens removed during the autopsy for histologic examination are placed immediately in fixing solution. Gross specimens which are to be preserved should be kept moist and placed in a preserving solution as early as possible.

481. Restoration of body.—At the conclusion of the autopsy all excess fluid should be removed from the cavities, the rectal, vaginal, and urethral openings closed, the organs and sternum replaced, and

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the incision sewed, using the "baseball" stitch. Begin at the upper end of the incision, sew from within out, taking liberal bits of skin and muscle, keep the string taut, and use uniform stitches about 2 cm apart. The body is then thoroughly washed, taking care to remove all blood stains especially from the hair, face, and hands. If one has been careful throughout the necropsy to keep the body clean so that the blood has not dried on the skin, the cleansing is a simple matter. If the head has been opened, the base of the skull is filled with plaster of paris, the skull cap replaced and the scalp sutured in a similar manner to that just described for the body. The brain is not replaced in the skull but with the other organs in the body cavity. If the spinal canal has been opened it is stuffed with cotton or oakum over which the spines are replaced and the incision then sewed as above.

482. Embalming.—*a. Preparation of body.*—The embalming of the head is readily done by the undertaker when the chest is open, but in his absence may be done very easily by anyone else. If shaving is necessary it must be done before the face is embalmed. The undertaker's pressure bottle with several tubes armed with long metal cannulae, which are tied into the carotids and subclavian arteries, is most convenient. Pressure is obtained with a pump. If this is not available an alpha enema syringe will suffice. The nozzle is tied into the upper thoracic aorta. The open end of the aorta as well as any leaking arteries (internal mammaries) must be closed with clamps or tied.

b. Technic.—Undertaker's embalming fluid or a 10 percent solution of formalin in water, to which a few drops of eosin solution are added to give it the faintest possible tinge of pink, may be used. As the fluid is pumped into the arteries and begins to drive blood before it out of the veins, the face and ears must be massaged and molded with a gauze sponge into a natural pose, with eyes and lips closed. The hands should also be massaged until white. When the tissue becomes blanched and firm the process is complete. The same process is applied to the legs, the fluid being injected through the femoral arteries. Some formalin should be allowed to stand for a time in the body cavity. It is well to soak the organs in a 10 percent formalin solution for several hours before replacing them in the body, making incisions in the solid organs and numerous punctures in the gastrointestinal tract if they have not been opened. Undertaker's hardening compound, oakum, or cotton should be spread over the organs after they have been replaced.

483. Fixation of tissue.—Pieces of tissue not more than 1 cm in thickness will be selected from representative parts of the various

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tissues and fixed in approximately 20 times their volume of 10 percent formalin. The formalin solution will be changed the following day and again immediately before packing for mailing. Such tissue is designated "wet tissue."

484. Shipping wet-tissue specimens.—For mailing small fragments of tissue the double mailing case (item No. 41270) is satisfactory. The wide-mouthed bottle (item No. 40590 taking a No. 20 cork, item No. 77700) will prove satisfactory as it will fit in the mailing case. For protection it should be carefully surrounded with absorbent cotton. The label should be marked "First class mail, rush, specimen for diagnosis." Shipments exceeding 4 pounds in weight will be made by express after having obtained procurement authority from the Curator of the Army Medical Museum, Seventh Street and Independence Avenue, SW., Washington, D. C.

485. Gross specimens.—*a. General.*—If preservation of color is not a factor, as when an organ is to be sent to the Army Medical Museum for examination, fix in abundant 10 percent formalin taking care that all parts are in contact with the solution. Bulky specimens, as a liver, should be sliced or injected. After fixation, this may be shipped in a minimum amount of solution, using any watertight container available.

b. Preservation of color.—(1) *Procedure.*—To preserve color in gross specimens, they should be quickly washed in water to remove excess blood and placed in Kaiserling's solution No. 1. It is necessary to arrange the specimens in this solution as it is intended for them to appear when finally mounted, as they will become fixed as placed in the solution and it is difficult to alter them after this fixation. The length of time in the No. 1 solution varies from 1 to 7 days, depending on the size of the specimen.

If the specimens are very large, it is advisable to inject fixative into the blood vessels, or to impregnate the tissues by means of a syringe and needle. The specimen should be supported by cotton or suspended by strings so that it will not be in contact with the container. Avoid direct light during all steps.

(2) *Fixative.*—(*a*) *Kaiserling's solution No. 1.*

Formalin	400 cc
Water	2,000 cc
Potassium nitrate	30 gm
Potassium acetate	60 gm

(*b*) *Kaiserling's solution No. 2.*

Alcohol	95 percent

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(*c*) *Kaiserling's solution No. 3.*

Potassium acetate	200 gm
Glycerin	400 cc
Sodium arsenate	100 gm
Water	2,000 cc

NOTE.—If sodium arsenate is not available some crystals of thymol, menthol, or sodium salicylate may be used instead, but the arsenate is preferable as a fungus deterrent.

(3) *Care after fixation.*—After fixation, the specimen is drained and blotted, and then placed in 95 percent alcohol (Kaiserling's solution No. 2). When the maximum color has returned, which it will do in a few minutes or an hour or so, the specimen is to be removed from the alcohol and thoroughly washed and then preserved in Kaiserling's solution No. 3. It is necessary to watch the development of the color, for after it has reached a certain point it will begin to fade and it is impossible to restore it again. Over-fixation in solution No. 1 is to be avoided; therefore, if the specimen is to be shipped some distance, it should be run through the alcohol and forwarded in solution No. 3. It is possible to develop some color in formalin-fixed tissue, but it is not satisfactory.

486. Preparation.—*a.* The preparation of museum specimens must be left largely to the ingenuity of the operator and only a few general principles can be given. The surface to be displayed should represent as large a section as possible of the whole organ and both the exterior and interior of the organ should be shown. In the case of solid organs such as the liver, a thick slice (5 cm) should be preserved, as it is impossible to fix a whole liver properly. The thickness of the slab should allow for the removal of a thin layer at a later date to freshen the surface. This is particularly true of the lung, in which case one-half or even the whole organ may be preserved. Nothing solid should be allowed to touch the surface of the fresh tissue until it is fixed and hardened.

b. It is injurious to pack cotton firmly into a cavity, since after fixation the lining of the cavity will appear merely as a mold of the cotton. If a hollow organ must be held open it is best to distend it with fixing fluid for a day or two before cutting into it. If this is no longer possible, and it must be propped open with cotton, this should at least be inserted very loosely.

c. The heart, after being opened, should be stretched on an improvised frame in such a way as to display to advantage the chief lesion, or it may be clamped together and held in its original form by a few temporary stitches during fixation.

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d. The stomach or portions of the intestine can be filled with Kaiserling's fluid or 10 percent formalin and ligated at the ends until hardened, after which they can be bisected longitudinally. Otherwise, they may be opened, stretched on a board with thread so that the mucosa is exposed, and immersed in the fixing fluid.

e. In the case of the kidney, one-half of the organ cleanly cut, forms a satisfactory specimen.

SECTION II

HISTOLOGIC TECHNIC

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Routine paraffin method 489
Rapid paraffin method (Mallory and Wright, method No. 3) 490
Special stains 491
Decalcification 492
Giemsa's staining method 493

487. Formulas.—*a. Mayer's albumin.*

White of egg	50 cc
Glycerol	50 cc
Sodium salicylate	1 gm

Shake well together and filter into clean bottle.

b. Acid-alcohol.—One percent of hydrochloric acid in 70 percent ethyl alcohol.

c. Kinyoun's carbol-fuchsin.

Basic fuchsin (rosaniline hydrochloride)	4 gm
Phenol crystals	8 gm
Alcohol, 95 percent	20 cc
Water	100 cc

d. Decalcifying fluid.—Ten percent nitric acid in 10 percent formol saline, or the following mixture:

Formic acid	50 cc
Formalin, 10 percent	50 cc

See directions for use at end of this chapter.

e. Eosin.

Eosin Y (di-sodium tetrabromfluorescein)	0.5 gm
Alcohol	25.0 cc
Distilled water	75.0 cc

This solution will keep indefinitely.

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f. Harris' hematoxylin.

Hematoxylin	1 gm
Alcohol	10 cc
Dissolve dye in alcohol.	
Alum (ammonium or potassium)	20 gm
Distilled water	200 cc

The alum is dissolved in water with the aid of heat, and then the alcoholic solution of the dye added. The mixture is brought to a boil rapidly and then 0.5 gm of mercuric oxide (red oxide) added. The solution at once assumes a dark purple color and as soon as this occurs it is cooled by plunging the flask into cold water. For use, 4 percent glacial acetic acid is added to the mixture, as this increases the precision of nuclear staining.

g. Oil red O (Sudan III or Sudan IV) fat stain.

Oil red O	1 gm
Acetone	50 cc
Seventy percent alcohol	50 cc

488. Frozen section method.—It is possible with this method to prepare a slide for examination in a few minutes so that it is particularly applicable to "operating-room diagnosis." It is also the method of choice when it is desired to stain for fat.

a. Materials.

Automatic freezing microtome, and sharp knife.
Tank of CO_2 and connection; the tank to be mounted inverted.
A shallow dish filled with water.
A mounted needle, glass rod drawn to dull point, or pair of fine, smooth-pointed forceps.
Two pyrex test tubes.
Bottle of 10 percent formalin.
Bottle of 1 percent ammonia water.
Bunsen burner or alcohol lamp.
Glass slides and cover glasses.
Seven small glass dishes.
Harris' hematoxylin.
Eosin.
Ninety-five percent alcohol.
Absolute alcohol.
Carbol-xylol.
Canada balsam.
Blotting paper.

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b. Technic.—If the tissue has not already been fixed, a block about 0.5 cm thick is boiled for 1 minute in 10 percent formalin in a test tube and rinsed in tap water. It is placed on the freezing stage of the microtome with a few drops of water and frozen, pressing it gently with the finger during this process. Do not freeze too hard; cut at 12 to 16 microns, remove the sections from the knife with the finger, and float them in water.

Select full sections and transfer in turn to the following, which are in small shallow glass dishes:

Harris' hematoxylin, 30 to 60 seconds.
One percent ammonia water, until blue.
Tap water, rinse.
Eosin, 5 to 15 seconds.
Ninety-five percent alcohol, rinse.
Absolute alcohol, 5 seconds.
Carbol-xylol, 5 seconds.
Mount on slide in canada balsam.

489. Routine paraffin method.—*a. Materials.*—The following are required in addition to those enumerated in paragraph 488:

Oven with automatic control, temperature 56° C.
Paraffin, refined, melting point 52° to 56° C.
Containers for paraffin in oven: beakers, or casseroles and Stender dishes.
Chloroform.
Acid-alcohol.
Xylol.
Paper or metal forms for molding blocks.
Two basins or photograph developing trays.
Ice.
Rotary microtome.
Sharp knife.
Hone and strop.
Camel's-hair brushes, one pointed, one 1 to 1½ inches wide.
Twelve Coplin jars.

b. Fixation.—Specimens of tissue which are to be examined microscopically should be fixed as quickly as possible after surgical removal or after death of the patient.

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TABLE XX.—*Tissue fixatives*

Fixative	Formula	Fixation time	After treatment
Formalin alcohol	Neutral formalin 10 cc 95 percent alcohol 90 cc	12 to 24 hours	Wash in water. Transfer to 80 percent alcohol.
Absolute alcohol	Absolute alcohol (changed after 3 to 4 hours)	12 to 24 hours	80 percent alcohol.
10 percent formalin	Neutral 40 percent formaldehyde 10 parts Water 90 parts Calcium carbonate (to neutralize).	24 hours	Store in same fluid.
Zenker acetic	Potassium bichromate 2.5 gm Corrosive sublimate 5 to 8 gm Distilled water 100 cc Glacial acetic acid (just before use) 5 cc	12 to 24 hours	Wash 12 to 24 hours in running water. Transfer to 80 percent alcohol.
Helly (Zenker-formol).	Potassium bichromate 2.5 gm Corrosive sublimate 5 to 8 gm Distilled water 100 cc Formalin (just before use) 5 to 10 cc	12 to 24 hours	Wash 12 to 24 hours in running water. Transfer to 80 percent alcohol.
Schaudinn	Mercuric chloride (saturated aqueous solution) 2 parts Absolute or 95 percent alcohol 1 part Glacial acetic (proportion of 5 percent added just before use).	48 hours. (Renew after 24 hours.)	70 percent alcohol. Transfer to 80 percent alcohol.

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Carnoy	Absolute alcohol Chloroform Glacial acetic acid (add just before use)	60 cc 30 cc 10 cc	1½ to 3 hours (not over 3 hours).	Absolute alcohol, 12 to 18 hours. Chloroform, 2 changes, 1 hour each. Chloroform-paraffin, 4 hours.
Formol bichloride	Corrosive sublimate (saturated aqueous solution, about 6.9 percent). 40 percent formalin	45 cc 5 cc	At least 48 hours (change after 24 hours).	80 percent alcohol.
Bouin	Picric acid (saturated aqueous solution, 1.22 percent). Formalin Glacial acetic acid	75 cc 25 cc 5 cc	18 to 24 hours	Wash in 50 percent alcohol. 70 percent to remove picric acid. Transfer to 80 percent alcohol.

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A 10 percent solution of formalin is the most convenient and generally practicable fixative. For finer cellular studies and some special stains, it is necessary to fix in one of the chromate solutions, of which Zenker's is the most popular. If it is desired to stain for glycogen, aqueous fixatives must be avoided as glycogen is soluble in water, and absolute alcohol must be used.

Blocks of tissue should be about 0.5 cm in thickness and they should be placed in an excess of the fixative agent to insure thorough impregnation. If formalin is used, the tissue may be kept in it indefinitely. If fixation is in Zenker's, the blocks are to remain in it but 24 hours, then washed in running water for 24 hours and preserved in 80 percent alcohol.

c. Embedding and cutting.—Blocks not more than 0.5 cm thick that have been fixed in 10 percent formalin or Zenker's fluid are placed in the following:

(1) Ninety-five percent alcohol, 2 to 4 hours.

(2) Absolute alcohol, 2 to 4 hours.

(3) Chloroform, 2 to 4 hours.

(4) Chloroform saturated with paraffin, overnight in warm place at about 37° C.

NOTE.—The above steps are carried out in wide-mouthed, tightly corked bottles.

(5) Paraffin, 2 to 4 hours in oven.

(6) Embed in paper or metal forms, with desired surface down, being sure to eliminate air bubbles. To prevent crystallization of paraffin, the mold should be immersed in ice water while paraffin is still melted.

(7) Trim block so that opposite edges about tissue are parallel, leaving narrow margin of paraffin.

(8) Mount on metal block holder by heating latter, pressing on block, and immersing all in ice water.

(9) Cut sections as thin as possible, under 10 microns. Be sure knife is sharp, tightly clamped in microtome, that its edge inclines toward block just enough so that block misses back surface of knife, and that lower edge of block and knife edge are parallel.

(10) Lay sections on surface of water sufficiently warm to insure complete spreading of section (40° to 50° C.). If sections are in ribbons, they may be separated by touching while in the water with the edge of a heated scalpel.

(11) Float section onto slide that has been very lightly smeared with Mayer's albumin.

(12) Drain off water and place slide in oven for ½ hour to fix albumin.

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d. Staining.—In the following steps, use a series of Coplin jars lined up in proper order.

(1) Remove paraffin by immersing slide in xylol for several minutes, then in absolute alcohol 1 minute.

(2) Ninety-five percent alcohol 1 minute. (If tissue has been fixed in Zenker's, add sufficient iodine to the alcohol to give it a light port-wine color. This is to remove the precipitate of mercuric chloride, and requires 5 to 10 minutes. Rinse in clear 95 percent alcohol.)

(3) Wash in tap water.

(4) Harris' hematoxylin, 10 minutes.

(5) Differentiate in tap water until nuclei are blue.

(6) Eosin, 2 minutes.

(7) Ninety-five percent alcohol, to remove excess eosin.

(8) Absolute alcohol, 1 minute.

(9) Xylol, two changes, 2 minutes each.

(10) Mount in Canada balsam.

490. Rapid paraffin method (Mallory and Wright, method No. 3).—Tissues already fixed in formalin or fresh tissues boiled 2 to 3 minutes in 10 percent formalin may be used. Blocks should not be more than 5 mm thick.

a. Acetone, two or three changes, 1 to 2 hours.

b. Benzene, two changes, 30 minutes.

c. Paraffin, two changes, 45 minutes each.

d. Following the above method, proceed as in step (6) under routine paraffin method (par. 489*c*).

491. Special stains.—*a. Stain for fat.*—(1) Stain frozen sections for 1 minute in oil red 0 solution.

(2) Wash in water.

(3) Counterstain in Harris' hematoxylin for 1 to 2 minutes.

(4) Develop blue of hematoxylin in tap water.

(5) Blot as dry as possible.

(6) Clear in aniline.

(7) Mount in glycerol.

The fat is stained red.

b. Stain for acidfast bacilli.—(1) Remove paraffin in xylol (2 changes) then place in absolute alcohol for 1 minute, 95 percent alcohol 1 minute, water 1 minute.

(2) Stain 1 hour in oven at 56° C. in Kinyoun's carbol-fuchsin.

(3) Rinse in water.

(4) Decolorize in acid alcohol until only thicker portions are pink (a minute or two).

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(5) Wash in water until a pale pink.
(6) Stain in Loeffler's methylene blue a few seconds.
(7) Wash quickly.
(8) Ninety-five percent alcohol-absolute alcohol-xylol, two changes.
(9) Mount in balsam.

c. MacCallum's stain for bacteria.—(1) Paraffin sections affixed to the slide are passed through xylol and alcohols to water, and stained for 10 minutes to ½ hour in the Goodpasture mixture prepared as follows:

Thirty percent alcohol	100.00 cc
Basic fuchsin	0.59 gm
Aniline	1.00 cc
Phenol crystals	1.00 gm

(2) They are then washed in water and differentiated in 40 percent formaldehyde. This requires only a few seconds, the bright red color being washed away and replaced by a clear rose. Sections are next washed in water and counterstained in a saturated aqueous solution of picric acid. The section remains in this until it assumes a purplish color (3 to 5 minutes); then it is washed in water and differentiated in 95 percent alcohol; the red reappears and some of it is washed out as is some of the yellow of the picric acid. The sections are then washed in water and stained for 3 to 5 minutes in Sterling's gentian violet prepared as follows:

Crystal violet	5 gm
Alcohol	10 cc
Aniline	2 cc
Water	88 cc

(3) Then washed in water and immersed in Gram's iodine solution 1 minute.

Iodine	1 gm
Potassium iodide	2 gm
Water	300 cc

(4) The slides are finally blotted dry without washing, treated in equal parts of xylol and aniline until no more color comes away, passed through two changes of xylol, and mounted in balsam.

Gram-negative organisms are stained red; Gram-positive, blue.

d. Wilder's stain for reticulum.—(1) Remove paraffin with xylol, then through absolute and 95 percent alcohol to water.

(2) Potassium permanganate, 0.25 percent, 1 minute. (May use 10 percent phosphomolybdic acid instead.)

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(3) Rinse in distilled water.

(4) Place in diluted hydrobromic acid, 1 minute. (Merck's concentrated 34 percent. 1 part; distilled water, 3 parts.) After phosphomolybdic acid this step may be omitted.

(5) Wash in tap water and then in distilled water.

(6) Dip for 5 seconds or less in 1 percent uranium nitrate (sodium-free).

(7) Wash 10 to 20 seconds in distilled water.

(8) Place in silver diamino hydroxid (foot), 1 minute. (Preparation: To 5 cc of 10.2 percent silver nitrate, add ammonium hydroxide drop by drop until the precipitate which forms is dissolved. Add 5 cc of 3.1 percent sodium hydroxide and just dissolve the resulting precipitate with a few drops of ammonium hydroxide. Make up to 50 cc with distilled water.)

(9) Dip quickly in 95 percent alcohol.

(10) Reduce in: distilled water, 50 cc; 40 percent neutral formalin (neutralized with magnesium carbonate), 0.5 cc; 1 percent uranium nitrate, 1.5 cc.

(11) Wash in distilled water.

(12) One minute in 1:500 gold chloride solution (Merck's reagent).

(13) Rinse in distilled water.

(14) One to two minutes in 5 percent sodium thiosulfate.

(15) Wash in tap water.

(16) Counterstain if desired with hematoxylin. (Overstain may be reduced with acid-alcohol and then neutralized with tap water; *do not* use ammonia water.)

(17) Dehydrate and mount.

e. Masson's stain (modified).—(1) *Solutions.*—(*a*) *Picric acid solution.*

Alcohol absolute	100
Picric acid to saturate (about 6 percent).	

(*b*) *Weigert's acid iron chloride hematoxylin.*

Hematoxylin 1.5 percent in 95 percent alcohol (aged)	1 part
Liquor ferri	1 part

Mix just before using.

(*c*) *Liquor ferri.*

Liq. ferri. sesquichlorate	4 cc
Distilled water	95 cc
Hydrochloric acid	1 cc

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(*d*) *Biebrich scarlet.*

Biebrich scarlet, 1 percent	9 cc
Acid fuchsin, 1 percent	1 cc
Glacial acetic acid	0.1 cc

(*e*) *Phosphomolybdic-phosphotungstic acid mixture.*—Equal parts of 5 percent solution of each acid.

(*f*) *Fiber stain.*

Fast green (FCF)	2.5 gm
Acetic acid, 2.5 percent solution	100 cc

To prepare: Boil 100 cc distilled water and remove from flame; add immediately 2.5 gm aniline blue or fast green (FCF). Boil a short time to dissolve dye. Add 2.5 cc glacial acetic acid. Cool and filter. (Aniline blue may be used instead of fast green in same quantity and prepared similarly.)

(*g*) *Salicylic acid balsam.*—A few crystals of acid salicylic added to an ounce of balsam. (Or a few drops of salicylic acid-xylol may be placed on the section just before mounting in neutral balsam.)

(2) *Technic.*—(*a*) Paraffin sections through xylol and alcohol to saturated alcoholic picric acid, 2 minutes.

(*b*) Wash 3 minutes in running water.

(*c*) Weigert's acid iron hematoxylin, 6 minutes.

(*d*) Rinse in water.

(*e*) Biebrich scarlet, 2 minutes (4 minutes if aniline blue is used in step *h* below).

(*f*) Rinse in distilled water.

(*g*) Mordant 1 minute in phosphomolybdic-phosphotungstic acid mixture.

(*h*) Stain 2 minutes in fiber stain (fast green or aniline blue).

(*i*) Differentiate 1 minute in 1 percent aqueous acetic acid.

(*j*) Rinse in water.

(*k*) Dehydrate and mount (alcohols, acetone, xylene, (xylene, 2 changes), salicylic acid balsam).

492. Decalcification.—Tissues containing bone are fixed in 10 percent formalin, then placed in the decalcifying fluid until the lime salts are removed, changing fluid daily. This requires from 1 to 7 days and may be determined by piercing the block with a needle. Suspend the tissues in neutral 10 percent formalin over magnesium carbonate until blue litmus paper is not changed by touching the tissue to it. Then wash in running water for 24 hours, after which the blocks are ready for dehydration and embedding, beginning with step (1) of routine paraffin method (par. 489*c*).

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493. Giemsa's staining method.—*a. Reagents.*—(1) *Stock acetone.*—To 500 cc of acetone add 0.1 cc of acetic acid, glacial.

(2) *Stock Giemsa's stain.*—(*a*) Dissolve 0.5 gm of Giemsa's stain (certified powder) in 33 cc of glycerin at 55° to 60° C. for 1½ to 2 hours. To this add 33 cc of methyl alcohol which has been heated to the same temperature.

Caution: Heat alcohol in a screw-top bottle in a paraffin oven or in hot water. Keep away from flame. When the solution has cooled, filter and keep in a tightly stoppered bottle.

(*b*) A satisfactory Giemsa's stain may be also prepared in the same manner, using 0.6 gm of Azure II eosin and 0.16 gm of Azure II in 50 cc of glycerin and 50 cc of methyl alcohol. The Azures replace the certified powder.

b. Technic.—(1) Bring sections (Zenker-, Schaudinn-, or formalin-fixed sections) to water in the usual way for each technic.

(2) Rinse in distilled water.

(3) Place in Giemsa's stain 3 to 4 hours observing depth of stain at the end of each hour (should be somewhat overstained).

(4) Remove from stain, drain, blot, and wave in air to dry slightly.

(5) Differentiate in the stock acetone, under observation.

(6) Drain slightly and place in xylol (2 changes); mount in cedarwood oil (immersion).

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APPENDIX

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