Laboratory methods of the United States Army / compiled by the Division of Infectious Diseases and Laboratories, Office of the Surgeon-General, Ear Department, Washington, D.C.

Contributors

United States. Surgeon-General's Office. Division of Infectious Diseases and Laboratories.

Publication/Creation

Philadelphia : Lea & Febiger, 1919.

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MEDICAL WAR MANUAL No. 6
LABORATORY METHODS
OF THE
UNITED STATES ARMY

MEDICAL DEPT. U.S.A.

<u>Best fixative</u>
for C N S studies
expec Pappenheims stain

Glycerine aceti 1 ⎫
Absolute Alc 3 ⎬ 24 hrs
                  ⎭
then one change
in alc and proceed
to paraffin embedding

Perchan March 5. 1931

M. H. Gordon

1920

Störi Sutur
Steins Sch
P 24

Acid Pyrogallol enrichment of Medium

Medium enrichment
with acid pyrogallol
as used by Nobby for
H.P. Glands 1930

Soln A

(1) Dissolve 0·1 gram pyrogallic
acid in 10cc aq dest   = 1:100

(2) Dilute this 1: 10cc
aq dest   = 1:1000

Soln B

(3) Dissolve 0·2 gram NaOH
in 10cc aq dest   = 1:50

(4) Dilute this 1: 10cc
aq dest   = 1:500

— o —

To 9 cc of medium after
(or before?) inoc add 0·5 cc
of (2) + 0·5 cc of (4) and
Seal c̄ Vaseline. Thus
the medium contains 1:10,000 Pyrog + 1:5,000 NaOH

Laboratory Methods

OF THE

UNITED STATES ARMY

COMPILED BY THE

DIVISION OF INFECTIOUS DISEASES AND LABORATORIES

OFFICE OF THE SURGEON-GENERAL, WAR DEPARTMENT
WASHINGTON, D. C.

SECOND EDITION, THOROUGHLY REVISED

Illustrated

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1919

FOREWORD.

THIS edition of the Manual of Laboratory Methods represents a complete revision of the first edition. The chapter on special bacteriological methods has been almost entirely revised, with the addition of certain methods which have proved practicable in the Army laboratories. The chapter on sanitary examination of water and sewage has been largely revised. A complete new section on autopsy technique, with directions for preparation and shipment of museum specimens, has been added. Many minor sections have been introduced, taking advantage of the experience gained in the laboratories both in this country and in France. Where a method adopted as a standard by the Central Laboratory of the A. E. F. has differed from that used in the Army in this country, both methods are given. Special acknowledgment is made to Dr. Donald D. Van Slyke, Dr. William G. MacCallum, Captain Oswald T. Avery and Major Edward T. Tucker, for aid in the preparation of some of the larger sections.

THE EDITOR.

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INTRODUCTION.

THIS manual is in no sense a text-book. It is a collection of formulæ and technical methods which will be useful in carrying out laboratory examinations which officers of the medical corps will be called upon to perform in stationary and in field laboratories. Since officers assigned to such work have all had bacteriological training, there is no need of giving detailed instructions for routine methods familiar to every bacteriologist. However, percentage strengths of solutions and staining fluids, methods of preparing special media, etc., are things easily forgotten and not memorized even by experienced men.

It is desirable, too, to establish so far as possible uniformity of procedure in the Army laboratories, at least in those phases of the work in which standard methods can be described. The technical procedures here given are those which, in the opinion of the Surgeon-General, are the best available at the present time. An experienced worker will, of course, vary these in detail, as subsequent improvements and local conditions may indicate. There is no desire, in other words, to obstruct originality or inventiveness on the part of the laboratory worker by prescribing a rigid technic.

It is the chief purpose of this bulletin to furnish the experienced officer with a standard of comparison and to give the less experienced a guide over the difficult places.

Suggestions for the improvement of this manual for future printings are invited.

THE RESPONSIBILITIES OF THE LABORATORY.

The functions of the laboratory consist chiefly in helping to safeguard the health of the troops by making rapid and accurate diagnosis of infectious and other diseases, by which the division surgeon and his staff may be guided in prophylaxis and treatment.

The laboratory director should bear in mind that military laboratories must combine the functions of Health Department laboratories with those of diagnostic hospital laboratories.

The directors of such laboratories are responsible directly to the commanding officer of the camp base hospital, through whom he will coöperate with the sanitary inspector of the camp in any sanitary examinations in which the laboratory can facilitate control of camp conditions.

The director of the laboratory is responsible not only for the proper conduct of the laboratory and the accuracy of the reports which are issued, but also for the preparation of requisitions for the supplies which are used in the laboratory.

Such requisitions are made up in accordance with the *Manual of the Medical Department*, paragraph 477 to 490, and he should note especially paragraph 485.

GENERAL RULES FOR THE CONDUCT OF THE LABORATORY.

1. Wear apron or other protective covering over uniform when in the laboratory. This avoids carrying infectious material to mess.

2. Keep your desk and floor area clean.

3. Wash and disinfect your hands before leaving the laboratory.

4. Preserve all cultures, except liquefied gelatin plates, for one week before discarding.

5. Place all discarded cultures and slides in receptacles provided for that purpose. If they are not provided, obtain them.

6. Label everything and do it legibly.

7. If a culture be dropped on the floor, breaking the glass, do not clean it up until everything has been disinfected for one hour; wet a towel in antiseptic solution and cover the entire infected area.

8. Clean the oil-immersion lens before leaving the laboratory.

9. Keep all room temperature cultures in dark, dust-free closets.

10. Do not keep gas burners lighted when not in use. Do not allow sterilizers and water baths to boil dry. Do not leave stoppers out of bottles. Do not take more media, stains, and other supplies than you need.

11. Clean oil from the condenser, lens, and mirror of the dark-field apparatus before leaving for the day. If you do not, it will have to be done in the morning before the apparatus can be used, and will then be much more difficult.

12. Remember that it is as important to keep accurate records as to carry on accurate tests.

The following tabulation is suggested as a general plan for the organization of the laboratory:

1. The Chief of the Laboratory should organize his department so that he will be relieved of routine work, will have time to act as consultant on the laboratory findings to the hospital and to the Camp Surgeon, as well as have time for administrative detail.

2. It is suggested that the laboratory personnel be divided into groups with an individual with special training along a specific line in charge of each group. For types of work requiring technical skill and experience, such as pneumo-

coccus grouping, it is considered advisable to have one person responsible and held to this, so as to obtain uniformity of results.

3. The following plan for division of work is suggested: The personnel should be divided as far as possible according to training and the amount of work to be accomplished in the different groups.

(a) Pathology:
> Autopsy.
> Section cutting (special technician).
> Preservation and shipment of museum specimens.

(b) Clinical microscopy:
> Routine examination of blood, sputum, urine, feces, spinal fluid (cell counts), urethral discharges, dark field examinations, etc.

(c) Bacteriology and serology:
> Preparation of media.
> Bacteriology of sputum, pneumonia and streptococcus typing (special technician).
> Nasopharyngeal cultures and detection of carriers of meningococcus and diphtheria.
> Bacteriology of feces and urine for detection of typhoid and dysentery groups.
> Bacteriology of food, milk, water and sewage.
> Blood cultures.
> Wassermann tests.

(d) Chemistry:
> Clinical chemistry, special examanition of blood, urine, etc.
> Chemical examination of food, milk, water and sewage.

(e) Records and reports:
> Files and librarian.
> Journals and books.

(f) Photographer:
> For cases, specimens, gross and microscopic.

COLLECTION AND SHIPMENT OF SPECIMENS AND MATERIALS.

SPUTUM.

THE sputum from the lower respiratory passages, and not saliva or the nasopharyngeal secretions, should be submitted. No disinfectant should be added to the specimen. It is necessary to explain this to the ward attendants, as they are usually instructed to add disinfectants to all discharges. Gross contamination should be avoided by keeping the specimen bottle tightly corked except during actual collection of the specimen. Plainly label the bottle with the patient's name, rank, organization, the station from which sent, and the examination desired. Each specimen must be accompanied by requests, *in duplicate*, on Form 550, M. D.

FECES.

The specimen bottles mentioned in this circular are for forwarding specimens of feces for examination for parasites and their ova and occult blood. If bacteriological examinations for typhoid fever, paratyphoid fever, or bacillary dysentery are desired, specimens should be forwarded in glass-stoppered bottles. Patients in whose cases an examination for occult blood is desired should be placed on a meat-free diet for at least two days prior to the collection of the specimen.

The specimens or feces are to be collected in large numbers for carrier examinations for typhoid, paratyphoid, etc. Time can be economized and a system for laboratory collections established by furnishing test-tubes containing

(13)

sterile swab sticks. The specimen feces can be taken up with a swab stick and inserted into the test-tubes, upon which are labels to be used for the name, rank and organization of the subject.

URINE.

Specimens of urine for bacteriological examination or animal inoculation must be collected under aseptic conditions. Catheterization should be resorted to and no disinfectant should be added. Specimens to be examined for organisms of the typhoid-paratyphoid group should be forwarded in bile medium. Each specimen of urine (except for typhoid) must be accompanied by requests, *in duplicate*, on Form 55m, M. D.

USE OF DIPHTHERIA CULTURE TUBES.

Good illumination of the throat is essential. Remove the swab from its container, and while depressing the tongue, pass swab into pharynx, avoiding contact with tongue or other parts of mouth, and rub firmly, but gently, over any visible membrane, or if no membrane is present, over the tonsils and pharynx. Withdraw swab and rub it over the whole surface of the Loeffler serum medium, being careful that the portion of the swab previously in contact with the membrane comes in contact with the medium. The inoculation of the medium should be thorough, but the surface of the medium must not be broken.

Replug the culture tube, return the swab to its container, pack securely in cotton, and mail to the officer in charge of the laboratory.

The culture tube must be plainly labelled with the name, rank and organization of the patient and the station from which the culture is sent. The nature of the examination desired—"for diphtheria"—must also be shown. Requests, *in duplicate*, on Form 55u must accompany each culture.

COLLECTING AND SHIPPING SAMPLES OF MILK FOR CHEMICAL AND BACTERIOLOGICAL EXAMINATION.

COLLECTION OF SAMPLES.—The surgeon should request, through the commanding officer, that the delivery wagon of each dairyman supplying the command be directed to report at the surgeon's office at a designated day each month for the purpose of delivering milk samples.

A one-quart bottle should be selected at random for analysis, the bottle being labelled with the name of the dairy.

PREPARATION FOR SHIPMENT.—If samples are delivered during the early morning hours they should be placed on ice immediately. All samples should be forwarded to the laboratory on the day of collection.

The following procedure should be carried out in preparing the samples for shipment: The quart of milk must be poured 25 times between the original container and a sterile bottle or flask in order that the milk and cream may be thoroughly mixed and that clumps of bacteria may be broken up. After thorough mixing add 1 c.c. of commercial formalin to the quart (1000 c.c.) of milk and agitate thoroughly to ensure inhibition of further growth of bacteria. Then fill the sterile 60 c.c. sample bottle. When it is desired to learn the type of organisms present the formalin should be omitted.

It is essential that the bottle containing the sample be filled flush to the lower end of the stopper to prevent churning of sample with formation of butter while in transit to the laboratory. Seal with paraffin or wax and cover with a square of muslin held in place by copper wire.

Label each bottle with the name of station or command, location and name of dairy, date of collection and date of shipment. Pack securely in absorbent cotton to avoid breakage.

Samples of milk for examination must reach the laboratory prior to the 25th of each month. Milk samples other than routine may be sent to the laboratory when occasion demands.

COLLECTING AND SHIPPING CEREBROSPINAL FLUID.

The container furnished is a sterile test-tube with constriction. Just before use the cotton plug is removed, the lip of the test-tube flamed and allowed to cool.

The first few cubic centimeters of the spinal fluid may be blood-tinged. Therefore, collect the fluid in two portions, allowing the first 2 or 3 c.c. to flow into one tube and the remainder into the specimen tube. At least 6 c.c. (90 drops) are required for complete serological examination.

The cotton plug is replaced. Now seal by melting the constricted portion of the tube in the flame, taking care not to heat the fluid.

Label the tube with the patient's name, rank, and organization, character of contents (spinal fluid), and nature of examination desired. Pack securely in absorbent cotton for shipment.

Duplicate requests on Form 55u must accompany the specimen.

COLLECTING AND SHIPPING SPECIMENS FOR THE WASSERMANN AND OTHER SEROLOGICAL REACTIONS.

1. WASSERMANN AND OTHER COMPLEMENT-FIXATION TESTS.—Much of the dissatisfaction and loss of time resulting from the necessity of reporting many specimens of whole blood received for the Wassermann test as hemolyzed, burned, or otherwise unfit for examination may be obviated by medical officers forwarding clear separated serum only, as required by paragraph 5, Circular No. 6, Headquarters S. E. D., D. S. O., August 1, 1917.

2. A simple technic for collection and forwarding the separated serum follows:

(*a*) Paint the area over the veins at the bend of the elbow with tincture of iodin. Apply a tourniquet to the middle of the arm and instruct the patient to extend the arm fully,

open and close the hand several times, and then make a fist. The veins will become prominent and can be entered easily.

(*b*) Insert a needle of about 20 gauge (the ordinary Luer syringe needle) into a vein, exercising care that the needle lies flat and enters the vein almost parallel to the skin surface, thus avoiding passing through the vein. Needles should be sterilized dry in glass tubes rather than boiled, because the moisture of the latter method may cause hemolysis.

(*c*) As soon as blood flows from the needle tilt the distal (to the patient) end and permit the blood to run into a sterile test-tube or centrifuge-tube. The flow of blood can be accelerated by the patient alternately opening and closing the fist. Permit at least 3 c.c., preferably more, blood to collect in the tube, replace the cotton plug and set aside at room temperature to allow separation of clot and serum.

(*d*) In many instances the clot adheres to the sides of the tube and the separated serum cannot be removed without disturbing the clot and clouding the serum. This may be controlled by separating the clot from the sides of the tube with a sterile wire or glass rod and then permitting the tube to stand until the clot settles and the supernatant serum becomes perfectly clear.

(*e*) The clear serum should be removed with a sterile capillary pipette. This procedure is facilitated by attaching a piece of rubber tubing, such as that on a blood-counting pipette, to the end of the capillary pipette. The serum is then transferred to a Wright's capsule, one end of which has been sealed in a flame previously, or to a special serum capsule. The empty portion of the capsule is then passed quickly through a flame once or twice to create a partial vacuum and the end sealed in the flame. Care should be exercised that sufficient heat is not applied to burn the serum.

(*f*) The sealed capsules containing the serum should be

labelled with the patient's name, rank, organization, and the station from which the specimen is sent. The test desired (Wassermann, gonococcus-fixation, etc.) should be designated also. The capsules should be wrapped securely in cotton to avoid breakage in the mails.

(g) Specimens for the Wassermann test must be accompanied by requests, *in duplicate*, on Form 55q, M.D., and the *first time* the serum of an individual is tested at *this* laboratory it must also be accompanied by a Wassermann card, Form 97, M. D.

AGGLUTINATION TESTS.—The institution of prophylactic inoculation against typhoid and paratyphoid fevers has very largely obviated the usefulness of the agglutination test (Widal) as a diagnostic procedure in these diseases. The results are of value in establishing a positive diagnosis in inoculated individuals only when there is a definite increase in the agglutinating property of the serum, as shown by repetition of the test with sera collected at intervals of a week or ten days. *For early diagnosis the blood culture should be resorted to in all cases.* In bacillary dysentery the agglutination test may be of value in establishing a diagnosis; but, as a rule, the serum possesses agglutinating properties only in the severe or moderately severe cases. Isolation of the causative organism by bacteriological examination of the feces is a surer and altogether more satisfactory diagnostic procedure.

Blood specimens forwarded for agglutination tests for typhoid and paratyphoid fevers should be collected in Wright's capsules, under aseptic precautions, in order that cultures may be attempted from the clot.

COLLECTING AND FORWARDING SPECIMENS FOR THE DIAGNOSIS OF GLANDERS.

CULTURES.—The glanders bacillus can be obtained easily, in pure cultures, from the interior of suppurating glands and

nodules, which *have not yet opened to the surface*. The discharges from the nostrils, or from an open lesion, are much less satisfactory, as very few bacilli may be present, and the detection of these is difficult because of the invariable admixture of numerous other microörganisms. Glycerin-agar slants should be used. The procedure of making cultures follows:

(*a*) Select a fluctuating gland or nodule which has not yet opened to the surface, shave the overlying skin, and sterilize this area with a thick coating of tincture of iodin.

(*b*) Incise the nodule with a sterile scalpel.

(*c*) Evert the edges of the incision with thumb and middle finger, introduce a sterile swab into the center of the lesion, and, exercising care that it does not touch the skin edge of the wound, rotate it gently so as to thoroughly impregnate it with the contents of the lesion.

(*d*) Remove the cotton plug from the culture tube, flame the neck of the tube and smear the material on the swab over the surface of the culture medium.

(*e*) Carefully replace the cotton plug in the culture tube, return the swab to the tube in which it was received and forward both tubes to the laboratory.

(*f*) The presence of B. mallei in material from open lesions, nasal discharge and sputum (pulmonary lesions in man) can be determined by guinea-pig inoculation (Straus reaction). Collect discharge and forward in sealed container; if necessary, add a small amount of saline to prevent drying.

(*g*) The tubes or containers should be plainly labelled with name (human case) or name or identification mark of animal, the nature of the material, the examination requested, together with the name, rank and station of the veterinarian to whom the reports of the examination are to be forwarded. Requests, *in duplicate*, on Form 55u, M. D., must accompany each culture.

COMPLEMENT-FIXATION OR AGGLUTINATION TEST.—A posi-

tive complement-fixation test may be obtained from the seventh to the tenth day and usually persists during the course of the disease. The agglutination test may be positive in four to seven days, the content in agglutinins increasing early in the disease, but decreasing if the disease becomes chronic. All blood-serum tests are influenced by the injection of mallein or vaccine, and blood should be taken before their administration.

Blood for these tests is collected from man as per instructions under Wassermann, page 16; from horse as follows:

(*a*) Sterilize a large-sized hypodermic needle *by boiling*. Do not use phenol or other antiseptics for this purpose.

(*b*) Shave and sterilize with tincture of iodin the skin over the jugular vein.

(*c*) Make the vein prominent by pressing with the thumb below the area selected for puncture, thrust needle into vein and permit blood to flow into sterile bottle furnished for this purpose. Tightly cork after collection.

Stand toward the side of the horse, while drawing blood, to avoid danger from sudden rearing.

Label specimen plainly with name (human case) or name or identification mark of animal, the nature of the specimen, the test desired and the name, rank and station of the surgeon or veterinarian to whom report is to be returned. Pack the bottle securely in cotton to avoid breakage. Requests *in duplicate*, on Form 55u, M. D., must accompany each specimen.

WATER ANALYSIS, BACTERIOLOGICAL. DIRECTIONS FOR COLLECTING AND SHIPPING.

These directions are a transcription of the instructions in the Manual of the Medical Department, 1916:

356. At the time of forwarding the water the officer to whom it is sent should be advised of the following particulars: (1) The date, place and mode of shipment; (2) the date

and place of the collection of the water; (3) the character of the watershed, its topography, and the uses to which the country is put if inhabited; (4) the proximity of houses, barns, privies, or other possible sources of contamination to the place of collection or the source of supply; (5) the proximity of fertilized land to such place or source and whether the said land is higher or lower than the adjacent land; (6) such other information as may suggest a possible deleterious influence on the purity of the water. If the water is from a well the letter should report the depth of the well, the strata found in digging or boring it, and the depth of the water in the well.

357. The specimen should, when practicable, be collected by a medical officer. If the water to be examined is delivered through pipes or is pumped from a well or cistern the local supply pipe and all pump connections should be emptied by allowing the water to run for fifteen minutes before taking the samples.

358. BACTERIOLOGICAL EXAMINATIONS.—Samples of water for bacteriological examination should be collected in bottles furnished for the purpose. Each bottle is sterilized before leaving the laboratory, and the glass stopper is protected by a piece of heavy sterilized muslin securely wired to the neck of the bottle. The stopper should not be removed until immediately before the bottle is filled.

(a) In taking specimens from a faucet or pump (after emptying the supply pipes and connections conformably to paragraph 357) a small, gentle stream should be allowed to flow, the stopper taken out, the bottle grasped near the bottom, held in an upright position, and the stream permitted to flow into the bottle until it is filled to the shoulder. The stopper should then be replaced; both it and the cloth should be secured by carrying the wire several times around the neck of the bottle and twisting the ends tight. The stopper must be handled only by the square cloth-covered top. The lip

of the bottle must not be brought in contact with the faucet or spout, nor should the neck of the bottle or naked part of the stopper be permitted to come in contact with any object during the manipulation. The projecting flange is designed to protect the plug of the stopper, which it will do if the stopper, after withdrawal, is held by the top in a vertical position. The stopper should not be laid down and the cloth should not be handled by the fingers except in the act of securing the wire about it. When well water is to be examined the bottle should be filled directly from the bucket constantly in use for drawing the water, and from no other vessel.

(*b*) On account of the labor involved and the possibility of error, bacteriological examinations of water collected in any other than the prescribed receptacles will not be made.

(*c*) Each package should be plainly marked to show the source from which the samples are taken and the date of collection.

(*d*) The case should be marked "Water for Bacteriological Examination," and it should be forwarded by mail at the earliest moment.

(See paragraph 355a: All bottles containing fluid material sent through the mails must be securely packed in cotton in double containers.)

PREPARATION AND SHIPMENT OF AUTOPSY SPECIMENS FOR THE MUSEUM.

SOLUTIONS AND STAINS.

PHYSIOLOGICAL SALT SOLUTION.

FOR bacteriological work, physiological solution is usually made up by adding 8.5 grams of sodium chloride to a liter of distilled water. When for reasons of speed and convenience tap water is used, one should have some idea of the salt contents of the water used before relying upon it.

SODIUM CITRATE SOLUTION.

For bacteriological purposes this contains 1 per cent. of sodium citrate and 0.85 per cent. of sodium chloride.

If sodium citrate is to be used for prevention of coagulation of blood without considerably changing the volume of the blood the solution is made up to contain 10 per cent. of sodium citrate and 0.85 sodium chloride.

FIVE PER CENT. SULPHURIC ACID FOR DECOLORIZING (AS USED IN TUBERCLE BACILLUS STAINING).

Slowly allow 2.7 c.c. of c. p. sulphuric acid of a specific gravity of 1.84 to flow into 80 c.c. of distilled water. After cooling, bring volume up to 100 c.c. (St. Luke's Manual.)

ACID ALCOHOL (ORTH).

HCl	1.0 c.c.
70 per cent. alcohol	99.0 c.c.

OXALATE SOLUTION FOR BLOOD CULTURE.

Ammonium oxalate	2.0 grams
Sodium chloride	6.0 grams
In distilled water	1000.0 c.c.

ZENKER'S FLUID.

Potassium dichromate	2.5 grams
Mercury bichloride	5.0 grams
Water	100.0 c.c.

Glacial acetic acid 5 per cent. is added to this stock solution just before use.

COPPER SULPHATE SOLUTION FOR CAPSULE STAIN.

20 grams of copper sulphate crystals are dissolved in 100 c.c. of water.

STOCK STAINING SOLUTIONS.

It is convenient in stationary laboratories to keep stock stains in the form of saturated solutions. The strengths of various saturated solutions are as follows:

	Saturation strengths.
Fuchsin in alcohol	3.0 per cent.
Gentian violet in water	1.5 "
Gentian violet in alcohol . . .	4.8 "
Methylene blue in water . . .	6.7 "
Methylene blue in alcohol . . .	7.0 "
Safranin	4.0 "

Saturated alcoholic solutions can be kept and aqueous staining solutions can best be made by adding 5 per cent. of the filtered alcoholic solutions to water.

LOEFFLER'S ALKALINE METHYLENE BLUE.

Saturated alcoholic solution of methylene blue	30.0 c.c.
1 to 10,000 solution potassium hydroxide in water	100.0 c.c.

ZIEHL CARBOL-FUCHSIN SOLUTION.

Fuchsin (basic)	1.0 gram
Alcohol (absolute)	10.0 c.c.
5 per cent. phenol	100.0 c.c.

To make up this staining solution by another method, 90 c.c. of a 5 per cent. aqueous solution of phenol is mixed with 10 c.c. of saturated alcoholic basic fuchsin.

CAPSULE STAINS.—*Hiss's Copper Sulphate Method.*—Cover-slip preparations are made by smearing the organisms in a drop of animal serum, preferably beef-blood serum.

Dry in air and fix by heat.

Stain for a few seconds with—

Saturated alcoholic solution of fuchsin or gentian violet, 5 c.c., in distilled water, 95 c.c. This combination is often too weak for good results. A gentian-violet or fuchsin solution twice as strong is advantagenous. Gram's gentian violet or carbol-fuchsin can be used.

The cover-slip is flooded with the dye and the preparation held for a second over a free flame until it steams.

Wash off dye with 20 per cent. aqueous copper sulphate solution.

Blot (do not wash).

Dry and mount.

NEISSER STAIN FOR POLAR BODIES.

Methylene blue	1.0 gram
Absolute alcohol	200.0 c.c.
Glacial acetic acid	50.0 c.c.
Distilled water	1000.0 c.c.

Preparations fixed by heat are immersed in this stain for five seconds and then washed in water and counterstained with Bismarck brown or, better, safranin.

CARBOL–THIONIN.

Saturated solution of thionin in 50 per
    cent. alcohol . . . .   10.0 c.c.
Two per cent. phenol   . . . . .   100.0 c.c.
Stain for two minutes.

GRAM'S METHOD AND MODIFICATIONS.—Preparations are made on cover-slips or slides in the usual way.

It is always necessary to control Gram stains with organisms of known type.

The preparation is covered with an anilin gentian-violet solution, which is best made up freshly before use.

The staining fluid is made up, according to Gram's original directions, as follows:

Five c.c. of anilin oil are shaken up thoroughly with 125 c.c. of distilled water. This solution is then filtered through a moist filter paper.

To 108 c.c. of this anilin water add 12 c.c. of a saturated alcoholic solution of gentian violet. The stain acts best when twelve to twenty-four hours old, but may be used at once. It lasts, if well stoppered, for three to five days. A more convenient and simple method of making up the stain is as follows:

To 10 c.c. of distilled water in a test-tube add anilin oil until on shaking the emulsion is opaque—roughly, 1 to 10. Filter this through a wet paper until the filtrate is clear. To this add saturated alcoholic solution of gentian violet until the mixture is no longer transparent and a metallic film on the surface indicates saturation. One part of alcoholic saturated gentian violet to nine parts of the anilin water will give this result. This mixture may be used immediately and lasts two to five days if kept in a stoppered bottle.

Cover the preparation with this; leave on for five minutes. Pour off excess stain and cover with Gram's iodin solution for two or three minutes.

GRAM'S IODIN SOLUTION.

Iodin	1.0 gram
Potassium iodide	2.0 grams
Distilled water	300.0 C.C.

Decolorize with 97 per cent. alcohol until no further traces of the stain can be washed out of the preparation. This takes usually thirty seconds to two minutes, according to thinness of preparation. Wash in water.

Counterstain with an aqueous contrast stain, preferably Bismarck brown or safranin.

Sterling's Modification of Gram's Method.—Two c.c. anilin oil and 10 c.c. 95 per cent. alcohol. Shake and add 88 c.c. distilled water. Five grams of gentian violet are ground in a mortar and the anilin solution added slowly while grinding. Filter. This solution keeps and stains in one-half to one minute.

CLASSIFICATION OF THE MOST IMPORTANT PATHOGENIC BACTERIA ACCORDING TO GRAM'S STAIN.

Gram-positive. (Retain the gentian violet.)	Gram-negative. (Take counterstain.)
Micrococcus pyogenes aureus	Meningococcus
Micrococcus pyogenes albus	Gonococcus
Streptococcus pyogenes	Micrococcus catarrhalis
Micrococcus tetragenus	Bacillus coli
Pneumococcus	Bacillus dysenteriæ
Bacillus subtilis	Bacillus typhosus
Bacillus anthracis	Bacillus paratyphosus
Bacillus diphtheriæ	Bacillus fecalis alkaligenes
Bacillus tetanus	Bacillus enteritidis
Bacillus tuberculosis and other acid-fast bacilli	Bacillus proteus
	Bacillus mallei
Bacillus aërogenes capsulatus	Bacillus pyocyaneus
Bacillus botulinus	Bacillus influenzæ
	Bacillus mucosus capsulatus
	Bacillus pestis
	Bacillus maligni edematis
	Spirillum choleræ
	Bacillus Koch-Weeks
	Bacillus Morax-Axenfeld

CARBOL-GENTIAN VIOLET.

Saturated alcoholic solution of gentian
    violet . . . . 90.0 c.c.
Five per cent. phenol in water . . 1000.0 c.c.

This solution retains its staining powers for the Gram method of staining for a longer period than does the ordinary Gram solution, but is not as permanent as the Sterling modification.

POLYCHROME STAINS.—The Romanowsky stain depends on the formation of methylene azure and methylene violet in alkaline solution of methylene blue. When this solution is mixed with a solution of water-soluble yellowish eosin, the eosinates of methylene azure, methylene violet, and methylene blue are thrown down, as these eosinates are insoluble in water.

Wright's stain consists of a solution of these eosinates in methyl alcohol.

Any methylene blue and any yellowish water-soluble eosin issued by the Medical Department can be used in preparing the stain.

WRIGHT'S STAIN.—Add 1 gram of methylene blue to 100 c.c. of a 0.5 per cent. solution of sodium bicarbonate in water and heat for one hour, after steam is up, in an Arnold sterilizer. The flask containing the alkaline methylene blue solution should be of such size that the depth of the fluid does not exceed two and a half inches. When cool add to the methylene blue solution 500 c.c. of a 1 to 1000 eosin solution (yellowish eosin, water soluble). Add the eosin solution slowly, stirring constantly, until the blue color is lost and the mixture becomes purple, with a yellow metallic luster on the surface and there is formed a finely granular black precipitate. The precipitate is the water insoluble eosinates of methylene blue and of methylene azure and other oxidation products

of methylene blue. The end-reaction is reached when enough eosin has been added to neutralize all of the methylene blue and its oxidation products. To determine this end-reaction, place a drop of the mixture on a piece of filter paper, a slight eosin halo appears around the drop, due to a slight excess of eosin. As the precipitate is soluble in eosin, add only enough excess of eosin to get the slight halo. Collect this precipitate on a filter paper and dry in the incubator at 38° C. When thoroughly dry, dissolve 0.06 gram in 20 c.c. of pure methyl alcohol (acetone-free). This is the stock solution. For use, filter the 20 c.c. and add to the filtrate 5 c.c. of methyl alcohol.

The dry powder keeps well: the alcoholic solution does not keep well. Therefore it is better to make only enough of the solution to last a couple of months.

Method of Staining.—1. Make films and dry in the air. The film must dry quickly and must be protected from dust and dirt.

2. Cover the dry film preparation with the stain for one minute (to fix).

3. Add distilled water to the stain on the preparation, drop by drop, until a yellow metallic scum begins to form (to stain). Add the drops of water rapidly in order to prevent precipitates on the stained film; practically add 1 drop of water for every drop of stain used. Allow to stain for five to ten minutes.

4. Wash off the stain with distilled water.

5. Wash in distilled water until the film has a pinkish tint.

6. Blot dry with filter paper. Do not put on a cover-glass or mount in liquid petroleum.

Red cells are orange to pink; nuclei, various shades of violet; eosinophile granules are red; neutrophile granules are yellow to lilac; blood plates are purplish; malaria parasites: cytoplasm is blue and chromatin is metallic red to rose pink.

Caution.—Never heat a preparation that is to be stained

by Romanowsky, and use only distilled water or rain water, in all Romanowsky methods. Old distilled water should be boiled to drive off CO_2, especially for Giemsa stain.

Fontana Method for Treponemata.

The following three solutions are used:

I

Acetic acid	1.0
Formalin	20.0
Distilled water	100.0

II.

Phenol	1.0
Acid tannic	5.0

III.

Twenty-five per cent. solution of silver nitrate	5.0 c.c.
Ammonia water	1.0 drop

Dry slide in air.
Wash in I for one minute.
Wash in water.
Pour on II and steam one-half minute.
Wash in water.
Pour on III, steam for one-half minute.
Wash in water.
Mount in balsam.
Immersion oil takes out the color.

Cleansing Solution for Glassware.

Potassium bichromate (powdered)	200 grams
Water distilled, up to	1500 c.c.
Sulphuric acid conc.	500 c.c.

This solution may be used for cleaning test-tubes and other glassware used in the preparation of the Colloidal Gold

Reagent and the Lange test. Microscopic slides cleaned with this solution may be used repeatedly.

Method for preparation of chemically clean glassware:

1. Boil for thirty minutes in hot Ivory soap solution.
2. Brush thoroughly under hot tap water.
3. Rinse in running water for ten minutes.
4. Immerse in hot bichromate solution for thirty minutes.
5. Rinse in running water for ten minutes.
6. Rinse in ordinary distilled water.
7. Rinse with triply distilled water.

KLOTZ'S FLUID.

The chief advantage of Klotz's method of preserving pathological specimens is that the specimens may be preserved and shipped in Fluid No. 1.

A practical method for a Base Hospital with a large pathological service is to make up a liberal supply of Carlsbad salts with which the fluid is readily prepared.

Modified Carlsbad salts:

Potassium sulphate	40 grams
Sodium or potassium nitrate	760 grams
Sodium chloride	360 grams
Sodium bicarbonate	400 grams
Sodium sulphate	440 grams

Fix tissues three to five days or longer in Fluid No. 1:

Carlsbad salts	375 grams
Chloral hydrate	375 grams
Formalin	375 c.c.
Water	15 liters

Wash three to five hours in running water. Preserve in Fluid No. 2:

Carlsbad salts . . . . 375 grams
Chloral hydrate . . . . 150 grams
Formalin . . . . 75 c.c.
Water . . . . 15 liters

KAISERLING'S FLUID.

For preservation of colors of gross pathological specimens the following method gives satisfactory results:

1. Fix the tissue in following solution for one to five days in Kaiserling's Fluid No. 1:

Formaldehyde . . . . 200 c.c.
Water . . . . 1000 c.c.
Potassium nitrate . . . . . . 15 grams
Potassium acetate . . . . . 30 grams

2. Drain and place in 80 per cent. alcohol for one to six hours.

3. Ninety-five per cent. alcohol for one to two hours.

4. Preserve specimen in Kaiserling's Fluid No. 3:

Potassium acetate . . . . . 200 grams
Glycerine . . . . 400 c.c.
Water . . . . 2000 c.c.

NEUTRAL SODIUM HYPOCHLORITE SOLUTION ("DAKIN'S SOLUTION").

PREPARATION FROM BLEACHING POWDER.—*Dakin's. Original Method.*—A strong solution of hypochlorite is prepared by decomposing 150 grams bleaching powder (about 25 to 35 per cent. available chlorine) with 105 grams dry sodium carbonate (122 grams monohydrate ($Na_2CO_3.H_2O$) or 284 grams washing soda ($Na_2CO_3.10H_2O$)). The mixture is very thoroughly shaken, both to make good contact and to render

the precipitated calcium carbonate granular and promote its settling. It is then allowed to stand quietly and after half an hour the clear liquid is siphoned off from the precipitate and filtered through paper or a cotton plug.

A 10 c.c. portion is rapidly titrated with $\frac{N}{2}$ boric acid solution (31 grams per liter), using powdered phenolphthalein as indicator (the usual alcoholic solution of phenolphthalein will not serve) in order to determine the amount of boric acid to be added to the rest of the filtrate. The end-point is the disappearance of the pink color. Each cubic centimeter of $\frac{N}{2}$ boric acid required for the 10 c.c. sample calls for the addition of 3 grams boric acid per liter of filtrate. An excess of boric acid should be avoided, as it favors the liberation of hypochlorous acid and renders the solution less stable. It is best to add slightly less than the calculated amount. The concentrated solution thus prepared contains about 4 per cent. of sodium hypochlorite, and before use should be diluted with about 7 parts of water and titrated with $\frac{N}{10}$ thiosulphate to determine its precise hypochlorite concentration. It is then accurately diluted to the required strength (usually 0.5 to 0.45 per cent.).

PREPARATION FROM CHLORINE AND SODIUM CARBONATE.— Chlorine may be obtained as the compressed gas in steel cylinders and is easily measured by a chlorine meter manufactured for the purpose. This is a stable, economical and convenient source of chlorine. A solution is prepared containing 15 grams of dry sodium carbonate per liter (= 17.6 grams monohydrate or 40 grams of washing soda.) A measured quantity, 4.8 grams (or about 1600 c.c.) of chlorine gas is allowed to run into the solution for each liter. Ten c.c. of the solution is then titrated. If the solution is too weak more chlorine is introduced. If the solution is too strong it should be diluted to a concentration of 0.5 per cent. NaOCl with 1.5 per cent. sodium carbonate solution, which at the same time serves to correct the unduly diminished alkalinity

caused by the excess of chlorine introduced into the solution. If the final solution fails to give a momentary flash of color with alcoholic solution of phenolphthalein it should be rejected. If the solution shows color with powdered phenolphthalein it must be titrated with boric acid as described above, for preparation from Bleaching Powder, until this defect is corrected, or it must be discarded. The solution should be titrated for hypochlorite concentration every twenty-four to forty-eight hours and discarded when it falls below the desired lower limit (usually 0.45 per cent.).

If a chlorine meter is not available, chlorine gas may be run into the 1.5 per cent. carbonate solution through any improvised diffuser until the hypochlorite concentration has reached 0.5 per cent. The amount of chlorine required to give a hypochlorite concentration of 0.5 per cent. is approximately twice the amount required to cause decolorization of powdered phenolphthalein. It is therefore convenient to add powdered phenolphthalein and note the amount of chlorine required to cause its decolorization. When almost twice that amount of chlorine has been introduced, frequent titrations of the hypochlorite content must be commenced to determine the proper point at which to stop the introduction of the chlorine.

TITRATION OF DAKIN'S SOLUTION.—To 10 c.c. of the Dakin solution, add approximately 5 c.c. of a 10 per cent. solution of potassium iodid and 3 c.c. of glacial acetic acid. Iodin is liberated and dissolves in the excess of iodid present. Dilute with water to about 50 c.c. A standard $\frac{N}{10}$ thiosulphate solution is then added from a burette until the solution is just rendered colorless. The number of cubic centimeters required to effect this result multiplied by the factor 0.0372 gives the percentage of sodium hypochlorite present in the Dakin solution.

PREPARATION OF STANDARD $\frac{N}{10}$ THIOSULPHATE SOLUTION.— Dissolve exactly 24.82 grams of pure crystalline sodium thiosulphate in water and make up to exactly 1 liter. One c.c. of

this standard solution is equivalent to 0.00372 gram of sodium hypochlorite.

TITRATION OF BLEACHING POWDER.—Bleaching powders vary considerably in their "available chlorine" content, so that it is desirable to determine the available chlorine in each large batch. Bleaching powders with less than 20 per cent. available chlorine should be rejected. Exceptional samples may contain as high as 35 per cent. available chlorine.

The available chlorine content may be determined as follows: Exactly 10 grams of bleaching powder made up of small samples from different parts of the jar, in order to obtain a representative sample, are well shaken with a liter of water. After standing about six hours the solution is filtered and a 10 c.c. sample of the filtrate is titrated in exactly the same manner as in the titration of Dakin's solution. In this case the number of cubic centimeters of decinormal thiosulphate required to decolorize, multiplied by the factor 3.55, gives the percentage of active chlorine in the bleaching powder.

OTHER CHLORINE ANTISEPTICS.

Antiseptics of the chlorine group when properly applied have proved to be the most efficient antiseptics used in the present war. We are indebted to Dakin for two other important chlorine antiseptics:

"CHLORAMIN-T.[1]—Chloramin-T is the abbreviated name for sodium-toluene-sulphon-chloramin. Chloramin-T is soluble in water and can be used in stronger solution (up to 2 per cent.) than the hypochlorites. It is more stable and exerts more prolonged action than hypochlorite when acting in the presence of much blood. It is not toxic and is less irritating than the hypochlorites, but it has but little solvent action on necrosed tissue. It is well suited for use on wounds previously cleansed with hypochlorites, and in suitably dilute

[1] Dakin and Dunham: Hand-book of Antiseptics.

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solutions may be used in the eye and on other sensitive parts. It may be applied in solution or as an impregnation of gauze, or in sodium stearate cream."

The concentration of chloramin-T may be determined by titrating with $\frac{N}{10}$ sodium thiosulphate solution in exactly the same manner as in the titration of Dakin's solution. In this case, however, if a 10 c.c. sample is used the factor is 0.141.

Preparation of Chloramin-T Paste.—Prepare in hot water a 7.5 per cent. solution of sodium stearate. A 10-liter lot of this may be prepared either from 750 grams of pure sodium stearate in the required amount of water or by neutralizing 696 grams of stearic acid with 100 grams of sodium hydroxide in the required amount of water. Neutralize the free alkali in this solution by adding a concentrated boric acid solution or a dilute hydrochloric acid solution until the addition of alcoholic phenolphthalein solution to a mixture of equal volumes of the solution and 95 per cent. alcohol gives no color. Then pour the hot solution into a mixing machine (an ordinary ice-cream freezer, for example), the inner container of which is surrounded by hot water. The inner container should not be more than half-full. A hole should be bored at the bottom of the outer wooden bucket for connection with the water tap in order to allow cold water to run in and gradually reduce the temperature of the water jacket. Let the cold water enter as soon as the machine starts and regulate the flow so that the complete cooling takes about one hour. Then open the inner container and add enough chloramin-T (chlorazene) to make a 1 per cent. solution (100 grams per 10 liters). Again allow the machine to beat the paste for twenty minutes. The paddles of the mixer must turn very slowly, about thirty revolutions per minute. The paste should look perfectly smooth and have no granulations.

The concentration of chloramin-T in this paste may be determined by treating a 10-gram sample of the paste with iodid and acetic acid, diluting and titrating exactly as in the

titration of Dakin's solution. The factor for percentage of chloramin-T is 0.141.

"DICHLORAMIN-T.[1]—Dichloramin-T is the abbreviated name for toluene-sulphon-dichloramin." It must be used in an oily vehicle, being insoluble in water. "Dissolved in oily media it may be sprayed upon wound surfaces or poured into accessible parts of deep wounds." The oily solution maintains a low concentration of antiseptic in the watery or serous media, such as secretions from wounds or mucous membranes, with which it lies in contact. "It is suitable for cases requiring prolonged antiseptic treatment and for first dressings of recent wounds which do not require irrigation. It is also used for nasal antisepsis."

Note Concerning Oil Solutions of Dichloramin-T.—"Chlorcosane," which is used as a solvent for dichloramin-T, is a heavy oil prepared from paraffin wax by replacing with chlorine part of the hydrogen in the compounds contained in the wax.

It is bland, almost tasteless, with a viscosity between that of olive and castor oils. The chlorine it contains is attached to carbon and is therefore inert, as is the chlorine in common salt, and is, hence, without antiseptic qualities. Owing to the high viscosity of chlorcosane it but slowly dissolves the antiseptic dichloramin-T at room temperature. To prepare a solution it is advisable to proceed as follows: Warm about one-quarter of the required amount of chlorcosane to about 80° C., add the dichloramin-T and stir or agitate until the solution is affected, then add the remaining three-quarters of the chlorcosane at room temperature and filter, if necessary, through a dry, fluted paper.

Chlorcosane will hold from 8 to 10 per cent. of dichloramin-T in solution at ordinary room temperature. For the treatment of wounds it is rarely desirable to exceed 5 per cent., and for nasopharyngeal spraying 1 or 2 per cent. will be

[1] Dakin and Dunham: Handbook of Antiseptics.

adequate. The solution may be applied directly to the surface of a wound by any convenient method. Spraying has been found satisfactory when all parts of the wound can be reached by this means. When this is not possible a grooved director, cotton swab, medicine dropper or glass syringe may be employed. The solution, prepared as described, may be sprayed without difficulty with a power atomizer under a pressure of 15 to 20 pounds, but is too viscous for use in a hand atomizer. For use in the latter, however, it can be rendered more fluid by the addition of from 3 to 5 per cent. of pure carbon tetrachloride. It is advisable to use a solution of as high viscosity as is practicable, since prolonged contact of the applied oil is thereby promoted.

Solutions of dichloramin-T in chlorcosane are remarkably stable, considering the high reactivity of the antiseptic. The most deteriorating influence is exposure to light. Solutions should be kept in amber-colored bottles and shielded from strong light. Heat and moisture also tend to decompose dichloramin-T. Solutions kept under favorable conditions suffer no material decomposition for several weeks. When decomposition takes place it is betrayed by the separation of insoluble substances (chiefly toluene-p-sulphonamide), and solutions which exhibit an abundant deposit should be discarded. Fresh solutions, if chilled, may temporarily become cloudy or even precipitate, owing to the separation of either dichloramin-T or of paraffin wax. On gentle warming the solutions will, in such cases, become clear, and are then suitable for use.

In ordinary wounds the application may be once in twenty-four hours and the dressings very light. In gangrenous or foul wounds a more frequent application should be made, since the active chlorine is more rapidly consumed.

As a solvent chlorcosane possesses so many advantages over eucalyptol, which was formerly used as a solvent for

dichloramin-T, that there is no longer any occasion to employ eucalyptol for this purpose.

The concentration of dichloramin-T in a chlorcosane solution may be determined by titrating with $\frac{N}{10}$ sodium thiosulphate. It is best to dilute the dichloramin-T-chlorcosane solution to five volumes with chloroform or carbon tetrachloride. Ten c.c. of this are then titrated as in the titration of Dakin's solution, except that no water (other than that in the iodid, acetic acid and thiosulphate solution) is added. The factor for the percentage of dichloramin-T in the solution from which the 10 c.c. sample is taken is 0.3. Therefore, if the dichloramin-T-chlorcosane solution has been diluted before sampling to five volumes, as suggested, the factor for the percentage of dichloramin-T in the original solution will be 1.5.

CLINICAL PATHOLOGICAL WORK.

ROUTINE METHODS.

URINE.

AMOUNT in twenty-four hours.

COLOR—odor—sediment.

SPECIFIC GRAVITY.—Read by a urinometer. The normal varies from 1.015 to 1.025.

REACTION.—Tested by litmus paper is satisfactory for routine clinical purposes.

ALBUMIN.—*Heat and Acetic Acid Test.*—Boil the upper level of a three-quarter filled test-tube, add 2 or 3 drops of 5 per cent. acetic acid, note whether a precipitate forms, and boil again. Examine by transmitted light against a black background.

Nitric Acid Test.—Urine is placed in a test-tube or small, conical glass. The concentrated nitric acid is carefully allowed to run down the side of the tilted tube or glass so as to underlayer the urine. A white ring forms at the surface of contact of the two fluids.

Quantitative.—*Esbach's Method.*—The reagent is composed of:

Picric acid . . . . 10.0 grams
Citric acid . . . . 20.0 grams
Distilled water to make . . . . 1000.0 c.c.

A special tube (Esbach albuminometer) is filled to the mark U with filtered urine and up to the mark R with reagent. The tube is closed, well mixed by inverting, but not shaking,

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and set aside for twenty-four hours. The sediment represents the albuminous bodies and is read off on the scale in grams pro mille.

The reaction of the urine should be acid, and if not so naturally it should be acidified by the addition of acetic acid. In case the specific gravity exceeds 1.006 to 1.008 or the albumin content is greater than 4 per cent. (as indicated by solidification on boiling the acid urine) the solution should be appropriately diluted with distilled water. In making comparative tests on the same patient the tubes must be read at as nearly the same temperature as possible each day.

This method gives only approximately accurate results, but is sufficient for most clinical purposes if used with the precautions noted.

SUGAR.—*Benedict's Test.*—Described under Special Determinations in the section on Quantitative Analytical Methods.

Fehling's Test.—Two solutions.

I.

Copper sulphate . . . . 34.64 grams
Distilled water . . . . 500.0 c.c.

II.

Potassium and sodium tartrate . 173.0 grams
Potassium hydroxide . . . . 50.0 grams
Distilled water . . . . . . 500.0 grams

Equal amounts of I and II mixed in a test-tube and diluted with four times as much water and boiled. Then urine is added (1 c.c.). Boil again. Sometimes the mixture turns green. This is not due to sugar, which is indicated only by a red-yellow precipitate.

INDICAN.—Reagent is Obermeyer's reagent, which is a 2 pro mille solution of ferric chloride in concentrated hydrochloric acid.

Four c.c. of this reagent is mixed with an equal quantity

of urine, about 1 c.c. of chloroform added, and the tube inverted several times. When indican is present it is broken down by the action of the reagent with liberation of indigo, which is taken up by the chloroform and shows blue.

BILE.—*Smith's Test.*—Five c.c. of urine are placed in a test-tube and overlaid with 2 or 3 c.c. tincture of iodin which has been diluted with alcohol in proportion of 1 to 10. In the presence of bilirubin a distinct emerald-green ring is seen at the zone of contact.

ACETONE.—1. *Legal's Test.*—To several c.c. urine, or better, distillate of urine to which has been added a little phosphoric acid solution (1 gram per liter), are added a few drops of strong solution of sodium nitroprusside and sodium hydrate. A red color appears which in the presence of acetone rapidly changes to purple or violet red when acetic acid is added.

2. *Acetone Test.*—Make a weak solution of sodium nitroprusside by dropping a crystal into 5 to 10 c.c. of distilled water. Add 1 to 2 c.c. of this solution and a few drops of glacial acetic acid to 5 c.c. of urine and stratify strong ammonia over the mixture. If acetone is present a purple ring will appear at the junction of the two fluids.

DIACETIC ACID TEST.—A few drops of 10 per cent. ferric chloride are added to about 10 c.c. of urine. If there is a precipitate, filter and add a few more drops of ferric chloride. If a Burgundy-red color develops the reaction is positive. In this test, color will also develop if the patient has been taking phenol, salicylates, antipyrin, or aspirin. In that case the color is purplish. If this happens, dilute the urine with equal parts of water and reduce the volume by boiling to the original volume of urine alone. Testing after this the color due to the drugs will be unchanged, whereas if it were due to diacetic acid it will have disappeared or be very much more faint.

EXAMINATION OF URINARY SEDIMENT.—All specimens should be centrifuged and a drop of the sediment used for

examination. Macroscopic examination of the sediment often gives an idea of the character of a deposit, but should not be depended upon for diagnosis.

For microscopic examinations all specimens should be centrifuged and a drop of the sediment observed immediately. It is well to use a cover-slip both for the purpose of spreading the objects in a thin layer and also to protect the lens from moisture. The low-power objective should be used.

Casts.—These are of three general classes, cellular, granular and the amorphous. *The cellular casts* are made up of the degenerated kidney cells or of leukocytes. *The granular casts* may have fine or coarse granules and are so classified. Every gradation may be found between the cellular and the coarsely granular casts. *The waxy casts* are highly refractive, homogeneous, and may be white or yellowish in color. *Hyaline casts* are very pale, have little refractivity and are difficult to see unless the light is almost shut off. It is sometimes advisable to stain them with Lugol's solution or with anilin violet.

Cylindroids.—These are bodies resembling hyaline casts, but distinguished from them by the fact that they have tapering end, often going on to a thread-like process.

Cells.—Epithelium of various types, leukocytes and finally red blood cells may be seen. The latter are difficult to recognize. They may appear as slightly cupped circles, often with a faintly greenish iridescence. One of the most helpful points in their recognition is the crenation of the cell margin.

Crystals.—Uric acid crystals have great variety of forms, the commonest being the "whetstone" form. As a rule the color is yellow, tawny or brown. Some of the forms may be confused with hexagonal cystin crystals and with the dumb-bell forms of calcium oxalate.

Calcium Oxalate.—Crystals of the so-called "envelope" form are very common. The dumb-bell shape also is often seen.

The large prismatic crystals of ammonia magnesium phosphate (triple phosphate) are common in alkaline urines.

SPUTUM.

To be examined for:

GROSS APPEARANCE.—Color—viscidity.

Blood.—Bright; tuberculosis, hemorrhage, cardiac disease. Rusty; pneumonia.

Pus.—Cheese particles.

MICROSCOPIC EXAMINATION.

Fresh sputum.

Curschmann's spirals.

Elastic Tissue.—Boil with NaOH 10 per cent. solution until solution is homogeneous, centrifugalize and examine sediment pressed out on slide. Indicates destruction of lung tissue (lung abscess, bronchiectasis, gangrene, infarct, tuberculosis), in which it is present in 90 per cent. of the cases.

Crystals.—Charcot-Leyden; hematoidin.

Tubercle Bacilli.—Examination for tubercle bacilli described under section on Tuberculosis.

Pneumococci.—Grouping. (See section on Pneumonia.)

Streptothrices.—Especially Streptothrix bovis communis (Actinomyces). It may be demonstrated in fresh sputum. Appropriate lumps selected are treated with 1 per cent. NaOH, crushed between cover-slip and slide; examined directly with high, dry lens. Or the sputum may be smeared and fixed by heat and stained for five to ten minutes with anilin-water-gentian violet. The smear so stained is washed in normal salt solution, dried between filter paper and transferred for two or three minutes to a solution of iodopotassic iodide (1 to 100). The smear is again dried between filter papers and decolorized by xylol-anilin (1 to 2), washed in xylol and examined. The mycelium assumes a dark blue color.

BLOOD.

COUNTING.—Apparatus needed consists of a counting chamber, ruled according to Neubauer; pipettes reading

1–101 and 1–11; the former for red blood cells, but it may also be used for white blood cells; the latter for white blood cells only. The latter form is also most convenient for counting the cells in spinal fluid.

Solutions for Counting Red Blood Cells.—Hayem's fluid is composed as follows:

Mercuric chloride	0.5
Sodium sulphate	5.0
Sodium chloride	1.0
Distilled water	200.0

For Counting White Blood Cells.—The diluting fluid is preferably 1.5 per cent. solution of acetic acid.

To make dilutions for red blood cells, draw blood up to the mark 0.5 and then fill to mark 101 with Hayem's fluid. Shake well, blow out two or three drops and then place a drop on the counting chamber and adjust cover-slip. Allow the preparation to stand, so that the cells will gravitate to the bottom of the film of blood and rest on the surface of the counting chamber.

Count cells in 100 small squares and multiply their number by 8000 to get the total count per cubic millimeter. This figure 8000 is derived from the facts that there are 400 small squares to a square millimeter; that the chamber is $\frac{1}{10}$ mm. deep; and that the dilution of the blood in the pipette is 1 to 200. Hence, 200 x 10 x 4 = 8000.

The white blood estimation can also be made in the 1 to 101 diluting pipette. In this case blood is drawn to the mark 1 and the diluting fluid to 101. The preparation is mixed and a drop made in the usual way. Count now all the leukocytes in the 9 large squares. Divide this figure by 9 to get the number of cells in one square millimeter. Then multiply by 1000 (cell depth 0.1 mm. and dilution 1 to 100) to get the number of cells per cubic millimeter.

If the white pipette be used, blood is drawn to the 0.5 mark and the pipette filled with the diluting fluid to the 11 mark. All large squares are counted and the average for one square taken. This is then multiplied by 20 for the dilution and 10 for the depth of the chamber.

DIFFERENTIAL COUNT.—Smears are made either between two cover-slips or on two slides. For making smears for differential blood counts the slide method is strongly advised against, as it greatly increases the error. It is of prime importance to have the cover-slips free from grease and grit. New covers should always be washed with soap and water, treated with acid and rinsed in water, and then stored in alcohol until they are desired for use. The approximate number for immedate use are removed and dried with a handkerchief or soft cloth, avoiding lint. It is desirable to have a flat, camel's-hair brush at hand to dust off each cover-slip immediately before using.

A small drop of blood is placed in the center of one cover-slip and quickly a second cover is placed over it until there is a suitable spread of the blood. They are then drawn apart with a rapid, sliding motion and allowed to dry in the air. The time allowed for spreading between the cover-slips can only be judged by experience. Too short a time gives a thick smear, while if too long is allowed the slips will stick together. An ideal smear is one in which the cells practically touch, but without overlapping.

Staining.—The smear is dried in the air and Wright's staining solution is dropped on the smear and allowed to stand for one minute. Then distilled water is dropped upon the stain until it becomes very dilute and a metallic iridescent scum appears on the surface. The diluted stain is allowed to act for three to four minutes, when it is washed off with distilled water. It is then blotted and examined. Sometimes if the preparation looks very bluish and under the microscope the red cells have a greenish-blue tinge the proper

pink color can be brought out by washing the smear quickly with a few drops of the original undiluted staining fluid.

A convenient method of using Wright's stain in the field is as follows: Four Copeland jars are set up. The first contains undiluted Wright's stain, the second distilled water, the third a 1 per cent. dilution of Manson's stain and the fourth water.

1. The blood slide is dropped into the undiluted Wright's stain for one to two minutes.
2. Transfer to distilled water for three minutes.
3. Transfer to dilute Manson's for one-half minute.
4. Wash in water.

The advantages of this method are its economy in staining fluid, the facility with which a large number of slides can be stained and the reënforcement of the blue cytoplasm stain by the use of Manson's borax methylene blue. Before the Romanowski stains were discovered, Manson's was the best single agent for staining malarial organisms, and it still is of great value in reënforcing the cytoplasmic stain, which is not as well brought out by the Romanowski methods.

Manson's stain is prepared by dissolving 2 per cent. of methylene blue, preferably medicinally pure, in a boiling 5 per cent. solution of borax. After the blue is dissolved the solution is cooled and filtered and is diluted up each day for use, the stock solution being permanent.

MALARIA.—Excellent preparations to demonstrate the malarial organism can be made by the method just described. Fresh preparations of blood can be examined by pressing a very small drop of blood between a cover-slip and a slide and observing with an oil-immersion lens. If large forms with pigment are present the pigment can be seen moving about.

FECES.—Macroscopic examination may reveal blood, mucus and pus, also gross particles of undigested food, the segments of tape-worms and other parasites. Microscopic examination should be made as soon as possible after the stool

is passed.   If amebic dysentery is suspected the stool must be kept warm until examined, and if a warm stage is not available the slide should be warmed from time to time over a gentle flame.   The specimen to be examined is fished with a large platinum loop, the drop placed on a slide and covered with a cover-slip.   The low power suffices to demonstrate the ova of parasites, but the ameba is seen best with the high, dry lens.

OCCULT BLOOD IN FECES.—PHENOLPHTHALEIN TEST.— *Principle.*—Phenolphthalein is reduced by zinc and alkali to its derivative, phenolphthalin, which is colorless even in alkaline solution.   By proper oxidizing agents the colorless phenolphthalin may be oxidized back to phenolphthalein. Hydrogen peroxide alone does not act as such an agent, but minute amounts of hemoglobin cause it to perform the oxidation.   Other matter of plant and animal origin may similarly activate the peroxide, but under the conditions used the occurrence of the reaction with fecal matter indicates the probable presence of blood.   Before carrying out this test it is essential that the patient be kept on a meat- and soup-free diet for at least two days, as these articles of diet give a positive test, as do also sodium salicytate and acetyl salicylic acid when given by mouth.

Reagent.—100 c.c. of a 20 per cent. solution of NaOH is mixed with 2 grams of phenolphthalein and 20 grams or more of zinc dust.   The bright rose-colored solution is heated gradually until it has become decolorized to a slightly yellowish tint as the result of reduction of the phenolphthalein to phenolphthalin.   The supernatant fluid is poured into a colored glass bottle and access of air prevented by addition of a little paraffin oil (20 to 30 grams), which floats upon the top.

Technique.—About 10 grams of the feces to be tested is boiled with 100 c.c. of distilled water and the mixture allowed to cool.   To 2 c.c. of the supernatant liquid is added 1 c.c. of the phenolphthalin reagent and 2 or 3 drops (not more) of 3 per cent. hydrogen peroxide.   If blood is present a pink

color will develop, owing to the reoxidation of the phenol-phthalin to phenolphthalein. Approximately o.2 mg. of blood per 10 grams of feces can be determined by this procedure.

BARBER'S METHOD OF HOOKWORM SURVEY.

Ring large slide with wax pencil.

Mix a good-sized lump of feces with glycerin and saturated sodium chlorid solution (proportions not important).

Place drop of mixture in ring on the slide.

Focus on the surface and in the center of the drop, *i. e.*,
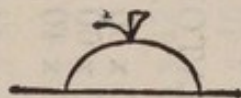
The eggs float on the surface and rise to center of the drop.

Advantages:

1. Large amount of feces easily examined.
2. Saving in cover-glasses.

THE DETECTION AND IDENTIFICATION OF ENTAMŒBÆ HISTOLYTICA.

Two species of parasitic amœbæ occur in the intestinal tract of man; one, *Entamœbæ histolytica*, is pathogenic, the other, *Entamœbæ coli*, is harmless. Neither has been cultivated.

COLLECTION OF SPECIMENS.—If active dysentery is present a freshly passed stool should be examined immediately. If no amœbæ are found a saline purge must be given (saturated magnesium sulphate), using perhaps ¼ ounce for a patient with active dysentery and the full dose (1 ounce) for a patient with little or no symptoms. Do not use castor oil, as the drop-lets cause delay in the examination for cysts. Also specimens obtained by passing a rectal tube or by giving enemata fail to reach the ascending colon, which is a frequent seat of amœbic lesions.

Both the first and second specimens of stool passed after purging must be saved for examination. Do not attempt to keep them warm during transmission to the laboratory, but

CLASSIFICATION OF OVA.

I.—CLASSIFICATION OF OVA OF CESTODES.

	Dimensions in M.	
No operculum { A single membrane { Thick and opaque { Ova spherical	21 – 56	Tenia solium.
Ova ovoid	35 x 25	Tenia saginata.
Thick and transparent	30 – 40	Dipylidium caninum.
Two thin and transparent membranes		Davainea madagascariensis.
Three transparent membranes	68	Hymenolepis.
Three transparent membranes, the third constituting the piriform apparatus	45 – 50	Bertiella satyri.
An operculum: ova brown {	75 x 45	Dibothriocephalus latus.
	60 x 41	Dibothriocephalus parvus.
	58 x 41	Diplogonoporus grandis.

II. CLASSIFICATION OF OVA OF TREMATODES.

	Dimensions in M.	
No operculum { Ova with a terminal spine	150 x 60	Schistosomum hematobium.
Ova with a lateral spine	150 x 60	Schistosomum mansoni.
Ova without a spine	75 x 40	Schistosomum japonicum.
An operculum { Large ovoid ova, 80 m. to 190 m. { Ova do not contain an embryo, when in the fecal matter	170 x 84	Fasciola gigantica.
	140 x 80	Fasciola hepatica.
	150 x 80	Fasciolopsis rathouisi.
	150 x 72	Gastrodiscus hominis.
	125 x 72	Echinostomum malayanum.
Ova do not contain an embryo	125 x 75	Fasciolopsis buski.
	125 x 75	Watsonius watsoni.
Ova do not contain an embryo	100 x 53	Echinostomum ilocanum.
Ova do not contain an embryo	95 x 55	Paragonimus westermanni.
Ova bulging on one side	40 x 25	Dicrocelium lanceatum.
Operculum prominent; prolongation at the non-operculated pole	30 x 16	Clonorchis sinensis.
Small ova less than 50 m. { Operculum prominent; no prolongation	30 x 11	Opisthorchis felineus.
Ovoid	34 x 21	Opisthorchis noverca.
Ovoid	29 x 11	Metorchis truncatus.
Ovoid	25 x 16	Heterophyes heterophyes.

III. CLASSIFICATION OF OVA OF NEMATHELMINTHES.

				Measurement	
Nematodes	Wall smooth	Thick	Bulging on one side, flat on the other	50 x 23	Oxyuris vermicularis.
			Regular ovoid	57 x 39	Physaloptera caucasica.
			Clear plug at each pole	55 x 25	Trichocephalus trichiuris.
			2 to 4 blastomeres	60 x 40	Ankylostomum duodenale.
				60 x 40	Ternidens deminutus.
		Transparent	2 to 4 blastomeres	70 x 40	Necator americanus.
			4 to 8 blastomeres	70 x 40	Esophagostomum brumpti.
				80 x 48	Hemonchus contortus.
			8 to 32 blastomeres	80 x 45	Trichostrongylus probolurus.
				83 x 45	Trichostrongylus instabilis.
				87 x 48	Trichostrongylus vitrinus.
	Wall ornamented	Ova with a single envelope,	Thick embryo, folded in two	54 x 32	Strongyloides intestinalis.
			Thin, twisted embryo	80 x 55	Metastrongylus apri.
			Mammillated, brown	60 x 44	Ascaris lumbricoides.
			Regularly cribbed with depressions, yellow	75 x 65	Ascaris canis.
			Cribbed with depressions, except at the poles, brown	66 x 42	Eustrongylus visceralis.
Acantho-cephalus	Ova with three envelopes; embryo with rows of spines		Ova have shallow lozenge-shaped depressions	85 x 45	Gigantorhynchus gigas.
				100 x 50	Gigantorhynchus moniliformis.

4

they should be examined immediately or at least within the first half-hour after being passed. If no gross blood or mucus is present a little mucus can usually be obtained by whipping the fluid stool with a wire.

A preliminary search should be made with the low power ($\frac{2}{3}$ objective). The organism is large (20 to 40μ) and its protoplasm possesses a peculiar index of refraction. The nucleus consists of a faint "ring" of protoplasm very small in proportion to the size of the cell and very difficult to see. In acute cases the amœbæ are always motile, and sluggish motility usually persists even at temperatures of 25° and 20°C. In cold weather, if no warm stage is available, after suspicious cells have been located, their motility may be tested by placing the microscope with its specimen in the incubator for several minutes and then examining promptly at room temperature.

Definite motility is a great aid in the identification of amœbæ. In acute cases it is an essential criterion. Occasionally chronic cases occur in which amœbæ are in a stage of "preëncystment," motility no longer being possible. However, the identification of amœbæ that are non-motile can only be made with safety after considerable experience.

There are two phases in the life cycle of amœbæ, namely, the motile trophozoite and the encysted stage. The cysts are small hyaline "discs" (about 15μ). Under the oil immersion several nuclei can be seen. Typical *E. histolytica* has 4 nuclei in its cysts though the number varies; *e. g.*, 2, 3, 4, 6 or 8 nuclei may be present in the rarer atypical forms. Typical *E. coli* has an 8-nucleated cyst, though the number may exceptionally vary from 2 to 16. Gram's iodin is of value in differentiating cysts from vegetable matter.

In the examination of pus from a liver abscess at operation it is not ordinarily to be expected that amœbæ will be found in the discharge during the first few days, but only after material from the walls of the abscess begins to discharge. The central portion of the abscess usually contains only thoroughly

necrotic material, the amœbæ being found in the active lesions at the edges.

Amœbæ are more readily demonstrated in fresh material than in stained preparations. If it is desired to study their morphology they may be stained as follows: Make a moderately heavy smear on a cover-slip and float at once face down while wet on Zenker's fluid. After five to ten minutes wash the cover-slips in water for at least two or three hours. Stain with Delafield's hematoxylin for five minutes and wash with tap water until very blue (ten to twenty minutes). Dehydrate in a series of alcohol (25 per cent., 50 per cent., 75 per cent. and 100 per cent.). Clear in oleum origani and mount in balsam. This method is a wet process. *The smear must not be allowed to dry at any stage.*

Differentiation of *E. coli* and *E. histolytica.* It is often very difficult to decide from microscopic appearances alone whether a given amœba is harmless or pathogenic. In borderline cases the differentiation of the two species is so difficult that it should be left to an expert protozoölogist.

The decision as to whether a given patient should be treated is usually not difficult. If active symptoms exist and if definite amœbæ are present specific treatment (emetine) should be used. If it is certain that no symptoms have ever existed and amœbæ are found it is usually safe to withhold treatment especially in northern climates provided the patient is kept under observation. If there is a history of dysentery, and even if it is *proved* that only *E. coli* are present in the stools, it is often safest to give specific treatment. The presence of *E. coli* does not exclude a latent infection with *E. histolytica.*

PREPARATION OF SALVARSANIZED SERUM FOR INTRA-SPINOUS INJECTION.

The purpose is to obtain serum which is sterile and as nearly as possible hemoglobin-free. For this reason scrupulous care should be exercised in the cleaning and sterilizing of

glassware which is used. It is advisable to wash all glassware thoroughly in soapsuds and, if thought necessary, with sulphuric acid-potassium bichromate mixture. If this is done, very thorough subsequent rinsing in tap water and finally in distilled water must be practised. Careful sterilization of the tubes is then carried out in the dry sterilizer as usual.

In taking the blood from the patient all the precaution should be exercised which make for sterility and for the avoidance of laking of the blood. The syringe should contain no water, and should not have been boiled in sodium carbonate solution. Also, one should avoid mixing air with the blood when taking it and when discharging it into the tubes. It is best taken in large sterile 50 c.c. centrifuge tubes. The tubes are left by the bedside until firmly clotted, then put it in the ice-chest; after several hours gently detach the clot from the sides of the glass with a sterile glass capillary rod. The blood should be withdrawn thirty minutes after the completion of the salvarsan injection.

The serum is removed, as usual, with a sterile pipette, preferably with a bulb attachment, and transferred to another centrifuge tube, so that, in case of need, stray red cells can be thrown down. In centrifuging, care should be exercised not to stop the centrifuge suddenly and disturb the sediment. The serum is then inactivated at 56° for twenty to thirty minutes and diluted with sterile salt solution, if so desired by the physician.

METHOD OF DETERMINING BLOOD GROUPS.

Before a blood transfusion is done it is necessary to make sure that the serum of the donor does not agglutinate nor hemolyse the corpuscles of the donor, or *vice versa*. Serious results may otherwise ensue. Since "hemolysis never occurs when agglutination is absent," the selection of a proper donor from the point of view of agglutination determine the other also.

Tests have shown that among human beings all individuals fall into four groups, having the following characteristics:

Group I (5 per cent. of individuals):
Serum agglutinates cells of no other group;
Cells are agglutinated by sera of Groups II, III, IV.
As donors may be used for Group I only.
As recipients may receive from Groups I, II, III, IV.

Group II (40 per cent. of individuals):
Serum agglutinates cells of Groups I, III.
Cells agglutinated by sera of Groups III, IV.
As donors may be used for Groups I, II.
As recipients may receive from Groups II, IV.

Group III (10 per cent. of individuals):
Serum agglutinates cells of Groups I, II.
Cells are agglutinated by areas of Groups II, IV.
As donors may be used for Groups I, III.
As recipients may receive from Group III, IV.

Group IV (45 per cent. of individuals):
Serum agglutinates cells of Groups I, II, III.
Cells are agglutinated by no Group.
As donors may be used for Groups I, II, III, IV.
As recipients may recieve from Group IV only.

Cells.	Sera.			
	1	2	3	4
1	O	+	+	+
2	O	O	+	+
3	O	+	O	+
4	O	O	O	O

It is, of course, preferable to have as donor and recipient members of the same group, but when it is impossible, members of another group may be chosen in accordance with the above. The agglutinative power of the donor's serum may be disregarded because it is so promptly diluted by the serum of the recipient.

MACROSCOPIC SLIDE TESTS.—As is shown in the two central columns of above table, two known sera, types II and III respectively, are sufficient for the determination of the group to which any blood cells belong. As the agglutinins resist drying it is possible to use either dried or fluid sera.

NOTE.—In the United States dried serum on slides may be obtained from the Army Medical School, Washington, D. C.

Sera are supplied to the laboratories of the American Expeditionary Forces upon application to the Director of Laboratories, A. P. O. No. 721.

When the fluid sera are used a drop of serum II and a drop of serum III are placed on right and left ends of a slide respectively, and about $\frac{1}{3}$ drop of blood from the ear or finger added to each drop in turn, using a clean glass rod for each transfer. Blood and serum are thoroughly mixed and the slide agitated. If agglutination occurs it will be evident to the naked eye as a granular or brick-dust appearance of the drop, in about a minute (Fig. 1).

Occasionally rouleaux formation may occur and cause confusion. This appears more slowly than true agglutination and disappears if the drop is stirred with a rod or loop. Too large a drop of blood also obscures the reaction. In cases in which the reaction remains doubtful the donor should not be used.

When using the dried sera a suspension of blood cells is employed. About 2 drops of blood from the person to be tested are taken up in 1 c.c. of a 1 to 2 per cent. sodium citrate solution. A loopful of this suspension is added to the cover-slip with dried serum Group II and a hanging drop made. The same is done with serum of Group III. In this case about ten minutes is required before a reading can be made.

When known sera cannot be obtained it is necessary to collect serum as well as cells for the tests, from both recipient
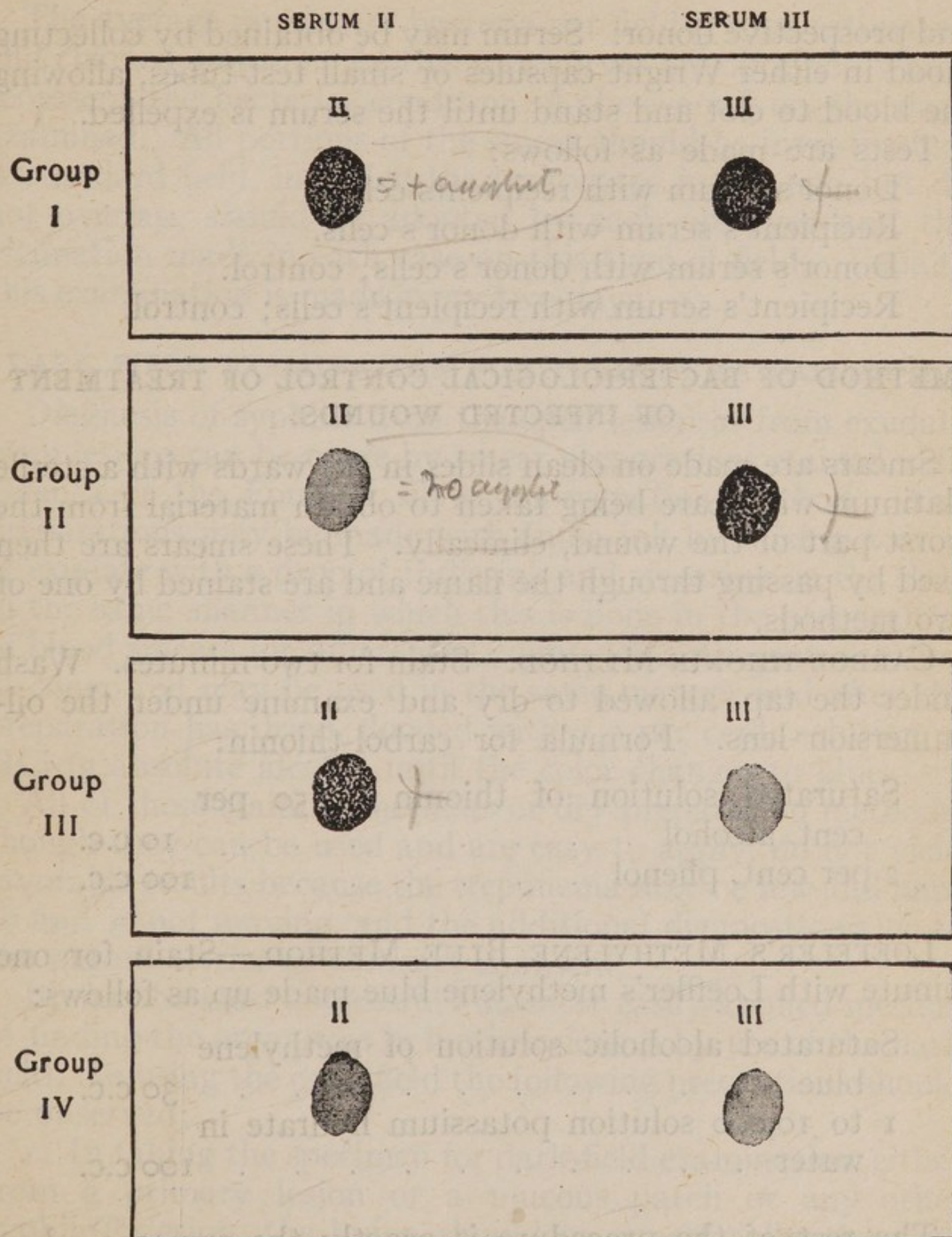
FIG. 1.—Graphic illustration of macroscopic agglutination test for blood groups. (Taken from Transfusion of Blood, published by the American Red Cross Society in France.)

and prospective donor. Serum may be obtained by collecting blood in either Wright capsules or small test-tubes, allowing the blood to clot and stand until the serum is expelled.

Tests are made as follows:

Donor's serum with recipients cells.
Recipient's serum with donor's cells.
Donor's serum with donor's cells; control.
Recipient's serum with recipient's cells; control.

METHOD OF BACTERIOLOGICAL CONTROL OF TREATMENT OF INFECTED WOUNDS.

Smears are made on clean slides in the wards with a sterile platinum wire, care being taken to obtain material from the worst part of the wound, clinically. These smears are then fixed by passing through the flame and are stained by one of two methods.

CARBOL-THIONIN METHOD.—Stain for two minutes. Wash under the tap, allowed to dry and examine under the oil-immersion lens. Formula for carbol-thionin:

Saturated solution of thionin in 50 per
 cent. alcohol . . . . 10 C.C.
2 per cent. phenol . . . . 100 C.C.

LOEFFLER'S METHYLENE BLUE METHOD.—Stain for one minute with Loeffler's methylene blue made up as follows:

Saturated alcoholic solution of methylene
 blue . . . . 30 C.C.
1 to 10,000 solution potassium hydrate in
 water . . . . 100 C.C.

The rest of the procedure is exactly the same as when carbol-thionin is used.

With Bausch & Lomb microscopes the No. 10 ocular and the 1.9 mm. objective are used.

The average number of bacteria per field is then estimated and charted on form. In this way curves are obtained which represent the bacterial condition of the wound on the days examined. All portions of the smear should be investigated. A standard field, in which the leukocytes just touch but do not overlap, should be adopted by each observer and the estimation made in each case on this type of field. Usually this examination is made every two days.

DARK FIELD EXAMINATION FOR TREPONEMA PALLIDUM.

Diagnosis of syphilis from the open lesion or from exudate from lesions can be made by smear preparations stained with Giemsa or the Fontana silver impregnation method. Also preparations may be made with India ink by mixing a drop of exudate with a drop of India ink and smearing on the slide in the same manner in which this is done in the preparation of blood smears for differential count. A 2 per cent. solution of congo red may be used in the same manner and after the preparation has dried, flooded with a 2 per cent. solution of HCl in absolute alcohol until the color changes to blue.

All of these staining methods or dry preparation methods, though they can be used and are easy to apply, do not yield favorable results because the treponema may be few and hard to find if not moving, and the additional diagnostic value to the nature of the movements is absent.

By far the most successful and most easily applied method of finding the organisms in luetic lesions is by use of the dark field. In using the dark field the following precautions should be observed:

1. In taking the specimen for dark-field examination, either from a primary lesion or a mucous patch or any other syphilitic lesion, the lesion should be superficially cleansed, in order to remove the many contaminating organisms on the surface. If taken from a primary lesion, it is well to cleanse rather roughly, remove most of the surface layer and

crust, and to squeeze out a little serum from the deeper tissues. It is sometimes convenient to improvise a little Bier's cup by attaching a rubber bulb or nipple firmly to a bit of glass tubing, the edges of which have been rounded in the flame, and applied with suction over the lesion. Or better, serum may be aspirated from the outer indurated edge of a primary lesion by means of a 5 to 10 c.c. syringe equipped with a sharp needle, 20 gauge $\frac{5}{8}$ inch. A small blunt curette is a convenient instrument for both mucous and primary lesions.

2. The drop or material obtained should be placed on a thin or medium slide (thick slides will not permit focussing of the dark field) and a thin cover-slip dropped upon it, so that the material spreads out evenly in a thin layer between the slide and cover-slip. It is not necessary—in fact it is not desirable —to have so much exudate that it spreads to the edges of the cover-slip, in which case little capillary streams are set up, which disturb the examination.

The condenser should first be centered by means of the lateral adjustment screws. This is accomplished by bringing the rings on the apex of the condenser to the center of the field as viewed through the low-power lens of the microscope. A drop of immersion oil is placed on the bottom of the slide, just under the preparation, and another drop on the cover-slip (this is preferable to putting a drop of oil on the condenser) and the preparation then placed on the condenser gently, so that the bottom drop of oil spreads evenly without bubbles between the bottom of the slide and the surface of the dark field condenser.

3. The apparatus is then examined to make sure that the light is at the proper distance and focussed on the mirror, and this is adjusted by moving the mirror while the examiner looks through the low power of the microscope and roughly determined whether the light is properly adjusted.

If the condenser is properly centered the correct focus of

light can be obtained by moving the condenser up and down until the center ring of light becomes a point of light in the middle of the preparation. This adjustment is necessary because of variations in the thickness of slides. After this has been accomplished, the oil-immersion lens is gently let down until it touches the oil on the top of the cover-slip. Further adjustment is made with the fine adjustment.

If these rules are observed, no trouble will be experienced in obtaining a satisfactory observation if the light is strong enough. If the bull'e-eye lens on the source of light is unsatisfactory, a 2-liter flask full of clear water just in front of it is useful. If an arc is not available a 100- or 120-watt nitrogen bulb will serve as a satisfactory source of illumination.

4. In examining primary lesions, a positive diagnosis can easily be made by one familiar with the appearance and characteristic movements of the Treponema pallidum, and it is easy for an experienced observer to distinguish them from other treponemata found in such places. When examinations are made from mucous patches or other lesions in the mouth and throat, care should be exercised not to be confused by Treponema mucosum, which are sometimes indistinguishable from pallidum. In such cases, the findings of the dark field must be tentatively interpreted together with the clinical appearance and the Wassermann reaction.

PREPARATION OF MICROSCOPIC SPECIMENS FOR DIAGNOSIS.

Specimens of tissue which are to be examined microscopically for diagnostic purposes should be preserved as quickly as possible after surgical removal from the patient or following the autopsy.

If cultures are desired they should be made immediately, and afterward suitable pieces of tissue should be removed and placed in fixative for microscopic study. Zenker's fluid is

the best routine fixative, but an aqueous solution containing 4 or 5 per cent. formaldehyde may be used. If bacteria are to be stained in sections, some specimens should also be placed in an abundant quantity of 95 per cent. alcohol. If the specimens are large, small samples should be taken from the material and the remainder wrapped in moist towels or gauze and placed in the ice-box to await description or photography. The date, the patient's name, and the nature of the specimen should be entered on the label of the bottle in which the samples are placed, and also written on a sheet of paper and enclosed in the wrapping of the gross specimen.

If color preparations are to be made either by the Kaiserling, Pick, or any other method, the fixation should be done promptly; otherwise the colors will not be well preserved. But as these methods do not furnish a good preservation for microscopic examinations, small fragments should be cut out and fixed in Zenker's or 4 per cent. formaldehyde before treatment with the special preservatives. The pieces removed should include characteristic portions, and if the lesion involves the skin or mucous membranes, the plane of the incision should be perpendicular to the surface. In all tumors, care should be taken to obtain portions of the adjacent lymph nodes or any distant nodules which may be suspicious of new growth. Unless otherwise requested by the surgeon, all large specimens should be divided by a series of incisions into slices of not over 5 to 6 cm. thick and separated by a thin layer of non-absorbent cotton, in order to permit the penetration of the preservative fluid.

Tissues containing bone or calcified areas should first be thoroughly fixed in 4 per cent. formaldehyde for twenty-four hours previous to decalcification. For such specimens as do not contain either bone or calcified areas at least from twelve to twenty-four hours, depending upon the thickness of the section, should be allowed for fixation by formaldehyde. In Zenker's fluid the tissues are fixed for twelve to twenty-

four hours and are then washed for twelve to twenty-four hours in running water. The blocks may then be placed in 80 per cent. alcohol until used.

There are three general methods for preparing sections after such fixation:

1. Freezing.
2. Embedding in paraffin.
3. Embedding in celloidin (Parlodion).

The paraffin method is preferable if the thinnest sections are to be obtained, but requires the use of an incubator.

The celloidin embedding is much slower than the paraffin, and does not give as good sections except under most expert handling.

Frozen sections may be made of certain of the more solid tissues which are almost as good as the celloidin preparations and the procedure is much more rapid. The method is unsatisfactory for fatty tissues or very friable structures.

FROZEN SECTIONS.—The blocks may be frozen either with ether, ethyl chloride spray, or liquid carbon dioxide. The technic for frozen sections is as follows:

Materials:

Microtome and sharp knife.

Photographic tray or large flat saucer with water or physiological salt solution (0.085 per cent.).

Lifting needles made either by steel or by drawing out the tip of a glass rod.

Smooth pointed forceps.

Glass slides and covers.

A couple of staining dishes or watch-glasses.

Saturated aqueous solution of thionin.

Blotting paper (Royal).

The specimen is frozen, and sections are made, some thick and some thin. They are dropped into water, and if they do not flatten promptly individual sections are transferred with a needle to 60 to 70 per cent. alcohol for a few seconds

and then retransferred to water, upon which they float and flatten out. The slide is then passed into the water and brought up under the section, which is steadied with a needle, and the whole is lifted out of the water, leaving the section flat. A few drops of the thionin solution are placed on the surface of the section and allowed to remain there for about half a minute; the cover-glass is then placed on the section still covered with dye, and the excess of dye is removed by touching a cloth or piece of blotting paper to the edge of the cover-glass. Polychrome methylene blue, either alkaline or acidified with glacial acetic acid, may replace the thionin if the latter is not obtainable. If preferred the sections may be stained in a dish of the dye and then rinsed in water and mounted. The staining is more even, but fresh tissues are apt to tear if much handled.

Such preparations allow of immediate diagnosis but do not keep any length of time. If great haste is not necessary, the following technic will give sections approaching those prepared by the more elaborate methods:

Slices of the tissue (not over 2 or 3 mm. thick and 1 cm. square) are cut with a sharp razor and dropped into 5 per cent. formaldehyde heated to about 40° C. After about ten minutes the preparation is removed from the formaldehyde and frozen, and the sections are placed in water and then on the slide as previously detailed. The water is drained off with blotting paper and the sections are covered with absolute alcohol and brought into close contact with the slide by careful pressure with a smooth piece of blotting paper, preferably that known to the trade as "Royal" Blotting Paper, which is used for drying photographic plates. A few drops of a very thin solution of celloidin dissolved in equal volumes of alcohol and ether are then flowed over the section. The excess of celloidin is drained off and the celloidin allowed to set. This takes only a few seconds, and under no circumstances should the preparation be

allowed to dry. As soon as the celloidin is set the slide is gently dipped in water to wash away the alcohol and ether. It is then placed in a solution of Delafield's hematoxylin diluted 1 to 10 with distilled water. In a few moments it is sufficiently stained, and is then rinsed in a jar of tap water until all excess of dye is washed away. The slide carrying the section is then placed in a closed jar containing an alcoholic or aqueous solution of eosin as preferred. A strength of 1 to 1000 is sufficient. After a few seconds it is rinsed either in alcohol or in water, depending upon the solvent used for the eosin, and transferred to 95 per cent. alcohol. After remaining about five minutes in this the slide is transferred to a fresh bath of 95 per cent. alcohol until all the water is removed. It is then cleared either with carbolxylol or with oil of origanum crotici. Care must be taken to wash out all the carbolxylol by treatment with several baths of xylol, as phenol decolorizes the specimen. When completely dehydrated and cleared the section is covered with balsam or a thick solution of gum damar in xylol, the cover pressed down upon it, and the preparation examined. The slide is marked with the number of the specimen, using a diamond-pointed pencil, so that no confusion can result while the section is passing through the staining mixtures.

PARAFFIN EMBEDDING.—For paraffin embedding the following are necessary:

Alcohol . . . . 80 per cent.
Alcohol . . . . 95 "
Chloroform,
Solid paraffin, with melting point 52° C.

The blocks of tissue are taken from the fixing or preserving fluid, trimmed and cut with parallel surface and treated as follows:

1. 80 per cent. alcohol . . . . 2 to 4 hours
2. 95 per cent. alcohol . . . . 6 to 24 "
3. Absolute alcohol . . . . . 6 to 24 "

If a large number of blocks are being carried the alcohol should be changed after a few hours.

4. Chloroform . . . . 6 to 24 hours
5. Chloroform saturated with par-
   affin at room temperature . . 6 to 24 "
6. Paraffin bath, two changes, 55° C. 2 to 4 "

This is in order to get rid of every trace of chloroform.

7. Fresh paraffin is melted in a flat tin or aluminum dish and cooled till a thin skin forms over the surface; the blocks are removed from the bottle in the incubator and placed in this paraffin, which is then cooled as rapidly as possible by floating the dish in cold water. If the walls of the dish have been rubbed with a trace of glycerin the cake paraffin containing the tissues will separate readily. A strip of paper bearing the number of the specimen should be embedded at the same time with the blocks. When the sections are to be cut the tissue blocks are trimmed from the paraffin with a sharp knife, leaving a rectangular block with a border of at least 3 mm. around the tissue. A wooden or fiber block is warmed over a Bunsen flame and the paraffin block containing the tissue placed on it. If properly heated a thin layer of paraffin will melt and hold the tissue on the block. This block is to be numbered immediately with a lead-pencil, with the number of the specimen.

As the sections are cut the edges should adhere, forming a ribbon. When a suitable number of these are obtained they are laid on the surface of a large dish of warm water at about 44° C., and, if necessary, gently stretched, so as to remove all wrinkles. Paint the surface of a slide with a thin layer of Mayer's glycerin-albumen mixture (equal parts of egg-white and glycerin with 1 per cent. sodium salicylate to prevent decomposition). The sections are floated on to this slide, allowed to dry in the air, after which

they may be put into an incubator at 55° C. for two to twelve hours. The paraffin may be removed by passing through several changes of xylol, followed by 95 per cent. alcohol.

EMBEDDING IN CELLOIDIN.—Two solutions are used: a thick solution which contains about 8 per cent. of celloidin (parlodion) dissolved in equal volumes of 95 per cent. alcohol and ether and a thin solution containing about 4 per cent. of the same. The blocks of tissue, properly trimmed, are dehydrated for about two hours in 95 per cent. alcohol, then for eight to twenty-four hours in fresh 95 per cent. alcohol. They are then placed in thin celloidin for several days, being shaken occasionally, and then for several days in thick celloidin. Finally, they are removed from the bottle with forceps and placed on fiber or wooden blocks; fresh thick celloidin is poured over, and the whole is allowed to harden slightly by drying in the air. The preparations are then dropped in 80 per cent. alcohol, in which they harden. If large sections are to be cut, the blocks are taken from the thick celloidin and arranged in the bottom of a paper tray which has been filled with thick celloidin. Such trays can be made by folding stiff writing paper about a wooden block of suitable size. The preparation is then placed under a bell jar and allowed to dry very slightly, the jar being lifted occasionally to allow access of air. The celloidin contracts down to a firm horny mass in the course of six to eight hours, and is then transferred to 80 per cent. alcohol, in which the final hardening takes place. The preparation may be ruined if this drying process is allowed to go too far. The tissue blocks are cut out of the celloidin and trimmed so as to leave 2 or 3 mm. of celloidin outside of the area of the tissue. They are moistened with alcohol and ether and placed on a block on which a drop of thick celloidin has already been poured. After drying in the air for a few minutes they are dropped in 80 per cent. alcohol, in which they are kept until

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cut.   After cutting the tissues should be removed from the block, as the alcohol dissolves the blocks and spoils the tissues.

STAINING OF SECTIONS.—For tissues fixed in Zenker's fluid the eosin-methylene-blue method is recommended for all general purposes.   The paraffin may be removed by immersing the slide in xylol and follow this with absolute alcohol and 95 per cent. alcohol.   Before staining sections cut from tissues fixed in Zenker's it is important to remove all precipitate of mercuric oxide.   This is done by treating with Lugol's solution or a 1 per cent. alcoholic solution of iodin for ten to twenty minutes, followed by 95 per cent. alcohol to remove the iodin.

The staining method is as follows:

1. Stain in 5 per cent. aqueous solution of eosin for twenty minutes or longer.

2. Wash in water to get rid of excess of eosin.

3. Stain in Unna's alkaline methylene-blue[1] solution, diluted 1 to 4 or 5 with water, for ten to fifteen minutes.

4. Wash in water.

5. Differentiate and dehydrate in 95 per cent. alcohol, keeping in constant motion, so that decolorization shall be uniform.   Control the result under the microscope.

6. Xylol.

7. Xylol balsam and mount.

For celloidin sections use 95 per cent. alcohol, blot and pour on xylol; repeat the last two steps until the specimen is clear.

Hematoxylin and eosin give a more permanent stain than the methylene-blue method.   The first part of the procedure

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[1] Unna's alkaline methylene blue:

Methylene blue	1 part
Carbonate of sodium	1 "
Water	100 "

is the same. After treating with Lugol's solution, the sections are washed in water and then treated as follows:

1. Hematoxylin for five to ten minutes.
2. Water.
3. Acid alcohol until brown.
4. Water.
5. Ammonia water until blue.
6. Water.
7. Eosin.
8. Water.
9. 95 per cent. alcohol.
10. Absolute alcohol.
11. Xylol.
12. Balsam and cover-slip.

Harris's and Delafield's hematoxylin are satisfactory, but the following modification of Ehrlich's hematoxylin devised by Dr. Hays Bullard allows the use of the American product, does not overstain and gives good results.

Alcohol 50 per cent. . . . . 144 c.c.
Acetic acid glacial . . . . 16 c.c.
Hematoxylin crystals (American) . . . 8 grams
Heat and add:
Distilled water . . . . 250 c.c.
Ammonia alum . . . . 20 c.c.
Heat to boiling and add slowly:
Mercuric oxide (red) . . . . 8 grams
Cool quickly, filter and add:
Alcohol 95 per cent. . . . . 275 c.c.
Glycerin . . . . 330 c.c.
Acetic acid glacial . . . . 18 c.c.
Ammonia alum . . . . 40 grams

Gram-Weigert Method for Demonstrating of Gram-Positive Bacteria in Tissue.—Carry section into water after removing paraffin as before.

1. Stain twenty minutes to one hour in lithium carmine.[1]
2. Acid alcohol (do not wash in water) until as seen under the microscope the nuclei alone are sharply stained.
3. Water.
4. Sterling's gentian voilet five to ten minutes. (See page 27.)
5. Wash quickly in water.
6. Gram iodin solution two to five minutes. (See page 27.) Blot dry on slide.
7. (Do not wash.)
8. Anilin oil and xylol (equal parts) until clouds of blue no longer wash out of the section.
9. Xylol two changes.
10. Balsam and cover-slip.

SPINAL FLUID.

Spinal fluid, taken by lumbar puncture, if normal, is colorless and clear, but when taken from cases of meningitis it is usually turbid and may be purulent. Routine bacteriological examination of such fluid should be done both by smear preparation and culture. The fluid should be taken into sterile centrifuge tubes by preference and a part of it immediately centrifugalized. From the sediment smears are made, and one of these, stained by the Gram method or a simple stain such as Peppenheim-Saathoff. A careful examination of these slides will show Gram-negative intracellular diplococci if the case is one of epidemic cerebrospinal meningitis. It should be remembered that, even in acute cases, a prolonged search may be necessary before organisms are found, and

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[1] Lithium carmine is made as follows:

Carmine	2.5 to 5 grams
Saturated aqueous solution of carbonate of lithium	100 c.c.
Thymol	a crystal

Dissolve the carmine in a small quantity of 95 per cent. alcohol, bring the lithium carbonate solution to a boil, mix and filter.

whenever a considerable number of polynuclear leukocytes are present in the fluid and no bacteria can be found for a long time, in an acute case, the chances are in favor of meningococcus rather than other organisms, since this organism is very apt to undergo autolysis. The same method will reveal pneumococci, in which case the spinal fluid is more apt to be fibrinous.

The cultivation of meningococci out of such spinal fluid is a matter which requires care, and it should be remembered that a considerable amount of fluid should be planted because many of the meningococci, visible under the microscope, are dead and a small proportion only capable of growth. It is not a bad plan, when plenty of fluid is available, to plant 1 or 2 c.c. of the centrifugate immediately and to set away another portion of the fluid in the incubator for four hours or more, since in the fluid occasionally a preliminary growth will take place. This incubated fluid when planted is apt to develop more colonies than the fresh fluid. This last method is very useful in many cases when immediate planting is without result.

The medium now recommended for the cultivation of meningococcus is a sheep serum water glucose agar, described in another place under Meningococcus Carrier Detection. This medium is excellent, but is by no means the only one which will grow the meningococcus. Ascitic agar, blood agar, sheep serum agar, or agar made with defibrinated whole blood of man and various animals can be used. Laked rabbits' blood and horse serum are very useful enriching additions, and glucose, 1 or 2 per cent., always helps. This medium may be used in slant tubes or plates, and it is essential to remember that quantities not less than 1 or 2 c.c. must be planted on the surface of the hardened plates or slanted tubes of medium.

TUBERCULOUS SPINAL FLUID.—Tuberculous spinal fluid is clear and usually contains only a moderate number of cells,

these being mostly lymphocytes. Such fluid may be centrifugated in part immediately and smears made from the sediment and stained in the usual manner for information concerning cells and the possible presence of tubercle bacilli. However, to find the tubercle bacilli it is much better to set away the centrifuge tube containing the fluid for a few hours in the incubator, when a thin thread-like coagulum will be found in the middle of the tube, soon sinking to the bottom. This clot can then be smeared, and has usually gathered the tubercle bacilli within itself. The smearing and staining by the ordinary method of successive clots usually shows the tubercle bacilli.

For the cultivation of other organisms found in connection with meningococcus, choice of media, etc., must be made according to the judgment of the laboratory office.

CELL COUNTS OF SPINAL FLUID.—These should be made as soon as possible after taking the fluid. The fluid is shaken, and a drop can immediately be placed on a counting chamber. Normal fluid contains about eight to ten cells to the cubic millimeter.

The cerebrospinal fluid in central nervous system syphilis is always clear. The number of cells, in rare instances, may reach as high as 600 or 700, but the usual extremes are 10 to 250 or 300. The cells consist of 85 per cent. or more of mononuclear elements, which look like small lymphocytes. There are also a variable number of larger mononuclear cells suggesting epithelioid types and still others with multilobed nucleus. This picture is characteristic in cerebrospinal syphilis and also in acute poliomyelitis.

In paresis the cell count may be very low, often within the limits of normal; whereas in syphilitic meningitis, or in the condition sometimes called "cerebrospinal syphilis" (as distinguished from paresis or tabes), the count may reach the high figures. Often in cases with the highest counts there are few or no clinical symptoms.

The intensity of the globulin reaction is variable but always positive; it is heaviest in cerebrospinal syphilis and paresis.

In acute poliomyelitis the fluid is clear in most cases. Occasionally there may be a faint opalescence and rarely a true grayness. The cell count may run as high as 2500 per c.mm. The usual extremes are 10 to 500.

During the very earliest hours after involvement of the cerebrospinal space the proportion of multilobed cells may be 50 per cent. or more of the total. This figure drops rapidly hour by hour, so that by the end of the first day the mononuclear forms predominate and make up 90 per cent. or more. Similar multilobed cells to those seen in cerebrospinal syphilis appear regularly, as do also the larger mononuclear types. After the first day of poliomyelitis the cell picture in the two conditions is indistinguishable.

The globulin reaction in poliomyelitis, on the other hand, is much more definite in its behavior than it is in syphilis. In the earlier hours and first day the reaction is absent or very slight. After that the test becomes increasingly strong. This early absent or weak reaction is an important differential point from the heavy globulin found at the same period of the disease in epidemic cerebrospinal meningitis. In poliomyelitis as the cells diminish from the fourth or fifth day on the globulin continues to increase and may persist for two or three weeks.

In the fluid of poliomyelitis the Wassermann reaction is regularly absent, but the Lange gold reaction shows a weak luetic type of curve.

In all acute conditions of the meninges the polymorphonuclear cells predominate. Albumin in spinal fluid may be roughly estimated by the following method, described in the manual of technic of St. Luke's Hospital, New York.

"*Albumin* may be roughly estimated by the following device: A narrow test-tube about 5 mm. in diameter is

strapped with adhesive to the side of an Esbach tube. Fluid is poured in to opposite the U mark and Esbach's reagent to R mark. The readings are in grams to the liter."

Globulins can be tested by the *butyric acid test of Noguchi*. 0.5 c.c. of 10 per cent. butyric acid solution, made up in normal salt solution, is added to 0.2 c.c. of clear spinal fluid. This is boiled for three seconds or better for two minutes in a boiling water-bath. Then 0.1 c.c. of $\frac{N}{1}$ NaOH is added and the tube is again boiled. A precipitate forms, which gradually settles to the bottom, and in strongly positive reactions appears within a few minutes. When only a small amount of globulin is present it may take an hour. Later readings are of doubtful significance.

Ross-Jones Method of Determining Globulin in Spinal Fluid.—Place 1 c.c. of spinal fluid in small test-tube and under this allow the same quantity of saturated ammonium sulphate to flow through a capillary pipette. A positive reaction shows as a white ring where the fluids meet and shows before the end of three minutes.

DETECTION OF MERCURY IN EXCRETIONS.

Principle.—The organic combination in which the mercury exists is broken down by oxidation with nascent chlorin. By electrolytic double decomposition the metal is then caused to become deposited on a piece of copper wire, and from this it is distilled on to a piece of dentists' gold-foil. The amalgam so formed is recognizable as a silvery patch of discoloration on the gold. In collecting the specimens and in carrying out the tests it is necessary to use only receptacles and apparatus that are chemically clean.

Reagents.—Potassium chlorate.
  Concentrated HCl.
  Bare copper wire (B. and S., No. 14, is a convenient size).
  Soft glass tubes, 2 mm. inside diameter, 10 cm. long, sealed at one end.

Small pellets of gold-foil as used by dentists.[1]
Microburner.

Technique.—As large a volume as possible of the material (urine, gastric lavage fluid, colon irrigation) is acidulated with 10 to 20 c.c. of concentrated hydrochloric acid; a few grams of potassium chlorate are added; and the whole is heated in a large porcelain evaporating dish. It is not advisable to use more acid or chlorate than is required for complete decolorization, the exact amounts being readily judged by experience. Stools, vomitus, blood, etc., are first diluted with several volumes of water, and require more of the oxidizing materials. The excess of acid and chlorine is eliminated by evaporation and the solution concentrated to about 25 c.c. Any solid matter, especially fat, is filtered off and into the filtrate, contained in an Erlenmeyer flask, is dropped a straight piece of the copper wire, 2 cm. long, preciously cleaned by a short immersion in concentrated nitric acid, followed by washing with distilled water. It is allowed to remain in the fluid for several hours or overnight, preferably in a warm place. In an emergency the time may be shortened by boiling the fluid with the wire for five minutes as a preliminary test, but if the result is negative the test must be repeated in the regular way. The wire is washed with distilled water by decantation, dried by rolling very gently on a filter paper, and slipped to the bottom of the glass tube, avoiding abrasion. It is then followed by a cylinder of gold-foil pushed to within 2 cm. of the wire.

Holding the tube horizontally, its closed end is carefully heated in the flame of the microburner almost to the softening point, after which the tube is gently warmed close to the gold cylinder, the edges of which should be frequently examined

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[1] Manufactured by J. M. Ney Co., Hartford, Conn. Sold by dentists' supply firms in $\frac{1}{16}$-ounce vials under the trade designation No. $\frac{1}{4}$ velvet cohesive gold cylinders.

during this operation for any trace of a silvery discoloration signifying the presence of mercury. A hand lens is useful in recognizing very small amounts of the metal. If chlorine is still present in the concentrated oxidized solution the wire may be completely dissolved, in which case the solution should be diluted, again concentrated by boiling, and another wire dropped in. If the gold is too close to the wire, or the tube is heated too strongly, the mercury may be driven into or beyond the gold, the discolorization then being transient.

By this method it is possible to recognize the presence of o.o1 mg. of mercury in the amount of material taken for analysis. Calomel in therapeutic doses occasionally gives a positive reaction in the urine.

TECHNIQUE FOR WASSERMANN TEST.

METHOD No. 1.—The following technique is used by the laboratory workers throughout the American Expeditionary Forces and is standardized so far as standardization of the two-tube Wassermann test is at present possible.

REAGENTS.

"Antigen:" Alcoholic extract of beef heart or calf heart, half-saturated with cholesterin at room temperature.

Hemolytic System: Amboceptor or Sensitizer: Antisheep serum. Red Blood Cells: Sheep.

Complement or alexin: Guinea-pig.

"Antigen" and amboceptor are supplied from the Central Medical Laboratory automatically each month to all laboratories where Wassermann tests are made. These reagents are titrated before being sent from the laboratory and the titer is marked on the label. Sheeps' blood is easily obtained from slaughter houses, or it is simple to obtain sheep for

permanent use; they are found in all locations in France. Guinea-pigs are kept at each laboratory. There have been arranged animal distributing centers, and such information as is wanted concerning the procuring of animals will be furnished on application to the Director of Laboratories, A. P. O., 721.

1. *Cell Suspension.*—5 per cent. suspension of sheep's erythocytes. Blood is obtained from the jugular vein of a sheep; it may be collected into a receptacle containing beads for defibrination or it may be collected into one containing a solution of sodium citrate, 1 or 2 per cent., in physiological saline. One should take at least an equal amount of solution for the amount of blood drawn. The cells should be washed by centrifuging several times with physiological saline; the number of washings will depend on the amount of cells being washed; three or four washings should do for 15 c.c. of blood-citrate mixture. It is important to mix the cells thoroughly with each new addition of saline after pipetting off the super-natant fluid, which is best accomplished by using a 25 c.c. bulb pipette, with a rubber bulb adapted to its end. After the last washing the cells should be packed well, then enough physiological saline added to bring total volume to a 5 per cent. suspension. In packing cells, one should allow centri-fuging to be done at the same speed and length of time for each preparation.

2. *Amboceptor or Sensitizer.*—The test is based on the "quarter unit" amount, *i. e.*, the amboceptor unit is that amount of serum causing complete hemolysis of 0.25 c.c. of the 5 per cent. sheep-cell suspension, in the presence of excess complement, after incubation in water-bath at 37.5° C. for one hour. The amboceptor is furnished in glass ampoules containing 0.1 c.c. inactivated antisheep serum. For con-venience the unit is expressed in terms of dilution rather than actual amount of serum. The dilution stated for any particu-lar lot of serum represents the dilution in the titration con-

taining the amount of serum determined as one unit. For example: It may be stated that a dilution of 1 to 3000 is one unit, meaning that this dilution contains the amount of serum which is one unit. Since two units are used in the test, in preparing the reagent a dilution of 1 to 1500 will be made, *i. e.*, 0.1 c.c. of serum diluted with 149.9 c.c. of physiological saline will give a reagent, each 0.25 c.c. of which represents two units of amboceptor.

3. *Complement or Alexin.*—Without entering into a controversy about the advisability of whether a preliminary complement or amboceptor titration be made, we feel that the variation in amboceptor is decidedly less than that of complement and that it is better to adjust the complement to a given amount of amboceptor.

Two or three guinea-pigs should be bled the night before the day the test is done. The blood should be taken from the heart by means of dry, sterile needle with syringe or suction apparatus and placed in a dry, sterile conical centrifuge tubes. After clotting has taken place a stiff, sterile wire should be run around the rim of the clot and the tube placed in an icebox until the following morning. The following morning the tube should be centrifuged and the clear serum drawn off. The serum is diluted 1 to 10 with physiological saline for use as complement. Each serum should be tested for hemolytic and complementary properties. For hemolytic properties, 0.5 c.c. of the dilution and 0.25 c.c. of 5 per cent. suspension of cells should be incubated in the water-bath at 37.5° C. for one hour. Providing each serum has good complementary properties and no hemolytic property the sera should be pooled and diluted. For determining complementary properties of serum the following protocol should be used:

PROTOCOL FOR COMPLEMENT TITRATION.

Tube.	G. P. serum 1 to 10. c.c.	Physiologi- cal saline. c.c.	2 units amboceptor c.c.	5 per cent. sheep-cell suspension. c.c.	
I	0.15	0.60	0.25	0.25	
2	0.14	0.61	0.25	0.25	
3	0.13	0.62	0.25	0.25	
4	0.12	0.63	0.25	0.25	
5	0.11	0.64	0.25	0.25	
6	0.10	0.65	0.25	0.25	
7	0.09	0.66	0.25	0.25	
8	0.25	0.75	0.0	0.25	complement control
9	0.0	1.00	0.0	0.25	saline control
10	0.0	0.75	0.25	0.25	amboceptor control

The *dose* for the test is twice the amount contained in the tube showing complete hemolysis after incubation in the water-bath at 37.5° C. for one hour. With a good serum 0.1 c.c. will usually be this amount, then 0.2 c.c. will be the dose for the test.

4. *Antigen.*—"Antigen" is adjusted so that 0.1 c.c. of emulsion in physiological saline will be the dose for the test. The proper dilution for each batch is stated on the label. It is very important that the "antigen" emulsion be prepared as follows: Place the amount of alcoholic extract to be emulsified in a flask; add physiological saline, drop by drop, shaking the flask vigorously between drops until at least 5 c.c. volume is obtained. The balance of the saline may be added in larger amounts, but the flask should be shaken well between each addition.

5. *The Test.*—One, front, tube for the test; one, back, tube for serum control. Patient's serum is inactivated by heating in water-bath at 56° C. for twenty to thirty minutes; when the amount of serum is little the shorter period should be used. The amount of patient's serum (inactivated) used in each test is 0.05 c.c. in each tube. In certain instances natural antisheep hemolysin is present in human serum and

at times in sufficient quantity to produce hemolysis of one unit of cells in the presence of the amount of alexin or complement used in the test. On account of this a unit of cell suspension, 0.25 c.c., may be added to all the tubes after the first incubation period and allowed to incubate fifteen minutes. At the end of this time complete or nearly complete hemolysis may have taken place in some of the serum control tubes (back tubes). If such be the case it will not be necessary to add amboceptor to those tubes. To all other tubes 0.25 c.c. of the amboceptor dilution representing two units is added to each tube.

It might be stated that although human serum does contain natural antisheep amboceptor or sensitizer, it is believed to be rather negligible in influencing the final result of the test except in rare instances. Moreover, the high hemolytic index frequently determined for human serum is influenced by the complement content of serum which varies more decidedly and widely than the amboceptor content. However, the method given allows for any material influence that may exist. When time is pressing the cell suspension and the amboceptor may be added simultaneously following the first incubation period, eliminating entirely the fifteen-minute period.

1st incubation period (for complement-fixation): one hour.

2d incubation period (for natural hemolytic activity): fifteen minutes.

3d incubation period (for hemolysis): one hour.

Too much emphasis cannot be laid upon the necessity of controls for every reagent and for their behavior with known negative and positive sera, *before* the actual test is set up.

The following protocol serves to illustrate the test:

(Sera for controls: one $+ +$ serum; one $+$ serum; one $\pm$ serum; one $(-)$ serum).

	Inactivated patient's serum.	Antigen emulsion.	Comple- ment.	Physiologi- cal saline.	5 per cent. sheep-cell suspension.	Ambocep- tor 2 units, if neces- sary.
	c.c.	c.c.	c.c.	c.c.	c.c.	c.c.
Back tube	0.05	0.0	0.2	0.50	0.25	0.25
Front tube	0.05	0.1	0.2	0.40	0.25	0.25

ANTIGEN CONTROLS.

I.

Known negative serum	0.05 c.c.
"Antigen" emulsion .	0.2 c.c.
Complement . . .	0.2 c.c.
Saline	0.25 c.c.

Incubation in water-bath at 37.5° C. for one hour.

5 per cent. suspension sheep cells . . .	0.25 c.c.

Incubate in water-bath fifteen minutes.

Amboceptor, 2 units if necessary.

Incubate in water-bath one hour.

II.

"Antigen" emulsion .	0.3 c.c.
Complement . . .	0.2 c.c.
Saline	0.25 c.c.

Incubation in water-bath at 37.5° C. for one hour.

5 per cent. suspension sheep cells . . .	0.25 c.c.
Amboceptor, 2 units .	0.25 c.c.

Incubate in water-bath, etc., for one hour.

PROTOCOL FOR SPINAL FLUID.

Tube.	1	2	3	4	5
	c.c.	c.c.	c.c.	c.c.	c.c.
Spinal fluid	1.0	1.0	0.5	0.25	0.12
"Antigen"	0.0	0.1	0.1	0.1	0.1
Complement	0.2	0.2	0.2	0.2	0.2
Saline	0.0	0.0	0.0	0.2	0.43

Incubate in water-bath at 37.5° C. for one hour.

	1	2	3	4	5
Amboceptor, 2 units	0.25	0.25	0.25	0.25	0.25
5 per cent. sheep-cells	0.25	0.25	0.25	0.25	0.25

Incubate in water-bath at 37.5° C. for one hour.

Another important control which should be run in regular test is one for serum specimens showing hemolysis when received:

Inactivated patient's serum . . . . 0.05 c.c.
5 per cent. suspension sheep cells . . 0.25 c.c.
Saline . . . . 0.95 c.c.

The tinge of red imparted to the supernatant fluid will serve as a comparison for reading the result on that particular serum

Too much care cannot be taken to be sure all glassware is absolutely clean, not necessarily sterile but free from all dirt and grease. This is best accomplished by personally supervising the cleaning of glassware used in serological work.

Interpretation of Results.—Four symbols will be used to designate results: Form 55 of M. D.:

++ complete fixation.
+ almost complete fixation.
± partial fixation.
— complete hemolysis.

Note.—No mention is made of method preparing and titrating "antigen" or amboceptor, because both reagents are furnished from the Central Laboratory.

Method No. 2.—This method is used in the Army laboratories of this country, and is recommended by the Army Medical School—from which antigen and amboceptor may be obtained.

REAGENTS.

Antigen: Supplied in the form of antigen papers.
Hemolytic system: Amboceptor or sensitizer: antihuman serum. Red blood cells: human.
Complement or Alexin: Guinea-pig.

1. *Cell Suspension.*—A 5 per cent. suspension of human cells. The human system is used to avoid the fallacy of anti-human amboceptor against sheep corpuscles which is naturally present in many human sera. It has apparently escaped the attention of serologists that isohemolysins are present in many human sera. This indicates that when the human system is used a number of donors should be typed out and blood for the cell suspension should be taken from only type four individuals, *i. e.*, those whose blood cells are unaffected by the sera of any other groups.

Blood from such an individual may be collected in small flasks filled with salt solution or in a graduated centrifuge tube filled with citrate solution. Centrifuge and wash cells thoroughly (three or four washings). At the last washing pack cells by running centrifuge for a given length of time at a certain speed, so that cells will always be packed to a similar density. Pour off supernatant fluid. To each 0.1 c.c. of packed cells is added 1.9 c.c. of salt solution.

A convenient method of making the salt solution is as follows: Make up several liters of 8.5 per cent. salt solution and keep it in a bottle with a siphon or a glass faucet. This need not be sterilized, as bacteria will not live in it. Have on hand flasks of sterile distilled water, and when it is desired to make saline solution for the tests, take 50 c.c. of the 8.5 per cent. solution and dilute to 500 c.c. with sterile distilled water.

2. *Amboceptor.*—The serum of rabbits that have been immunized to human red cells.

The difficulty in producing antihuman hemolysin has been the chief drawback to the human system. It has generally been supposed that rabbits immunized with human cells died of anaphylaxis. This may occur, but they usually die after the second or third injection from embolism caused by the strong agglutination of the injected cells. This may be avoided and practically 100 per cent. of successful immuniza-

tions secured by the following method: Select good healthy rabbits and test their blood against human cells for agglutination. If agglutination is absent or slight, give the rabbit 3 or 4 c.c. of packed human corpuscles intravenously on three successive days. Wait about two weeks and then give very slowly daily injections of 0.5 c.c. packed cells. The titer of the serum rises quickly, so that in a week or ten days a usable amboceptor is secured.

Titration of Amboceptor.—Before killing the rabbit a preliminary titration is made. The rabbit is bled into a Wright capsule, the serum inactivated (heated to 56° C. for one-half hour) and one drop of this serum is diluted with 19 drops of salt solution. One drop of this 1 to 20 dilution should give complete hemolysis in one hour when mixed with 0.1 c.c. cell suspension and one unit of complement. If this fails the rabbit is given another injection. Otherwise the animal is bled and the serum separated and inactivated. This serum is then placed on paper (Schleich & Schull's No. 597 paper is used) and cut in pieces 8 cm. square. To each sheet $1\frac{1}{3}$ c.c. serum is added, an amount that just saturates the sheet, leaving no excess. These pieces of paper are dried on a strip of unbleached muslin by an electric fan and constitute the amboceptor, which must then be accurately titrated.[1]

A complement which has been previously standardized by titrating against an old and known amboceptor is used. The amboceptor paper is cut in strips, 5 mm. wide, and various lengths of this width are titrated against the known complement as follows:

Tube.	1	2	3	4	5
Complement	1 unit	1 unit	1 unit	1 unit	1 unit.
Amboceptor	1 x 5 mm.	1½ x 5 mm.	2 x 5 mm.	2½ x 5 mm.	3 x 5 mm.
Cell suspension	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c
Salt solution	q. s. ad 1.3	c.c. to each	tube.		

[1] When not in use amboceptor paper must be kept in a dry cold place.

Incubate one hour in water-bath.

Suppose that the last tube in which hemolysis is complete is tube 4. Then for this paper a piece $2\frac{1}{2}$ x 5 mm. is the unit, and as two units are used in the Wassermann reaction a piece 5 x 5 mm. would be used in each tube in the actual tests. This titration must be repeated several times with different complements before a new amboceptor is used.

3. *Complement.*—The pooled serum of several guinea-pigs that have been freshly bled as in previous method. To each 1 c.c. of serum add $1\frac{1}{2}$ c.c. of 0.85 per cent. salt solution, making a 40 per cent. complement.

Complement that is left over from one day's tests is usually wasted. Complement may be preserved for several weeks by Rhamy's method. Dilute the fresh guinea-pig serum to 40 per cent. as usual, but use 10 per cent. sodium acetate in 0.85 salt solution as the diluent. This does not interfere with the tests.

Titration of Complement.—Each complement must be titrated before use, using the cell suspension, which should not vary from day to day, and an old amboceptor which has been tested often previously with one unit of a known complement. The amboceptor changes in titer so slowly that for all intents and purposes it may be regarded as a non-variable. The new complement is therefore titrated against an amboceptor and a cell suspension which does not vary in strength, as follows:

Tube.	1	2	3	4	5	6	7	8	9
Complement	0.02 c.c.	0.03 c.c.	0.04 c.c.	0.05 c.c.	0.06 c.c.	0.07 c.c.	0.08 c.c.	0.09 c.c.	0.1 c.c.
Amboceptor	1 unit	1 unit	1 unit	1 unit	1 unit	1 unit	1 unit	1 unit	1 unit
Cell suspension	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.
Salt solution	1.18 c.c.	1.17 c.c.	1.16 c.c.	1.15 c.c.	1.14 c.c.	1.13 c.c.	1.12 c.c.	1.11 c.c.	1.10 c.c.

Total volume in each tube 1.3 c.c. Incubate for one hour in water-bath at 37.5° C., shaking every fifteen minutes, the unit of complement being the last tube in which hemolysis is complete. If complement and amboceptor are both of normal strength, this should be tube 4, making 0.05 c.c. the unit of complement. As two units of complement and amboceptor are used in the Wassermann reaction, the amount to be used in the actual tests would therefore be 0.1 c.c.

4. *Antigen.*—A normal human heart is obtained from a recent necropsy, is washed free from blood and all fat carefully removed. To 100 grams of finely minced heart muscle add 1000 c.c. of absolute alcohol and shake continuously in a machine for twenty-four hours. Filter and keep the alcoholic extract in the ice-box in well-stoppered bottles. When needed for use 25 c.c. of this extract is fortified by adding 100 mg. cholesterin, thus making a 0.4 per cent. solution. Allow this to stand for several days and filter. Keep in the ice-box. When it is to be used it is diluted with 9 parts of normal salt solution, making a 1 to 10 solution of the original stock antigen.

Titration of the Antigen.—Tests for hemolytic power, antigen power and anticomplementary action must be made. As a matter of fact, having never found an antigen prepared in this way to be hemolytic, this test is confined to one tube in which the antigen is used in five times the amount used in the test, with cell suspension and salt solution. No hemolysis should occur. Should the antigen be hemolytic alone it must be discarded.

Test for Antigenic Power. — A known positive serum is titrated with the new antigen and an old antigen of known strength as follows:

Tube.	1	2	3	4	5
Positive serum . . .	0.1 c.c.	0.05 c.c.	0.025 c.c.	0.012 c.c.	0.006 c.c.
Complement	2 units	2 units	2 units	2 units	2 units
Antigen	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.	0.1 c.c.
Salt solution	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.	1.0 c.c.

Incubate one-half hour in water-bath and add to each tube 0.1 c.c. cells and two units of amboceptor. Incubate one hour, shaking every fifteen minutes, and read.

An antigen to be usable should give complete fixation in tube 2 at least, viz., with half the amount of serum to be used in the tests. Most cholesterinized antigens will give a double plus in tube 3, with quarter the amount to be used in the tests. By comparing the new antigen with the old in this titration and discarding antigens that are either too weak or too strong, the reaction will be standardized; that is, an antigen of the same strength will always be used.

The antigen should also be tested with a known negative serum. Twice as much antigen as is used in the tests must give a negative reaction with a known negative serum.

Anticomplementary Test.—All antigens possess to a greater or less extent the power of uniting with complement even in the absence of a positive serum. If this power were at all marked the antigen would obviously be unsafe to use, as it would cause false positive reactions. The extent of this anti-complementary action is determined as follows:

A dilution of the stock antigen is made with salt solution 1 to 5 instead of the customary 1 to 10 dilution, and this antigen is titrated.

Tube.	1	2	3	4	5
Antigen	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.4 c.c.	0.5 c.c.
Complement	2 units	2 units	2 units	2 units	2 units
Salt solution	0.9 c.c.	0.8 c.c.	0.7 c.c.	0.6 c.c.	0.5 c.c.

Incubate in water-bath for one-half hour and add to each tube two units of amboceptor and one unit of cell suspension (0.1 c.c.). Incubate for one hour, shaking every fifteen minutes. Should hemolysis be complete in all tubes the antigen is not anticomplementary in ten times the amount used in the test. A good antigen should fulfil this requirement.

5. *Performance of the Test.*—All sera used are inactivated by heating to 56° C. for one-half hour. Two tubes are used for each test, the front tube being the test proper and the back tube, an anticomplementary control to show that the serum alone without antigen cannot fix complement. Should this back tube be positive the test must, of course, be thrown out. Each day the tests are made a known positive serum and a known negative serum must be included as controls. These must be positive and negative respectively or the tests are valueless. The manner in which the tests are set up is indicated as follows:

Back tube.	Unknown. 1	Known positive. 2	Known negative. 3
Serum to be tested	0.15 c.c.	0.15 c.c.	0.15 c.c.
Complement (2 units)	0.1 c.c.	0.1 c.c.	0.1 c.c.
Salt solution	0.9 c.c.	0.9 c.c.	0.9 c.c.

Front tube.	1	2	3
Serum to be tested	0.1 c.c.	0.1 c.c.	0.1 c.c.
Complement (2 units)	0.1 c.c.	0.1 c.c.	0.1 c.c.
Antigen	0.1 c.c.	0.1 c.c.	0.1 c.c.
Salt solution	0.9 c.c.	0.9 c.c.	0.9 c.c.

Incubate for one-half hour and add to every tube, both front and back, two units of amboceptor and one unit cell suspension (0.1 c.c.). Incubate for one hour, shaking every fifteen minutes. Then remove from the water-bath and read the reactions.

Formerly it was customary to keep tubes in the ice-box overnight and make final readings then. This practice has been discontinued because it is believed that the reaction obtained by reading immediately after incubation is more accurate than the later ice-box reading, and also because it saves at least twelve hours in getting out reports.

Interpretation of Results.—*As in Method 1.*

GENERAL AUTOPSY METHODS.

THE performance of autopsies is not merely for the purpose of confirming the clinical diagnosis or determining the cause of death in connection with the adjustment of legal claims. Its more important object is the complete study of the effects of disease so as to render possible the more intelligent and efficient control of the diseases that affect the troops.

Records of autopsies should be written, not in a form which might be sufficient to recall to the pathologist what he observed at that autopsy but rather with such minuteness and clearness of description as to convey to another person a precise idea of the condition found.

The record should be dictated during the autopsy while the tissues are fresh and unchanged before the eyes of the pathologist, and not from memory after its completion. Relations of organs to adjacent structures are described as the examination is made and weights and measurements noted. It is better, however, in the case of a complicated organ such as the heart or lung, to proceed with the dissection, examining each new surface exposed by incision, and when it is finished to reconsider the whole and give a systematic description of the organ and its changes. Organs and tissues which are normal should be specifically recorded as being normal.

When complete the whole record should be immediately revised and corrected or amplified in the light of the conclusions finally reached with regard to the nature of the pathological changes and their relation to one another and type: written in triplicate.

The *anatomical diagnosis* should constitute a brief summary of these conclusions, the lesions being named in their order of sequence. This expresses the opinion of the pathologist, but the protocol itself should be a rigidly objective description of what is seen without the use of words which serve to avoid the trouble of constructing a vivid picture by merely stating a diagnosis.

The investigation of organs and tissues should be sufficiently complete to satisfy the pathologist that no important lesion has been overlooked and to allow him to give a clear demonstration and report of the conditions to the clinicians who have studied the case with interest. This need not in any way interfere with the very important obligation of the pathologist to preserve carefully representative museum specimens from each case for shipment to the Army Medical Museum in Washington, where they form the most valuable archives of the medical history of the war. The preservation of tissues for microscopic study and their shipment to the Army Medical Museum with the gross specimens and the protocol are equally important.

A plan of procedure, departing here and there from classical and long-established usage, but only for well-considered reasons, is given below for the average case, indicating the order of dissection calculated to ensure the examination of every organ and to give the best exposure of each without interfering with the next. Its adoption will tend to secure uniformity in the results and records of autopsies throughout the army.

Nevertheless, in any individual case in which the normal relations of the organs are greatly altered the pathologist should improvise a course which will suit the changed conditions. Much then depends upon the exercise of forethought in planning a dissection which will most clearly reveal and preserve the essential relation of lesions, and this plan can often be derived from the preliminary inspection of the organs or from the clinical diagnosis.

Throughout the dissection great care must be taken to avoid mutilating or disfiguring the body. No incision should be made which cannot be closed and covered from view when the body is prepared for burial. It is the duty of the pathologist to assure himself in each case of the restoration of the body, and especially of the face and hands to a presentable appearance. This should not be easily entrusted to a strange undertaker, and in general it is safest to have the embalming performed in the morgue of the hospital and the body inspected after its completion. Directions are given below for carrying out this procedure.

Knives should be kept really sharp. All incisions through organs and tissuses should be made with a drawing motion without undue pressure and using the whole blade of the knife. Tissues should be handled with gentleness and never squeezed or pinched. The consistence of the organ is not really appreciated except by a very light touch. Tissues intended for microscopic study should be sliced off and laid in the fixing fluid without being otherwise touched at all.

THE AUTOPSY.

The protocol should begin with the serial number of the autopsy, name of the individual—with data, such as the designation of the army organization to which he belonged—age, race, date, hour and place of autopsy, etc., and the name of the pathologist making the examination.

It is desirable to include an abstract of the clinical history, giving the date of onset of illness and entrance into hospital, a brief outline of family and personal history, a more detailed account of the present illness, a summary record of the symptoms and the results of physical examination, bacteriological and other laboratory studies, in the order in which they occurred. The day and hour of death should be noted, together with the clinical diagnosis.

EXTERNAL EXAMINATION.—The record begins with a description of the external appearance of the body, noting the length in centimeters, state of nutrition, the presence or absence of rigor mortis, together with the extent of livor mortis and any evidences of decomposition. The condition of the skin and exposed mucosæ, the eyes, ears, teeth, hair and external genitals are noted. Accurate descriptions and measurements of operative or other wounds and scars are given.

PRIMARY INCISION.—An incision has usually been made from the suprasternal notch to the symphysis pubis. Much better, as affording freer access to the organs of the neck and a better appearance after closure, is an incision from the point of one shoulder passing in a curve downward across the lower half of the sternum and up to the point of the other shoulder. From this a median incision extends to the symphysis pubis. The bib-like flap is stripped with the muscles from the front of the thorax, above the clavicles the skin is freed from the muscles of the neck up to the lower jaw and the whole flap turned back over the face. The lateral parts of the body wall are turned back from the thorax and the rectus abdominis muscles and tendons cut transversely at their insertion upon the os pubis. In this way the abdominal cavity is freely exposed.

If the peritoneum contains an inflammatory exudate, cultures and smears are made and the fluid afterward collected for examination and measurement. Fluid exudate is taken up with a loop or a sterile pipette from a protected corner of the peritoneal cavity not contaminated by the first incision.

ABDOMINAL ORGANS.—The external appearance and relations of the organs in the abdominal cavity are noted at once before exposure and handling have altered their color and relative position. At this point such a note may advantageously be made with regard to the spleen, intestines and stomach, mesentery, omentum, appendix, liver and gall-

bladder, while the remaining organs can be well seen only after dissection.

THORAX.—The dissection proceeds at once to the thorax. Its shape is noted and the position of the diaphragm measured by ascertaining on each side which rib or intercostal space can be reached by pressing it upward with the finger tips from the abdominal cavity. This must be done before any opening is made in the thorax. The information thus acquired is rough and not very valuable.

The thorax is opened by cutting through the costal cartilages along a line 5 mm. within the costochondral junction. The line turns outward to reach the cartilage of the first rib.

With the beginning of the incision through the costal cartilages on each side and before contamination can take place the thorax should be carefully pulled open to observe whether either pleura contains an inflammatory exudate. Cultures can be made at this point in the way described for the peritoneum, with best chance of cleanliness. The clavicles are disarticulated by means of a narrow knife at the sternoclavicular joints. Before removing the sternum it should be turned upward until the contents of the pleural cavities are examined, because the severing of the internal mammary vessels and the accidental wounding of the subclavian vessels are sure to soil the pleural contents with blood.

Upon opening the thorax, note the condition of the *left* pleural cavity, describing its serous surfaces and any exudate or adhesions which may be present. The surfaces are mentioned in order, beginning with the convexity of the lung and its corresponding parietal surface, passing from the apex to base and noting the condition of the interlobar pleura, then the mesial and finally the diaphragmatic surface. In this way the whole lining of the pleural sac is systematically examined. Operative incisions entering the pleura are described, with reference to the rib involved.

This is followed by a similar examination of the *right* pleural cavity.

The tissues of the *anterior mediastinum* are dissected and the details of size, shape, weight and consistence of the *thymus* ascertained. Retrosternal and mediastinal lymph nodes are examined.

THE PERICARDIAL SAC.—If it is obvious from without that the pericardium contains an inflammatory exudate, its surface is seared and fluid aspirated with a sterile syringe through the seared area or removed with a pipette through a hole made in the seared area with a sterile scalpel.

The sac is opened by a T-shaped incision, the vertical line extending in the median line from top to bottom, the transverse line across the bottom. The contents and the condition of the pericardial surfaces and adhesions are noted.

THE HEART.—The size, shape and relative position of the heart are noted, together with the degree of distention of the chambers. At this point a culture is made from the heart's blood through the seared surface of the right auricle.

The pulmonary artery is incised *in situ* and opened far enough to determine whether it contains an embolus.

The condition of the ductus arteriosus, which runs from the pulmonary artery at the left of the point of bifurcation to the descending portion of the arch of the aorta, is ascertained by isolating it or passing a probe into the orifice exposed in the lining of the pulmonary artery. This is because it plays an important part in malformations of the heart and is too frequently mutilated. If there is any reason for suspecting a cardiac malformation the whole arch of the aorta should be removed with the heart. Otherwise the heart is removed by cutting successively through the inferior vena cava, the left and right pulmonary veins, the superior vena cave, the aorta and the pulmonary artery as near the pericardial sac as possible without actually cutting through it.

The heart is opened by incisions, which in general follow

the course of the blood; but in making these it is important to bear in mind the possibility that bacteria-laden vegetations may exist on the valves, and the earliest opportunity should be taken to look into these orifices and to pick off with cold sterile forceps fragments of such vegetations for culture and smears. After this the incisions through the valvular orifices may be completed.

They run as follows: The openings of the two venæ cavæ are joined and the right auricular appendage opened to its tip. The tricuspid valve is thus exposed and any vegetations on the valve become accessible for culture. Then an incision through the tricuspid orifice along the posterior border of the interventricular septum to the apex of the right ventricle, followed by another from this point through the anterior wall of the ventricle a little to the right of the anterior border of the interventricular septum through the pulmonary orifice opens the whole right ventricle and exposes fully the tricuspid and pulmonary valves.

The left auricle is opened by uniting the apertures of the pulmonary veins and laying open the auricular appendage to its tip. The mitral valve is then examined and measured, as was the tricuspid. The left ventricle is opened by an incision which passes from the auricle through the mitral orifice to the apex and runs in the midline of the ventricle as the heart lies with the septum flat on the table. This cut is carried back along the left side of the anterior edge of the interventricular septum through the aortic orifice, avoiding the pulmonary artery on the right and the auricular appendage on the left.

It is most convenient to make those portions of the incision which pass through thick muscular walls with a knife, while the valves and thin parts of the wall are best cut with scissors.

The left coronary artery is found quite close to the last incision, and this artery is opened from that point. The right

coronary artery is cut into outside the aorta, being made palpable, if necessary, by a probe inserted into its orifice and opened from that incision with scissors.

The protocol records the weight of the empty heart, the measurements of the length of at least the two ventricles and of the thickness of their walls, together with measurements of the circumference of the valvular orifices at the valves. The plan of thrusting the fingers into each orifice to measure it gives untrustworthy results and destroys and contaminates any vegetations which may be on the valve. The contents of the chambers and the condition of the endocardium, including the valves, are noted. The chambers with their valves should be described in their order and then the state of the coronary arteries and of the myocardium.

THE LUNGS.—The posterior wall of the pericardium is incised and turned back, revealing the bifurcation of the bronchi and the bronchial lymph nodes, which may be dissected at this point. In some cases it is advantageous to remove both lungs in continuity with the trachea, larynx and pharynx, but when this is not necessary it is important to leave the organs of the neck until the head is embalmed through the carotid arteries.

The *left* lung is removed by pulling it forward and cutting along its mediastinal attachment, the knife passing through the main branches. When there are dense adhesions between lung and parietal pleura the latter may be stripped from the ribs and removed with the lung, otherwise adhesions should be cut through instead of being torn with force, which may lacerate the lung.

At this point a culture is made from the lung if indicated. It is best made by searing the surface, seizing the central point of the seared area with sterile forceps and cutting out a long cone of tissue with cold sterile scissors. The point of this cone is touched to the culture medium and smeared, or the whole is carried to the laboratory in a Petri dish.

It is frequently advantageous to distend the lung with air by means of a cannula and atomizer bulb before cutting into it. This is disturbing in so far as it destroys the condition of atelectasis, but is helpful, especially in bronchopneumonia, in aërating the blood in the alveolar walls of those alveoli which become distended and thus marking out the consolidated areas by their darker color.

When this is completed the lung is laid on the table, resting on its convex surface with the bronchus uppermost. The bronchus is opened with scissors far into the upper lobe and in the same way far along the opposite bronchus into the lower lobe. The main bronchus is thus bisected and the edge of a long knife is laid in these opened bronchi, assuming at once the best line for the main incision through the whole lung. When this is complete either half presents a cut surface, giving a good view of the bronchial tree, and one should be preserved as a museum specimen. Further incisions in the other half should be parallel with the first. Large thin (8 mm.) representative slices of the lung extending from pleura to hilum should be laid in fixing fluid for topographical histological study.

Bronchi and bloodvessels are opened on the cut surface and the lymph nodes embedded at the hilum of the lung examined. The description of the lung is made for each lobe separately after its thorough examination beginning with the upper lobe. It should detail the exact topography of the lesions as well as their character.

The *right* lung is treated in the same manner.

Mallory and Wright give the following directions: "The primary or main incision into a lung is a long deep cut from the apex to the base and from the convex surface to the root, slitting the primary bronchus and thus not cutting it off from its branches to the upper and lower lobes. To incise the left lung place it with its inner or median surface and root downward on a board and with its base toward the operator.

The left thumb steadies the lower lobe; the first finger reaches between the two lobes almost to the primary bronchus, and the rest of the fingers should hold the upper lobe.

"The right lung is most easily incised by placing it in the same position but with the apex toward the operator; in other words, always place the anterior edge of a lung beneath the palm of the hand. Secondary cuts into the lung are to be made parallel to the main incision."

ORGANS OF THE NECK.—The removal of the organs of the neck is deferred until the head has been embalmed. A long knife is then passed around the inside of the lower jaw, loosening the attachments of the tongue. The organs of the neck beginning at the bifurcation of the trachea are stripped from the vertebral column. The knife is passed around the back of the pharynx, cutting it at the base of the skull. After pulling down the tongue the soft palate is cut across and the whole mass from the posterior nares down is removed in one block. This allows of a survey of the tongue, tonsils, uvula and fauces. The *pharynx* is split posteriorly in the midline and the incision carried through the whole length of the *esophagus*. The *larynx* and *trachea* are similarly opened in the posterior median line. *Thyroid, parathyroids* and *cervical lymph nodes* are dissected clear.

THE ABDOMINAL ORGANS.—The *spleen* is pulled forward and removed after inspection of the bloodvessels at the hilum by their division. It is measured in all diameters and weighed, and the shape, consistence and external appearance noted. The organ is cut in its long diameter and the condition of the bloodvessels, trabeculæ, Malpighian bodies and splenic pulp described.

The Intestines.—The external appearance of the intestines and mesentery with its lymph nodes and bloodvessels having been described the intestine is divided at the duodenojejunal junction and removed by cutting the mesentery at its insertion into the intestinal wall. This is most satisfactorily done

if the pathologist holds the intestine gently with the left hand while the assistant follows the strokes of the knife, holding the mesentery taut.

At the ileocecal region the free mesentery is left and the cecum and ascending colon freed by sweeping incisions through the peritoneum and gentle traction. In the same way the transverse and descending colon are drawn free after appropriate incisions in the mesocolon. The rectum is cut across low in the pelvis.

The whole intestine is opened with long scissors, which in the case of the small intestine pass along the mesenteric border. The washing of the mucosa for examination is done under a gentle stream of water without rubbing the fingers or a sponge over its surface, and it is scrutinized from end to end.

The mesentery is drawn up and severed at its root, exposing the duodenum throughout its course.

Stomach and Duodenum.—The duodenum is opened *in situ* along its convex border and this line of incision is continued along the greater curvature of the stomach or along its anterior surface. The contents of these organs are evacuated and their nature noted. If poisoning is suspected the stomach and intestines are preserved in chemically clean containers so as to keep their contents unchanged. The mucosa of both stomach and duodenum is described.

Bile duct and pancreatic ducts opening into the duodenum require attention at this point. The squeezing of the gall-bladder to test the patency of the ducts may give deceptive results, but if there is any suspicion that the bile duct is obstructed the connection with the liver should be preserved until the dissection is complete; otherwise the portal vein, bile duct and hepatic artery are cut and the stomach and duodenum removed from the body, together with the pancreas, after cutting through the celiac axis and superior mesenteric arteries.

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The *pancreas* is exposed and measured. Its weight can be ascertained only after it is carefully dissected away from the intestine, which is not always desirable. It is inspected by making transverse incisions at the tail and near the middle. Upon the latter cut surface the duct of Wirsung is visible and is opened with blunt-pointed scissors to its entrance into the intestine. The common bile duct is also opened to its outlet in the intestine and the relation of these ducts in the ampulla of Vater described. The lesser pancreatic duct may be similarly traced out. A third transverse incision through the head of the pancreas may then be made.

The *liver* is removed by cutting through the falciform ligament in front and the lateral and coronary ligaments behind, cutting the vena cava below the organ. Care is taken not to injure the right adrenal, which is usually closely applied to the under surface of the right lobe. When the liver is free it can be lifted by a finger passed through the vena cava. It is weighed and measured and its external appearance and consistence described. Several transverse slices are made through the whole organ and the appearance of the cut section described, noting the size of the lobules and the color of each of their zones as well as the condition of bile ducts and bloodvessels.

The *gall-bladder* after external examination is opened and the character of contents and mucosa noted. The cystic and hepatic ducts are also opened.

The *left adrenal* is dissected free and removed and weighed and measured. Upon longitudinal section the thickness, color and definition of the cortex and medulla are recorded. The *right adrenal* is similarly examined.

The Genito-urinary Apparatus.—In many cases in males it is desirable to remove all these organs together, preserving their connections. In females it is usual to do so. When there is no indication of disease which would require this continuity to make the examination clear, the kidneys

may be removed separately after uncovering and examining the ureters before they are cut.

The Left Kidney.—The organ is weighed and measured after the removal of the capsule. The appearance of the capsule and external surface of the kidney and, after longitudinal section, the appearance of the cortex, medulla, pelvis and ureter are described. The thickness of the cortex is measured and the general appearance of the striations recorded. Glomeruli, bloodvessels, convoluted and straight tubular portions of the striations are separately described.

The *right kidney* is similarly studied.

The testicles are exposed by incision through the tissues about the inguinal canal, which allows their being drawn out of the scrotum. The size, shape, consistence, appearance externally and on longitudinal section are observed for testis and epididymis, together with the condition of the tunica vaginalis. The testes may be left in connection with the pelvic organs if this is advantageous.

The Pelvic Organs.—The bladder and rectum are readily removed from the pelvis by a sweeping incision which passes through the urethra just below the prostate and the rectum just above the anus. It is better, however, to strip forward the skin from the penis cutting across its substance at a short distance from the glands. By cutting through the ligaments beneath the symphysis pubis the whole penis can be removed with the bladder and rectum. The rectum is opened along the posterior median line, the penis and bladder along the anterior median line, although this involves the separation of the corpora cavernosa.

Bladder.—Size, thickness of wall and condition of mucosa are recorded. Special attention is directed to the ureteral orifices and ureters. The prostate is examined in several transverse incisions, which are made from the posterior surface if the urethral mucosa is to be preserved intact. The seminal vesicles behind the bladder are dissected clear and opened by a single oblique incision.

In the female the pelvic organs are treated in a similar way. The bladder and urethra are opened by an anterior median incision, the rectum by a posterior median incision. Upon turning aside the bladder, the vagina, uterus and tubes are opened by anterior incisions. The ovaries are measured, incised longitudinally and inspected externally and internally.

There remains in the body the aorta with its branches, the retroperitoneal tissues and the thoracic duct. The thoracic duct is dissected out and opened. The condition of the retroperitoneal lymph glands is ascertained and the aorta with its branches finally removed or opened *in situ*. The iliac, femoral and pelvic veins should be examined for thrombi, and in cases of pulmonary embolism this should be done before the pelvic organs are disturbed.

Voluntary muscles, peripheral and splanchnic nerves and ganglia, peripheral bloodvessels, bones, cartilages and joints are dissected and described whenever necessary. A routine examination of ribs, vertebræ and pelvis should be made.

BONE-MARROW. — As the most important blood-forming tissue the bone-marrow should be the subject of routine study and is best obtained from the sternum or a long bone.

It is relatively easy to dislocate one humerus at the shoulder-joint, push it upward, stripping back the muscles and remove a mass of marrow from its shaft without breaking it. The arm falls back into shape and position without disfigurement. Ordinarily, however, the marrow is removed from the shaft of the left femur. A transverse incision is made on the outer side of the thigh and the muscles cut to the bone and retracted. Two saw-cuts at an interval of 3 cm. are made deep enough to go through the cortex, *i. e.*, about 1 cm. One sharp blow of a mallet on a chisel applied at the end of the upper saw-cut will chip off the 3 cm. length of cortex, exposing a cylinder of marrow, which is easily cut out. The color and consistence of the marrow are recorded.

BRAIN.—The proper embalming of the face is so important that the removal of the brain must be postponed until this is finished, although it is possible to ligate the carotid arteries within the skull or to pack the base of the skull with plaster of Paris and thus make embalming of the face practicable after the brain has been removed. When it is necessary to make cultures from the meninges the calvarium may be removed and a small flap of dura lifted so that a culture can be obtained, after which, upon replacing these structures, embalming can proceed satisfactorily in spite of slight leakage. A great advantage in the embalming lies in the fact that it gives the most satisfactory fixation of the brain.

The incision through the scalp begins behind one ear and passes vertically over the top of the head to a similar point behind the other ear. It is best to insert the knife under the scalp and to cut outward to save the hair. The scalp is stripped forward and backward to the orbital ridge in front and to the occipital protuberance behind. The calvarium is opened by saw-cuts. "The first cut begins just above and behind the left ear and is carried over the forehead just back of the edge of the hair or over the frontal eminences to a corresponding point above and behind the right ear. The other two cuts begin at each end of the first incision, forming there an obtuse angle, and are carried back to meet in the median line behind at an angle of about 160 degrees a little in front of the occipital protuberance." (Mallory and Wright.) The saw-cut need not go quite through the skull, but the incision may be completed by cracking with a chisel, except in cases of fracture of the skull, when no cracking is allowable and the saw-cut must be complete. The calvarium is pulled off with a hook, leaving the brain covered with the dura.

The longitudinal sinus is opened and inspected. The dura is cut with scissors along the line of the saw-cut and laid back. The falx cerebri is cut near its anterior insertion and the dura pulled back, exposing the brain. In this process the

Pacchionian granulations must usually be torn through as they project through the dura along the longitudinal sinus. When the dura is densely adherent to the skull, as in old men and children, it is incised as just described and removed with the calvarium.

The brain will be found hardened by the formalin injection. Nevertheless, it is such a soft tissue that it must be handled with extreme gentleness. After inspection of the surface it is removed by gently pulling up the frontal lobes, lifting the olfactory lobes against them so that they adhere. The optic nerves and internal carotid arteries and the stalk of the hypophysis are cut through and the anterior ends of the temporal lobes lifted up. This exposes the tentorium cerebelli, which is incised with the point of the scalpel along its insertion on the petrous portion of the temporal bone. This frees the cerebellum. From this point the cranial nerves and vertebral arteries are most satisfactorily cut with scissors unless one has an extremely sharp knife. The brain comes to lie in the hollow of the left hand. It must be protected from overstretching in the region of the pons and medulla, and when everything else is clear the spinal cord is cut across with the tip of a long knife or much better with a myelotome, which is a little blade set nearly at right angles on a long handle. The further study of the brain begins with inspection of the meninges, bloodvessels and cranial nerves.

The brain should be suspended for a week in 10 per cent. formalin until it is hard. It may be hung by a cord tied about the basilar artery so as not to touch the bottom of the jar. Then the pathologist should section the organ enough to learn what gross lesions there are and demonstrate them. The plan which has been used by many is to separate the cerebellum with the pons and medulla from the cerebrum by cutting through the crura cerebri and upper peduncles of the cerebellum and then to make several parallel frontal sections through the cerebrum.

THE SPINAL CORD.—The spinal cord is also an extremely soft structure and in its removal the utmost gentleness must be observed to avoid compression, twisting or stretching. To secure all of it, it is advisable to saw a wedge-shaped piece out of the occipital bone opening into the foramen magnum, after which the whole spinal canal is laid open. This operation is begun by making a dorsal median incision and cleaning back the muscles from the spine. The spines of the vertebræ may be clipped off with a large bone cutter. A saw with a round edge is used and on each side a cut is made at about the outer end of the lamina from the midlumbar region up to the neck. It is difficult to saw the laminæ in the neck, and the spinal canal is wide enough there to allow of their being cut with a chisel or bone cutter. The loose portion in the third or fourth lumbar vertebra is now pried up and the whole covering of the spinal canal removed in one piece. By cutting out the adjacent articular processes of the vertebræ the ganglia may be removed in continuity with the cord. Representative ganglia from lumbar dorsal and cervical regions on both sides should be obtained.

The dura is cut across with the cauda equina between the third and fourth lumbar vertebræ and seized with forceps or artery clamp. The nerves are divided on each side with a sharp scalpel and the cord carefully lifted out of the canal by the dura held in the clamp.

It should be laid out straight on the ventral surface, the dura incised in the dorsal median line with scissors and the cord cut across once in the middle without cutting the dura anteriorly. It is then so suspended over a thread that the upper and lower halves of the cord hang straight in a jar of 10 per cent. formalin.

Inspection of the pia covering the cord and of the blood-vessels is completed, but except in the case of gross lesions the further study of the cord may be postponed until it is hardened.

BASE OF CRANIAL CAVITY.—After the removal of the brain the *venous sinuses* of the *dura mater* may be opened and inspected. The superior longitudinal having already been opened there remain, especially the lateral, the petrosals, the cavernous and the ophthalmic on each side.

The *hypophysis* is set free by breaking away the posterior clinoid process on each side after which it can be dissected out with ease. It should be weighed and if not enlarged fixed *in toto.* Little is to be learned by an incision while fresh, but if such incision be made it should be sagittal, at one side of the stalk.

At this point the whole dura covering the base of the skull is peeled off to expose the bones. This is especially important in cases in which fracture of the skull is suspected.

There remain for examination the nose, with its sinuses, the ear and the eye. In connection with the prevalent respiratory infections it is especially important to secure cultures from the middle ear and nasal sinuses.

THE NASAL SINUSES.—One can with relatively little chiselling lay open all the sinuses widely enough to inspect their lining and take cultures from their contents. The examination may begin with the *frontal sinuses,* which can be entered just above the anterior margin of the orbit and opened laterally as far as necessary. Next, the *ethmoid cells* are opened through the cribriform plate. The indirect communication of the frontal sinus with the anterior group becomes visible. By chiselling into the body of the sphenoid behind this the *sphenoid sinus* is opened on each side with its canal entering the nares anteriorly. The antrum may be entered by pulling up the upper lip and chiselling into the front of the superior maxillary above the second premolar tooth or by removing the roof of the orbit and the contents of its posterior part and then cutting a hole in its floor, which is the roof of the antrum.

When the sinuses on both sides have been opened and

examined and cultures and smears obtained the cavity of the nose may be exposed by chiselling through the floor and front wall of the sphenoid sinuses.

MIDDLE EAR.—For the examination of the *middle ear* and the *mastoid cells* it will generally suffice to chip off with the chisel the middle of the petrous portion of the temporal bone or to bite it off with heavy bone forceps. This exposes the cavity of the tympanum or middle ear with its ossicles, the opening into the mastoid cells on the posterior wall and that of the Eustachian tube on the anterior. It is easy to open more of the mastoid cells by further chiselling. Cultures can be obtained if the roof of the tympanum is split off cleanly.

THE EYE.—The roof of the orbit is chiselled off and the contents of the orbit examined. It is possible to examine the anterior of the eye by cutting with sharp scissors around the eyeball and dropping the posterior half of the eye into formalin after inspection. The anterior half must be carefully fixed in place with a plug of blackened cotton.

RESTORATION OF THE BODY.

The statements made above that the body must leave the morgue in a presentable condition, verified by the pathologist, may be repeated with advantage here. Aside from the ordinary justice of this demand, it is to the interest of the pathologists to exercise extreme care in this regard, since the shipment of a neglected body may result in an interruption of pathological studies or legal complications.

EMBALMING.—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 any one else.[1] The undertaker's pressure bottle with several tubes armed with long metal cannulæ, which are tied into the carotids and subclavian

[1] If shaving is necessary it must be done before the face is embalmed.

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. Of course, the open end of the aorta as well as any leaking arteries (internal mammaries) must be closed with clamps or tied.

Undertaker's embalming fluid or a 10 per cent. 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.

Closure of Incisions.—After the brain has been removed, sawdust in a bag should be put into the cranium or it may be filled with plaster of Paris. In the removal of the cranium the temporal muscles should be pushed back and not cut away. The calvarium is fitted in place and held by stitches taken through these muscles. Or if the saw-cuts are allowed to cross for an inch at the point where they meet behind the ear, bandages may be worked into these slits and pinned together over the top of the calvarium.

The greatest care must be devoted to obtaining an exact and lasting adjustment of the calvarium before the scalp is pulled into place and molded over it. The incision is most carefully sutured and the hair made to cover the suture as completely as possible.

The body cavity is sponged out dry and filled with sawdust, oakum or cotton waste. This should be packed tightly into the pelvis to prevent leakage. The place of the neck organs

must be filled with some substance, such as cotton, which will allow of the neck's being molded into a natural form. Paper is laid over the material in the body cavity and the sternum replaced. The incision is closed by a continuous suture, the needle passing from within out and the twine being held tight after each stitch. It is knotted at both ends and finally buried by taking a long stitch to one side and cutting it off close to the skin.

The incision in the back through which the spinal cord was removed and that in the thigh for the removal of bone-marrow are packed with cotton waste and tightly sutured in the same way. All blood stains are removed before the body is handed over to the undertaker. While it is the business of the undertaker to make the body presentable, the pathologist is held personally accountable for making sure that no body is allowed to leave the autopsy room until this is done.

PRESERVATION OF MUSEUM SPECIMENS.

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 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 slice should allow for the removal of a thin layer at a later date to freshen the surface. This is practically true of the lung, in which case a 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.

It is injurious to pack cotton 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 that is no longer possible, and it must be propped open with cotton, it should at least be inserted very loosely.

The heart after being opened in the manner described should be stretched on a glass 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 during fixation by a few temporary stitches.

The stomach or portions of the intestine can be filled with Klotz fluid or 10 per cent. formalin and ligated at the ends until hardened, after which they can be bisected longitudinally. Otherwise they may, of course, be opened, pinned out on a board with thumb tacks, so that the mucosa is exposed and floated face down on the surface of the fixing fluid.

In the case of the kidney, one-half cleanly cut forms a satisfactory specimen.

The brain and cord are preserved intact in formalin.

There is no antagonism between the aims of the autopsy and those of the museum. The best museum specimen is that which is most intelligently dissected and then fixed. For a pure display a rather thick slice of an organ if taken in the right direction and complete should be as good as a whole organ. For the museum, broad smooth-cut surfaces are desired. Numerous incisions in conflicting directions defeat this object, and for that reason it is suggested that at least until a slice or half of an organ has been reserved as a museum specimen the incisions be bold and slashing to produce a smooth surface and parallel.

FIXATION OF MUSEUM MATERIAL.—The Army Medical Museum recommends Klotz's fluid for the preservation of museum specimens. Its chief advantage is that specimens can be preserved and shipped in fluid No. 1. For preparation of Klotz's fluid see page 31.

The chief limitation of this method is that it does not give

fully satisfactory results for histological studies, especially after long fixation. Hence, it is necessary to fix additional material in formalin or other fluid.

In many army laboratories the materials for Kaiserling's fluid are on hand, and in such cases this fluid is recommended. Specimens should be shipped in fluid No. 3.

When the use of neither Klotz's nor Kaiserling's method is practicable, specimens should be well fixed and shipped in 10 per cent. formalin; and in general, unless formalized tissues for histological purposes can be sent in addition to Klotz's or Kaiserling's material it is better to fix and ship the entire specimen in formalin. If forwarded promptly the colors can be partly restored by appropriate treatment after reaching the Museum.

All neurological specimens should be fixed and shipped in formalin.

Materials of this sort should be forwarded through the Quartermaster's Department to the Army Medical Museum in Washington. They should be accurately tagged with the autopsy number written in lead-pencil and should be accompanied by all possible clinical and bacteriological data and the protocol of the autopsy. (Boxes of galvanized iron or tin, measuring 9 x 9 x 12 inches, should be made by the tinsmith so that the top may be soldered on.) The specimens are packed in layers of cotton with enough fluid to keep them moist. The can should be enclosed in a wooden box for shipment. Histological material should be sent in a separate container if it has been fixed in Zenker's fluid, since this tends to stain museum specimens.

HISTOLOGICAL METHODS.

In general, slices of tissue selected for microscopic study should be about 5 or 6 mm. thick. Slices 2 or 3 mm. thick curl in the fixing fluid, so that it is almost impossible to secure

a section, while slices 8 to 12 mm. thick are not completely penetrated except by formalin.

Tissues are fixed in Zenker's fluid to which 5 per cent. of formalin instead of 5 per cent. of acetic acid is added just before it is used. Slices such as described are laid flat in a dish of this fluid and not crowded into a bottle. They are left twelve to twenty-four hours and then washed in running water for twenty-four hours, after which they are preserved in 80 per cent. alcohol; 5 per cent. formalin for twenty-four hours followed by washing and preservation in 80 per cent. alcohol gives fair results, but is not to be compared with Zenker's fluid.

Both in selecting these slices and in subsequently cutting blocks of the hardened tissue from them for embedding, it is most important to bear in mind that microscopic study is primarily concerned with rather gross changes in the tissues and not merely with the condition of the single cells. For example, a microscopic section of a part of the lung should show the pleura, two or more interlobular septa, a bronchiole with its branches, bloodvessels and lymphatics, as well as the surrounding substance of the lung. It must be cut from a block taken from a slice of the lung, which is large and carefully chosen with the naked eye, for the purpose of including all these things. A section of the kidney must be cut from a slice which is made through the whole cortex and pyramid at right angles to the main median incision and from a particular slice, not one which passes through the columns of Bertini and therefore cuts all the tubules crosswise, but one which passes through a papilla and therefore shows the cortical striations in their full extent as well as the medullary tubules.

The point is labored because most of the histological material sent in for examination consists of minute fragments so small that no satisfactory section can be made or of sections cut in any direction regardless of the structure of the organ. Practically nothing can be made out of such sections

except possibly in the case of the liver or spleen. Care of this point must begin therefore at the autopsy, when the direction and size of the slice of tissue is determined, in the proper relation to the architecture of the organ and to the extent of the lesion.

For methods of preparation, sectioning and staining of histological material see page 68.

GENERAL BACTERIOLOGICAL METHODS.

STERILIZATION.

INSTRUMENTS and everything else that can stand it without injury can be sterilized by prolonged boiling. The addition of a pinch of sodium carbonate prevents the rusting of instruments and increases the efficiency of sterilization.

Prolonged boiling in water is a convenient method of sterilizing syringes for taking the blood, but no soda should be added in this case, since it might lead to laking of blood; also, if dried, interferes with smooth operation of syringe. Syringes can also be sterilized conveniently in autoclave.

Dry glassware, cotton stoppered, should be sterilized in the dry oven at 160° C. for one hour. If in a hurry, 180° to 190° can be used for a half-hour; 200° and over browns the cotton stoppers.

Salt solution, and media, if they do not contain carbohydrates, gelatin, or glycerin, can be sterilized in the autoclave. Autoclave sterilization is the most thorough sterilization that can be applied. Fifteen pounds' pressure for fifteen minutes should be employed. In using any kind of an autoclave, it is extremely important that all the air be let out of the autoclave before the valve is closed and pressure allowed to develop. If there is air in the autoclave, insufficient sterilization will follow because the interior of the autoclave will not be saturated with water vapor. Superheated steam will result, and the conditions approach those which take

place in the dry sterilizer. The temperature of the auto-clave should be 100° for a few minutes before the steam outlet is closed.

Media containing carbohydrates and other substances which are subject to injury by the high pressure can be sterilized by live steam in the Arnold sterilizer.

In using the Arnold sterilizer, fractional sterilization must be employed. This consists in giving the materials to be sterilized twenty to thirty minutes in the Arnold after the Arnold is hot, on three successive days, keeping in a warm place in the intervals between exposures. The purpose of this is to allow the development of any spores which may be present in the materials.

BASIC MEDIA.

MEAT-EXTRACT BROTH.—To 1 liter of clear tap water add:

Meat extract . . . . 5 grams
Pepton . . . . 10 grams
NaCl . . . . 5 grams

Dissolve and weigh with containing vessel.

Heat over free flame until dissolved, weigh again, make up loss, and measure volume.

Titrate and adjust, heating over flame for five minutes. Filter through paper. Sterilize.

If medium cannot be cleared by filtering through paper, clear by white of egg and filter through cotton.

MEAT-INFUSION BROTH.—500 grams of lean meat are infused twelve hours in 1 liter of water. While infusing, place in ice-box.

Strain through cheesecloth and add water to make up to 1 liter.

Add:

Common salt . . . . 5 grams
Pepton . . . . 10 grams

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Weigh this mixture with vessel and note weight.

Warm, preferably in a water-bath, stirring until the peptone is dissolved. It is important not to allow the temperature to go above 50° C.

Now, for the purposes of adjustment, measure the volume, titrate and bring to neutral with normal sodium hydrate.

Heat in the Arnold sterilizer for half an hour, stir thoroughly, and heat again for fifteen minutes.

Weigh and make up for evaporation.

Titrate and adjust to the desired point, which for ordinary work is best kept between 0.5 and 1 acidity.

If adjustment has been necessary, heat again for about ten minutes and filter through paper until clear.

If paper filtration does not clear the medium, then the medium can be egged by the method described below and filtered through absorbent cotton.

Finally, titrate to determine reaction of medium as finished. Fill into sterile flasks or test-tubes and sterilize in autoclave.

SUGAR-FREE BROTH.—A liter of finished infusion broth can be used. If there is none available, make a liter of meat infusion broth by the method described, bringing it to the point before final clearing and adjustment of reaction. Filter through a thin cotton filter to remove the larger particles, and allow to cool in a flask.

Inoculate with Bacillus coli communis.

The flask is incubated for one or two days, during which time the sugars are fermented out by the colon bacillus.

Bacteria are killed by heating in the Arnold.

Determine volume, titrate and adjust to an acidity of 0.2. Heat thoroughly and filter through paper until clear. (If paper filtration does not suffice, the medium can be egged.)

In careful work the media should be tested with B. coli to be sure that no sugar is present.

Divide into portions of 250 c.c. and add the sugar desired in proportions to 1 per cent.

Sterilization should be done by the fractional method, not heating for more than twenty minutes at a time after the Arnold is hot, since heat is likely to hydrolize the polysaccharides.

PEPTONE-SALT SOLUTION (Dunham's solution):

Distilled water	1000.0 c.c.
Peptone (Witte)	10.0 grams
NaCl	5.0 grams

Heat until ingredients are thoroughly dissolved.

Filter through filter paper until perfectly clear.

Tube twenty-five tubes, and store remainder in 250 c.c. flasks.

GELATIN.—Meat-extract Gelatin.—To 1 liter of distilled or tap water add:

Meat extract	5.0 grams
Peptone	10.0 grams
NaCl	5.0 grams
Finest French sheet gelatin	120.0 grams

Weigh with vessel and dissolve by warming.

Bring back to original weight, determine volume, titrate and adjust.

Cool to 60° C., add whites of two eggs and stir.

Heat for half an hour, stir and heat again fifteen minutes.

Adjust weight, filter through cotton and sterilize.

Gelatin should not be subjected to too prolonged heating. Sterilize by fractional sterilization. Never in autoclave.

MEAT-EXTRACT AGAR.—To 1000 c.c. of distilled or tap water add:

Thread agar (according to purpose)	15 to 20.0 grams
Peptone	10.0 grams
Meat extract	5.0 grams
Common salt	5.0 grams

Put in autoclave, 15 pounds' pressure, for 15 minutes.

The agar can also be dissolved over the free flame, but this takes a long time and is unsatisfactory for ordinary laboratory purposes. However, if so done make up wherever lost by evaporation.

Take out of autoclave, determine the volume, and adjust to desired reaction.

Cool to 60° C. and add the whites of two eggs. Stir thoroughly.

Heat either in the autoclave, fifteen pounds, fifteen minutes; or if no autoclave is available, thirty minutes in the Arnold. If the heating is done in the Arnold, take out after half an hour, stir and replace for fifteen minutes more.

Determine volume, titrate, and adjust again if reaction has changed.

If a correction in reaction is made, heat again for ten minutes, filter through cotton, tube, and sterilize.

MEAT-INFUSION AGAR.—I. Infuse 500 grams of lean meat twelve hours in 500 c.c. of distilled water in ice-box.

Strain through wet cotton flannel or wet cheesecloth, and make up volume to 500 c.c.

Add:

Peptone . . . . 10.0 grams
Common salt . . . . 5.0 grams

Weigh with vessel.

Warm carefully over water-bath until peptone and salt are dissolved, and do not allow temperature to exceed 50° C.

Determine volume, titrate, and neutralize.

II. Add 15 grams of thread agar to 500 c.c. of distilled water and dissolve in autoclave as above.

Cool to about 60° C.

III. Then to solution I of meat infusion (at 50° C.) add solution II of agar (at 60° C.). After the two are mixed, stir thoroughly, take out a specimen for titration, and lose no time in getting the mixture into the Arnold or water-

bath to keep it from cooling below the congealing temperature of the agar.

Before doing this, quickly measure volume.

Titrate the specimen removed, calculate the amount necessary to bring the entire volume to 0.5 acidity, add normal sodium hydrate solution as required and place the mixture in the autoclave.

After this, treat it like meat-extract agar.

LACTOSE-LITMUS AGAR.—Lactose-litmus agar is a 1.5 per cent. to 2 per cent. meat-extract agar, to which 1 per cent. lactose has been added and sufficient litmus solution to give it a purple color when cool.

DORSETT EGG MEDIUM.—This medium is used for the cultivation of tubercle bacilli.

Four eggs thoroughly cleansed with water, washed in 5 per cent. carbolic solution and allowed to partially dry. The ends are then gently dried in the flame and the shell pierced with a flamed sharp forceps, breaking the membrane in one end and leaving it intact in the other. By blowing through the latter opening the contents of the egg are expelled into a sterile Erlenmeyer flask.

Break up yolk with a platinum wire and mix thoroughly the whites and yolks.

Add 25 c.c. of distilled water and strain through sterile cloth.

Pour 10 c.c. each into sterile test-tubes and stand the tubes in water at 45° for ten minutes or so, leaving them undisturbed, so that the air may gradually come out of the egg mixture. Slant in an inspissator and expose to 70° to 75° C. for four or five hours on two days.

On the third day, gradually raise temperature above this.

The sterilization may be finished by a single exposure to 100° C. in an Arnold sterilizer. Before inoculation, add two or three drops of sterile broth to each tube.

Five per cent. to 6 per cent. glycerin can be added to this medium when human tubercle bacilli are to be cultivated.

PETROFF'S MEDIUM.—This medium consists of 2 parts of egg (white and yolk), 1 part of meat juice and gentian violet sufficient to the proportion of 1 to 10,000; 500 grams of beef or veal are infused in 500 c.c. of a 15 per cent. solution of glycerin in water. Twenty-four hours later the meat is squeezed in a sterile meat press and collected in a sterile beaker. Sterilize the shells of the eggs by immersion for ten minutes in 70 per cent. alcohol or by pouring hot water upon them. Break the eggs into a sterile beaker and after mixing the eggs well, filter through sterile gauze. Add 1 part by volume of meat juice. Add sufficient 1 per cent. alcoholic gentian violet to make a dilution of 1 to 10,000.

Tube about 3 c.c. in each sterile test-tube and inspissate for three successive days: on the first day at 85° C., until all the medium is solidified, changing the places of the tubes if necessary; on the second and third days for not more than one hour at 75° C. For the bovine type omit the glycerin and infuse the meat for twenty-four hours in water. Bovine tubercle bacilli grow in this medium even if it contains glycerin, but on account of the popular belief and the lack of data a medium without the glycerin is used.

LUBENAU'S GLYCERIN-EGG MEDIUM.—Prepare 1 liter of veal broth (2 per cent. peptone) and 5 per cent. glycerin. Make neutral to litmus. To each 200 c.c. add ten fresh eggs, mix well, avoiding froth, tube and heat in slanted position for two and one-quarter hours at 70° C. on three successive days.

STARCH AGAR.—Beef-infusion (not extract) agar is made in the usual way except that salt and peptone are omitted. Adjust to very slight acidity, that is, 0.2 to 0.5+. Add 10 grams of corn starch to each liter. Cook in autoclave for thirty minutes at ten pounds' pressure. Tube and sterilize.

This medium is especially useful for gonococcus cultivation.

For a description of Petroff's medium for the isolation of tubercle bacilli see page 167.

MILK MEDIA.—Fresh milk is heated in a flask for fifteen minutes in an Arnold sterilizer. It is set away in the ice-chest for twelve hours to allow the cream to rise. The milk is then separated from the cream by siphoning it into another flask. Old milk that is acid should be thrown away. It is not necessary to adjust reaction ordinarily. The milk is tubed with or without litmus as desired, and sterilized by fractional sterilization.

LOEFFLER'S MEDIUM.—Beef blood is collected at the slaughter house in high cylindrical jars. Attempts should be made to avoid contamination as much as possible by sterilizing the jars, keeping them covered, and exercising care in collecting blood.

In camps, beef serum often is hard to get, but through the veterinary of the camp blood may be obtained from horses and mules in the veterinary hospital. To get the maximum amount of serum a wire frame of two decks and such dimensions as to set in the pail may be used. The blood is drawn in the pail, allowed to clot and then the frame is raised about three inches and held there until all the serum has been squeezed out.

Allow the blood to coagulate in the jars, and do not move from the slaughter house until coagulated. As soon as the coagulum is formed, adhesions between the clot and the sides of the jar should be carefully separated with a sterile glass rod or wire. Set away in the ice-chest for twenty-four hours. Pipette off clear serum, preferably with a large pipette of 50 to 100 c.c. capacity.

One part of a 1 per cent. glucose veal or beef infusion broth is added to three parts of the serum. The mixture is filled into small test-tubes used for diphtheria and inspissated. Here, as in the making of the egg medium, it is desirable to set the tubes for a short time in hot water, at not above 50°, to drive out the air. The temperature of the inspissator should be raised very gradually to 75° and a little casserole

of water or some wet cotton placed inside to keep the medium from drying up. The temperature is maintained for about three hours on the first day, and this process is repeated for five successive days. If haste is desired the medium can be heated in an Arnold sterilizer after the first and second inspissation if well coagulated. If the sterilizing and coagulating processes are hurried without judgment, the medium will be broken up by the expansion of air bubbles. In using the Arnold for this medium, the heat should be brought up very gradually and not allowed to rise as rapidly as ordinarily done in Arnold sterilization. If the inspissator is not available an Arnold sterilizer may be used, covering the top with a cloth instead of the usual cover.

SERUM-WATER MEDIA FOR FERMENTATION TESTS.—The serum-water media of Hiss are used for the determination of sugar-splitting powers and acid formation of various bacteria.

Beef or sheep serum is obtained from clotted blood, and this is diluted with three times its volume of distilled water. The mixture is heated in the Arnold for fifteen minutes, and to parts of it are added the various sugars which it is desired to use in the proportion of 1 per cent. and enough litmus to give it a deep purple color. Sterilization is done by the fractional method.

Inulin serum water is the medium for the differentiation of pneumococcus and streptococcus. The inulin is first dissolved in the water and sterilized in the autoclave for fifteen minutes at fifteen pounds. The serum is then diluted with this sterile inulin solution and sterilized as usual.

CLEARING OF MEDIA.—Solid media, in order to be of any use, must be clear. This is accomplished by precipitation and filtration. The simplest method of precipitation is by the addition of eggs. The technic is as follows: The white of egg, using one egg to the liter, is broken into a small tin measure and a few cubic centimeters of warm water added, and then beaten slightly in order to mix the egg with the water.

This egg water is then added to the medium. Care should be exercised in the case of such media as agar, etc., which are hot when in the liquid form, that the egg is added only after the medium has been cooled to about 50° C.

Unless this is done the egg will immediately coagulate and exercise no clearing action.

After the egg has been added, mix thoroughly with medium and heat in Arnold sterilizer for forty-five minutes or in autoclave for fifteen minutes at fifteen pounds' pressure.

Filter through cotton.

Broth can sometimes be cleared by filtration through paper, but egg is better.

To Make a Cotton Filter.—Put a little copper wire spiral into the bottom of a large funnel. Take a piece of absorbent cotton and split it horizontally, that is, into two thicknesses. One of these pieces, about four by four inches, is pushed down into the funnel against the copper spiral and the other laid over it so that its fibers are at right angles with the piece underneath. Stick the edges of the cotton layers against the funnel with hot water dripped from the faucet, and plaster these edges against the inside of the funnel by stroking with the fingers. Filtration is improved by giving the entire filter thirty minutes in the steam sterilizer before use.

When pouring in the medium, be careful not to break through the cotton by the rush of the first fluid and not to dissect the edges of the cotton away from the glass sides of the funnel with the stream. It is best to pour against a glass rod held gently against the center of the filter.

TITRATION OF MEDIA.—Standard solutions of $\frac{N}{1}$ sulphuric acid and $\frac{N}{10}$ sulphuric acid may be obtained from the Department Laboratory.

To make $\frac{1}{20}$ NaOH: Weigh out 5 grams of pure NaOH; dissolve in distilled water and dilute up to 1100 c.c. in a standard flask. Titrate against the standard $\frac{N}{10}$ sulphuric

acid and dilute so that 2 c.c. of the NaOH solution shall balance against 1 c.c. of the acid.

In titrating, use a porcelain casserole, carefully washed out with distilled water, and stirring rod, washed with distilled water. Put in 40 c.c. of distilled water and 1 c.c. of 1 per cent. alcoholic phenolphthalein solution. In titrating media, add 5 c.c. of the medium to the 40 c.c. of distilled water and the phenolphthalein in the casserole, bring to a boil, and run in the NaOH until the first noticeable faint pink which remains on boiling has been reached.

Calculations from titrations of media: If it takes 1.5 $\frac{N}{20}$ to neutralize 5 c.c. then 1.5 $\frac{N}{1}$ will neutralize 100 c.c. and 15 $\frac{N}{1}$ will neutralize 1000 c.c.

Now, if it is desired to make this 0.2 per cent. acid to phenolphthalein, as in Endo's medium, then add 13 c.c. to the liter.

For the adjustment of media $\frac{N}{1}$ NaOH may be used, made in the following way: Dissolve 41 grams of NaOH in water and gradually fill up accurately to one liter.

TITRATION OF MEDIA TO DEFINITE HYDROGEN ION CONCENTRATION.—This method is somewhat more exact than the preceding for adjusting the reaction of media.

Materials:

1. Chemically clean 100 c.c. Erlenmeyer flasks.
2. Freshly distilled water.
3. M/5 KH_2PO_4 solution, 27.234 grams to liter of water.
4. M/5 NaOH solution, 8.002 grams per liter.
5. Phenolsulphonephthalein (phenol red), 0.05 per cent. water solution.
6. N/20 NaOH and N/1 NaOH.
7. N/20 HCl and N/1 HCl.

Phosphate Mixtures for pH Values 7.0 to 8.0.

pH value.	Amt. of M/5 KH_2PO_4 in c.c.	Amt. of M/5 NaOH in c.c.
7.0	50	29.63
7.2	50	35.00
7.4	50	39.50
7.6	50	42.80
7.8	50	45.20
8.0	50	46.80

Each of the above mixtures is diluted to 200 c.c. with distilled water.

Procedure.—Place in one of the Erlenmeyer flasks 20 c.c. freshly distilled water, 5 drops phenol red and 5 c.c. of the medium to be titrated. Compare with standard, which contains 5 c.c. of standard phosphate of the desired pH, 20 c.c. of water and 5 drops of phenol red. Titrate with N/20 NaOH or N/20 HCl to match tint of standard. Calculate the amount of normal alkali or acid to be added to the medium to give the proper reaction. For most pathogenic bacteria the optimum pH is 7.2 to 7.8.

Example.—If it requires 2 c.c. N/20 sodium hydroxide to bring 5 c.c. of the medium to pH 7.6, then to bring a liter of medium to the same reaction will require the addition of

$$\frac{200}{20} \times 2 = 20 \text{ c.c. of N/1 NaOH.}$$

ANAËROBIC METHODS.—For anaërobic work a large number of methods are available, a few only suitable for use in laboratories not elaborately equipped.

HYDROGEN DISPLACEMENT METHODS.—If these are used the hydrogen should be produced in a Kipp apparatus with zinc and dilute sulphuric acid and the hydrogen should be passed through a series of Woulfe bottles containing solutions of lead acetate and of pyrogallic acid respectively. It is also recommended to interpose one containing silver nitrate solution

to take out any hydrogen arsenide which may have resulted from impurities in the zinc. A closed jar can be improvised from a museum jar which has a hole in the lid or from a large, wide-mouthed bottle if no Novy jar is at hand, and this jar can be alternately exhausted and refilled with hydrogen.

Such a procedure can also be combined with the pyrogallic method.

All methods depending upon hydrogen are more or less complicated.

Nitrogen can be used in a similar way if it can be obtained in cylinders of compressed liquid nitrogen. This is very convenient.

For methods in which no elaborate apparatus is desired, pyrogallic acid and KOH can be used.

The easiest and most efficient way to apply this for anaërobic cultivation of cultures in test-tubes is the following:

Buchner tubes, that is, large test-tubes about ten inches in length and one inch inside diameter, are fitted with rubber stoppers. Into the bottom of these, about half a teaspoonful of pyrogallol is placed. This is gently packed down with a small tab of absorbent cotton. When the test-tube has been inoculated, 4 or 5 c.c. of a 15 to 20 KOH or NaOH solution is run into the tubes, and while this is in the process of dissolving the pyrogallol, the inoculated tube is gently slid into the larger tube and the rubber stopper inserted.

Blood cultures and other cultures which it is desired to take anaërobically may be taken in long tubes covered with agar, over the top of which a little sterile paraffin oil is allowed to flow. Better anaërobiosis, however, is obtained if such cultures are taken in the ordinary size test-tube, filled within an inch and a half of the top with agar, ascitic agar or whatever other medium is used, and these tubes then placed in the larger pyrogallol tubes, as described above.

It is unnecessary to describe at length details of the

various modifications that can be made in the methods of application of the hydrogen and nitrogen displacement, the exhaustion, the deep tube, and the pyrogallic methods. A little laboratory ingenuity will adapt one or the other or a combination of these methods to the definite purpose desired.

HANDLING BACTERIOLOGICAL MATERIAL FROM PATIENTS.

SURGICAL MATERIAL.—Cultures taken at operation must be taken with care, in order to be of any value. If a case is important from the point of view of infection, it is very desirable that a bacteriologist or someone familiar with the taking of the culture should be present at the operation. The custom of leaving a basket of agar tubes in the operating room to dry up before used is to be discouraged. Fresh media, chosen with discrimination in reference to the type of infection expected, should be furnished. It is best to take a culture directly from the lesion into the culture media at the time of operation. If this is not possible, it is a good plan to take a specimen of the pus on a sterile swab, insert into a test-tube, and send it to the laboratory immediately. It is very important that freshly taken cultures of such materials should not lie around the operating room before being planted and incubated. When such a specimen is received at the laboratory, plants should first be made upon slant tubes or plates of suitable media; also immediately a smear should be made and examined by Gram, so that the bacteria originally present in the pus morphologically can be checked up with those appearing in culture.

In addition to stock medium when the kind of infection is unknown, use Loeffler's medium, glucose infusion agar, with or without ascitic fluid.

For staphylococci: Simple media will do.

For streptococci: Blood agar plates, see page 143.

For pneumococci: Use glucose ascitic agar or Loeffler's or blood agar.

For meningococci: Use defibrinated or laked blood agar plates.

For gonococci: Use human ascitic glucose agar in plates.

For influenza bacilli: Use whole blood agar in plates, preferably human or pigeon blood.

For colon-typhoid group: Any simple medium will do.

For anthrax: Any simple medium will do.

For tuberculous material: Use Dorsett's glycerin egg. Tuberculous material contaminated can be treated by Petroff's method, described in another place. Tuberculous material that it is hopeless to cultivate should be injected into a guinea-pig. See also antiformin method.

For gas bacillus: Use deep anaërobic tubes of glucose agar.

For diphtheria bacilli: Use Loeffler's medium.

For glanders: Use glycerin potato agar 2.5 per cent. acid.

For tetanus: Use glucose broth fermentation tube with addition of a piece of tissue.

AUTOPSY MATERIALS FOR CULTURE.—It should be remembered that cultures taken from a heart's blood are useless unless taken very soon after death. If taken more than three or four hours after death, it should be remembered that the findings must be bacteriologically analyzed, since contamination takes place, probably from the bowel and the portal circulation, very soon after death, and the finding of gas bacilli, colon bacilli, etc., if cultures are taken late, mean very little. In taking heart's blood from a fresh autopsy, it is advantageous to puncture the heart with a long needle on a syringe before the autopsy has been begun.

Material such as pleural and pericardial exudate should be taken with a sterile glass pipette, drawn to a capillary point at the tip and fitted with a nipple, with which the pleural sac can be penetrated. Cultures from organs should be taken in the following way: The surface of the organ should be thoroughly seared with a hot knife, and with the

same hot knife a small slit made into the seared area. A hot platinum wire can be then shoved through the sterile area and material for smears and cultures taken.

GENERAL DIRECTIONS FOR BLOOD CULTURES.—A blood culture to be of any value must be taken with rigid sterile precautions. Although some of these precautions are onerous, they should all be observed, because a contaminated blood culture might just as well not have been taken. The following procedure may be taken as a standard, which experienced officers may vary according to local conditions.

The bacteriologist taking a blood culture should familiarize himself with the nature of the infection suspected, for in order to take a blood culture intelligently it is necessary to vary the media used. Thus when typhoid or paratyphoid bacilli are suspected a tube or two of bile broth should be added to the media used. When streptococci are suspected, as in malignant endocarditis, glucose agar should be used, and for infections like gonococcus endocarditis it is well to add sterile ascitic fluid to the media. Variations in individual cases must be made according to the general bacteriological judgment of the laboratory officer.

Preparation of the patient is important. It is usually sufficient to paint the antecubital space with strong tincture of iodin after a thorough scrubbing with soap and warm water. Others prefer a thorough soap and water cleansing followed by ether and alcohol with a 1 to 1000 bichloride dressing left on the arm for half an hour before the culture is taken. In all but a very few cases it is possible to plunge the needle directly into the vein without incising the skin.

Just before taking blood a piece of rubber tubing can be used to constrict the upper part of the arm in order to fill the veins. This should be loosened just as soon as the syringe is full. It is most convenient to clamp the rubber with artery forceps. Too much stress cannot be laid on the necessity of using the sharpest possible needle. A dull

needle causes much distress to the patient and usually results in several failures to enter the vein, thereby increasing the chances of contamination.

Materials Used.—In stationary laboratories it is convenient to prepare a tray from an old box to which a basket handle can be fitted, in which the following materials are assembled for the taking of blood cultures:

1. 10 c.c. Luer Syringe.—This Luer syringe is boiled with one or two needles in water to which no antiseptic has been added. It is sometimes convenient to sterilize the syringes and needles in the dry sterilizer, taking the syringe apart and putting piston and plunger and needles into separate large test-tubes, stoppered with cotton. However, boiling for twenty minutes just before use is sufficient for most purposes.

2. Small Enamelware Pint Measure. — In this the agar is melted and cooled to 50° C. just before taking to the ward. Just before blood is added this water should be cooled to 42° C., carefully controlled by:

3. Thermometer.

4. Six Sterilized Petri Dishes.—Preferably put up in a towel or piece of cloth, fastened with a safety pin and so sterilized in hot-air sterilizer.

5. Two or Three Erlenmeyer Flasks.—Or other small flasks of 50 c.c. capacity, containing plain broth and glucose broth.

6. Alcohol lamp.

7. Bottle of alcohol.

8. Bottle of tincture of iodin.

9. Package containing sterile cotton and gauze bandage.

When blood culture is taken, it is desirable to have the assistance of a nurse or other assistant. The arm is painted with iodin or otherwise sterilized, the needle plunged into the vein and about 6 to 10 c.c. of blood withdrawn. Meanwhile the assistant has lighted the alcohol lamp. The

blood is then distributed into tubes and flasks, great care being exercised to work quickly so that blood will not clot. When stoppers are removed from flasks and tubes, assistant flames lips of these in alcohol flame, rapidly, before blood is poured in, and again just before agar tubes are poured into plates. It is not a bad plan to pass point of needle very rapidly through alcohol flame between successive inoculations. In pouring agar into Petri plates the usual precautions in lifting lid, etc., must be observed.

Plates must be left undisturbed on a flat surface, preferably a sterile towel on table, until well hardened. The lid is then fastened by passing a thin strip of plaster around plate, and on this the name of the patient can be marked. The inoculated flasks should be quickly mixed so that blood is equally distributed. Care in mixing the agar and blood before pouring should also be exercised, and this can be done most easily and thoroughly if the inoculated agar tubes are set away in the water in enamel measure at 42° C. before pouring. Plates and flasks should be transferred to incubator without delay and daily readings made.

It is a good plan in many cases to make anaërobic cultures in a few tubes. This should be done in high test-tubes (8-inch test-tubes), containing glucose or glucose ascitic agar. They must be quickly cooled in cold water after mixing. It is also a good plan to take some of the anaërobic blood cultures in the ordinary test-tubes, placing these in Buchner tubes with pyrogallol and KOH in the manner described elsewhere.

Observation period is five to seven days in slow-growing organism like streptococcus.

SPECIAL BACTERIOLOGICAL METHODS.

DETERMINATION OF TYPES OF PNEUMOCOCCUS (AS CARRIED OUT AT THE ROCKEFELLER INSTITUTE).

1. COLLECTION OF SPUTUM.—Care should be exercised in the collection of sputum to obtain a specimen from the deeper air passages as free as possible from saliva. This can be done in practically all cases, even the most difficult, with a little persistence. The specimen of sputum should be collected by a trained man, preferably the bacteriologist, or someone trained by him for this particular duty, since the success of diagnosis and treatment of the cases depends on the collection of a proper sample. The sputum is collected in a sterile Esmarch dish or other suitable container and should be sent at once to the laboratory for mouse injection. When delay is unavoidable, the specimen should be kept on ice during the interval.

2. MICROSCOPIC EXAMINATION OF THE SPUTUM.—Direct smears are made from the sputum and stained by Gram's method, using 10 per cent. aqueous safranin as a counter-stain, by Ziehl-Neelson and by Hiss's capsule stain. This serves to give an idea of the nature of the organisms present and an indication of the source of the sputum. Suitable lung specimens are relatively free in most instances from contaminating mouth organisms. It is frequently possible to identify Type III (Pneumococcus mucosus) organisms when they are present, as they possess a very large distinct capsule stained by both Gram's and Hiss's method.

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3. MOUSE INOCULATION.—A small portion of the sputum, about the size of a bean, is selected and washed through three or four changes of sterile salt solution in sterile Esmarch or Petri dishes, to remove the surface contaminations. The washed sputum is then transferred to a sterile mortar, ground up and emulsified with about 1 c.c. of sterile bouillon or salt solution, added drop by drop, until a homogeneous emulsion is obtained that will readily pass through the needle of a small syringe. 0.5 to 1 c.c. of this emulsion is inoculated intraperitoneally into a white mouse with a sterile syringe. The pneumococcus grows rapidly in the mouse peritoneum while the majority of saprophytic mouth organisms rapidly die off with the exceptions noted: B. influenzæ and occasionally M. catarrhalis, staphylococcus and streptococcus.

Pneumococcal invasion of the blood stream also occurs early. B. influenzæ likewise invades the blood stream if present; other organisms, as a rule, do not. The time elapsing before there is a sufficient growth of the pneumococcus in the mouse peritoneum for the satisfactory determination of type varies with the individual case, depending upon the abundance of pneumococcus in the specimen of sputum and the virulence and invasiveness of the strain present. This may be anywhere from five to twenty-four hours, averaging six to eight hours, with the parasitic fixed types I, II, and III. As soon as the injected mouse appears sick a drop of peritoneal exudate is removed by means of peritoneal puncture with a sterile capillary pipette, spread on a slide, stained by Gram's method and examined microscopically to determine whether there is abundant growth of the pneumococcus present. If there is an abundant growth of pneumococcus, the mouse is killed and the determination of type proceeded with. If the growth is only moderate or if other organisms are present in any quantity, further time must be allowed until subsequent examination of the peritoneal exudate shows an abundant growth of pneumococcus. It

should be emphasized that undue haste in killing the mouse is time lost in the end.

4. MOUSE AUTOPSY.—As soon as the mouse is killed or dies the peritoneal cavity is opened with sterile precautions, and cultures are made of the exudate in plain broth and on one-half of a blood-agar plate. Smears are made and stained by Gram and Hiss's capsule stain for microscopic examination. The peritoneal exudate is then washed out by means of a sterile glass pipette with 4 or 5 c.c. of sterile salt solution, the washings being placed in a centrifuge tube. Cultures are then made from the heart's blood in plain broth and on the other half of the blood-agar plate, and the mouse is discarded.

5. DETERMINATION OF TYPE.—(a) *Agglutination Technic.* —When the pneumococcus is present in pure culture in the peritoneal exudate the determination of type may be satisfactorily made by macroscopic agglutination tests as follows: The peritoneal washings are centrifugalized at low speed for a few minutes until the cells and fibrin contained in the exudate are thrown down. The supernatant bacterial suspension is decanted into a second centrifuge tube and centrifugalized at high speed until the organisms are thrown down. The supernatant fluid is discarded and the bacterial sediment taken up in sufficient sterile salt solution to make a moderately heavy suspension. The concentration of bacteria should be similar to that of a good eighteen-hour broth culture of the pneumococcus. This suspension is used directly for macroscopic agglutination tests, being mixed with immune serum in small test-tubes in equal quantities of 0.5 c.c. each. To obviate the difficulty that occasionally arises from the occurrence of Group IV strains that show cross-agglutination in all three types of immune serum, the optimum dilutions of serum and the optimum incubation time that will surely identify all type strains and fail to give any cross-agglutination reactions have been determined on a large series of

strains. (This work applies only to the antipneumococcus immune serum prepared at the Hospital of the Rockefeller Institute for Medical Research.) The results are shown in the following table:

DETERMINATION OF PNEUMOCOCCUS TYPES BY AGGLUTINATION.

Pneumococcus suspension, 0.5 c.c.	Serum I (1 to 20), 0.5 c.c.	Serum II (undiluted), 0.5 c.c.	Serum II (1 to 20), 0.5 c.c.	Serum III (1 to 5), 0.5 c.c.
Type I	++	—	—	—
Type II	—	++	++	—
Subgroups II, A, B, X .	—	+	—	—
Type III	—	—	—	++
Group IV	—	—	—	—

Incubation for one hour at 37° C.

From the above table it will be seen that a 1 to 20 dilution of Type I serum, making with the addition of an equal amount of pneumococcus suspension a final dilution of 1 to 40, a 1 to 20 dilution of Type II serum, making a final dilution of 1 to 40, and a 1 to 5 dilution of Type III serum, making a final dilution of 1 to 10, serve to agglutinate Types I, II and III pneumococci respectively, and fail to show any cross-agglutination reaction with strains belonging to Group IV. It will further be seen that by the use of a tube containing 0.5 c.c. of undiluted Type II serum, as well as the 1 to 20 dilution pneumococci belonging to the various II subgroups may be identified and rapidly differentiated from Type II pneumococci in that they show partial to complete agglutination in undiluted Type II serum, but not in the 1 to 20 dilution at the end of one hour's incubation at 37° C.

For the determination of types on the peritoneal washings such serum dilutions give the most satisfactory and clear-cut results. Five small test-tubes are set up as follows: Tube 1: 0.5 c.c. Serum I (1 to 20) + 0.5 c.c. bacterial suspension. Tube 2: 0.5 c.c. Serum II (undiluted) + 0.5 c.c. bacterial

syspension.   Tube 3: 0.5 c.c. Serum II (1 to 20) + 0.5 c.c. bacterial suspension. Tube 4: 0.5 c.c. Serum III (1 to 5) + 0.5 c.c. bacterial suspension. Tube 5: 0.1 c.c. sterile ox bile + 0.3 to 0.5 c.c. bacterial suspension to determine the bile solubility of the strain for differentiation from the streptococcus. The tubes are incubated in the water-bath for one hour at 37° C. Agglutination of Types I, II, and III pneumococci in such serum dilutions is practically always immediate in the homologous serum and no agglutination occurs in the heterologous sera. Rapid clumping of the organisms is seen to take place and may be brought out clearly by gentle agitation of the tubes. For the identification of subgroups II pneumococci incubation is necessary, such strains showing partial to complete agglutination in undiluted Type II serum at the end of one hour's incubation. If no agglutination occurs and the organism is bile-soluble, it is classified as a Group IV pneumococcus.

(*b*) *Precipitin Method*.—It has been stated above that the determination of pneumococcus types by macroscopic agglutination tests with the peritoneal washings is interfered with when other organisms are present with the pneumococcus in the peritoneal exudate with a resultant delay of eighteen hours or more before the type of pneumococcus present can be established. To obviate this difficulty the following method has been devised: Dochez and Avery have shown that the pneumococcus produces in broth cultures during the period of active growth a soluble substance which gives a specific precipitin reaction with the homologous antipneumococcus immune serum. It seemed probable that this soluble substance or precipitinogen would be present in the peritoneal exudate of the mouse in sufficient quantity to give a specific precipitin reaction with the homologous serum, and such has proved to be the case. The method to be described is dependent upon this phenomena. The technic is as follows:

The peritoneal exudate is washed out with 4 or 5 c.c. of sterile salt solution by means of a sterile glass pipette and placed in a centrifuge tube. The peritoneal washings containing cells, fibrin, and bacteria are immediately centrifuged at high speed until the supernatant fluid is water-clear. The supernatant fluid is then pipetted off, with care not to disturb the sediment which is discarded, and is mixed with quantities of 0.5 c.c. each with an equal amount of the anti-pneumococcus immune serum in a series of small test-tubes as follows: Tube 1: 0.5 c.c. Serum I (1 to 10) + 0.5 c.c. supernatant peritoneal washings. Tube 2: 0.5 c.c. Serum II (undiluted) + 0.5 c.c. supernatant peritoneal washings. Tube 3: 0.5 c.c. Serum II (1 to 10) + 0.5 c.c. supernatant peritoneal washings. Tube 4: 0.5 c.c. Serum III (1 to 5) + 0.5 c.c. supernatant peritoneal washings. An immediate specific precipitin reaction occurs in the tube containing the homologous immune serum, the other tubes remaining clear (see Table). No incubation is necessary. Two tubes of Type II serum are used for the purpose of distinguishing between Type II pneumococci and members of the II sub-groups—the former giving a precipitin reaction in both tubes, the latter only in the undiluted Type II serum. A negative reaction, in all tubes, indicates a pneumococcus belonging to Group IV.

The method has been tested with a large number of strains and has been consistently positive and specific with pneumococci of Types I, II and III and consistently negative with pneumococci of Group IV. The presence of other organisms together with the pneumococcus in the peritoneal exudate does not interfere with the reaction, and other organisms than the pneumococcus produce no substance that might give a false positive reaction.

DETERMINATION OF PNEUMOCOCCUS TYPES BY THE PRECIPITIN METHOD.

Supernatent peritoneal washings, 0.5 c.c.	Serum I (1 to 10), 0.5 c.c.	Serum II (undiluted), 0.5 c.c.	Serum II (1 to 10), 0.5 c.c.	Serum III (1 to 5), 0.5 c.c.
Type I	+ +	—	—	—
Type II	—	+ +	+ +	—
Subgroups II, A, B, X .	—	+	—	—
Type III	—	—	—	+ +
Group IV	—	—	—	—

The results with Subgroup II pneumococci have not been so satisfactory. Reference to the table will show that pneumococci belonging to these groups give a precipitin reaction with undiluted Type II serum but not with the 1 to 10 dilution, thereby being distinguishable from Type II pneumococci. A number of Subgroup II organisms, however, have been encountered in which the peritoneal washings have failed to give a precipitin reaction with undiluted Type II serum. In the identification of the fixed parasitic types of pneumococci this occasional difficulty is of little practical importance from the point of view of treatment, as there is at present no specific therapy for cases of pneumonia caused by pneumococci of the II subgroups. For purposes of classification and statistics these organisms can readily be identified subsequently when the organism has been obtained in pure culture.

The precipitin method possesses the following distinct advantages. It is available as soon as satisfactory agglutination tests can be made; incubation of the tubes is unnecessary; it is not interfered with by the presence of other organisms in the exudate; it is specific and shows no cross-immunity reactions; it is applicable to mice which through unavoidable circumstances have been dead for some time before the determination of type can be made and in which

autolysis of the pneumococci or postmortem invasion of the peritoneal cavity by other organisms has made the agglutination method impracticable. For these reasons it is recommended as the method of choice in all cases.

(c) *Identification of Type III Pneumococci by Morphological and Cultural Characteristics.*—If Type III antipneumococcus immune serum is not available for diagnostic purposes, Type III pneumococci may be identified in most instances by cultural and morphological characteristics. Pneumococcus mucosus is usually somewhat larger, rounder, and less lanceolate than other types of pneumococci. It possesses a arge distinct capsule which stains readily with Hiss's capsule stain and usually retains the pink counterstain with Gram's method. The peritoneal exudate produced on mouse inoculation is usually quite mucoid and colonies on solid media are moist, mucoid, and spreading. It is always bile-soluble. These characteristics usually serve to differentiate Type III pneumococci from other types. Occasional strains of pneumococci which agglutinate in Type III serum, however, are encountered which do not show well-developed mucoid characteristics and cannot be distinguished on cultural grounds from other types. Furthermore, Type II strains are occasionally found that exhibit fairly well-developed mucoid characteristics. For these reasons the identification of Type III pneumococci by morphological and cultural characteristics is not always absolute and the diagnosis should be established by immunological methods when Type III serum is available.

6. Confirmation of Type.—The determination of type on the peritoneal washings should be confirmed by macroscopic agglutination tests with a pure bouillon culture of the pneumococcus obtained from culture of the heart's blood at the time of mouse autopsy. The technic is the same as that employed in the agglutination tests on the bacterial

suspension obtained from the peritoneal washings and should include a test for bile solubility.

7. DETERMINATION OF TYPES ON BLOOD CULTURES, SPINAL FLUIDS, EMPYEMA FLUIDS AND BY LUNG PUNCTURE.—(*a*) *Blood Culture.*—The usual technic in routine blood culture is carried out. From a positive bouillon blood culture 10 c.c. is removed by pipette and centrifugalized at low speed to remove the blood cells. The supernatant fluid is pipetted off and the bacteria thrown down by centrifugalization at high speed, the supernatant fluid is discarded and the bacterial sediment is suspended in sterile salt solution. The pneumococcus type is then determined by macroscopic agglutination tests following the same technic described above.

(*b*) *Spinal Fluid and Empyema Fluid.*—Cultures are made by the methods ordinarily employed in culturing fluids and the type of pneumococcus determined when the culture has grown out by the use of the same technic as that applied to blood cultures. If desired, in addition to culturing spinal fluids, a portion of the fluid may be centrifugalized at high speed to throw down the pneumococci present and the sediment taken up in 1 c.c. of sterile salt solution, inoculated intraperitoneally into a mouse.

(*c*) *Lung Puncture.*—This procedure should be resorted to only when it is impossible to obtain a suitable specimen of sputum or a positive blood culture. Cultures are made in bouillon of the lung puncture material and the determination of type is made by the same technic as that employed in the case of blood cultures.

8. CULTURAL METHOD FOR GROUPING THE PNEUMOCOCCUS.—Avery has recently devised a method for grouping the pneumococcus which may be used when mice are not available.

Preparation of Media.—I. Meat infusion broth, 0.3 to

0.5 acid to phenolphthalein. This should not be sterilized under pressure.

II. Prepare also a sterile 20 per cent. solution of dextrose and a flask of defibrinated rabbit's blood.

III. Every 100 c.c. of medium should contain 90 c.c. of the above broth and 5 c.c. each of dextrose and rabbit's blood. The medium is tubed in 4 c.c. quantities and should not be reheated.

Sputum.—I. Care should be taken to obtain a specimen from the deep air passages and to avoid mouth secretions.

II. A portion the size of a bean should be selected and washed even more carefully than for mouse inoculation, passing it three or four times through sterile salt solution.

III. The sputum is then ground in a sterile mortar, adding 0.5 to 1 c.c. of broth drop by drop. This emulsion is then introduced directly into the medium.

Classification.—After inoculation the tubes are incubated for five hours at 37° C. A smear is then made, stained by Gram and a blood-agar plate is inoculated.

The tube is now centrifuged at low speed for two minutes, just enough to throw down the red cells but not enough to bring down the bacteria, and the supernatant fluid is transferred into a second tube.

There are two methods of proceeding from this point.

I. *The Precipitin Method.*—To the above supernatant fluid add 1 c.c. of sterile bile and place the tube in a waterbath at 37° C. for twenty minutes. If not clear at the end of this time, centrifuge. The clear fluid is then used for a precipitin test according to the table on page 138.

II. If bile is not at hand an agglutination test may be made directly on the fluid after removal of the red cells.

The growth of the pneumococcus in this medium is not dependent upon virulence, as in the mouse. Therefore, greater care must be taken to avoid contamination by mouth types by careful selection and washing of the sputum.

DETERMINATION OF TYPES OF PNEUMOCOCCUS.—*Coagulation Method for Type Determination of Pneumococcus (Krumwiede)*.—In about 75 to 85 per cent. of the average sputa containing fixed types the type can be determined by this method in from fifteen to thirty minutes. The technique is as follows: From 3 to 10 c.c. of the sputum, depending on the amount available, is poured from the sputum container into a test-tube. This is placed in boiling water for several minutes or longer until a more or less firm coagulum results, which will occur if the specimen is a suitable one, *i. e.*, if the sputum is of pulmonary origin. The coagulum is due to the presence of albumins. The coagulum is then broken up with a heavy platinum wire or glass rod and saline is added. Just enough saline should be added so that, on subsequent centrifuging, there will be sufficient fluid to carry out the test. If too much is added the resulting antigen may be too dilute. In some instances little or no saline is necessary, as sufficient fluid separates from the coagulum.

After the addition of the saline the tube is again placed in boiling water for a few minutes to extract the soluble antigen from the coagulum, the tube being shaken several times during the heating. The broken clot is then thrown down by centrifuge and the clear supernatant fluid used for the test. To hasten the appearance of the reaction and to obtain a reaction even should the antigen be dilute, layer or stratify the antigen over the "type" serums, using the latter undiluted. 0.2 c.c. of the three "type" serums are placed in narrow-test tubes and the antigen added from a capillary tube with a rubber teat. If the tubes containing the serum are tilted and the antigen dropped slowly on the side of the tube just above the serum no difficulty will be encountered in obtaining sharp layers, as the undiluted serum is sufficiently higher in its specific gravity. The tubes are then placed in the water-bath at from 50° to 55° C. and observed after several minutes.

If a fixed type is present in the sputum, and should the sputum be rich in antigen, a definite contact ring is seen in the tube containing the homologous serum. With sputums less rich in antigen the ring may develop more slowly, and it will be less marked. Some experience is necessary in detecting the less marked contact rings and in differentiating them from an apparent ring, which may be confusing if one of the serums is darker in color, giving thus a sharper contrast with the supernatant antigen. The true ring is more or less opaque, and this quality can be seen by tilting the tubes and looking at the area of contact against a dark background; for example, the lower edge of a dark shade raised to just above the level of the eyes. The advantage of the ring test is that a ring may be evident, whereas definite clouding or visible precipitate may appear only after longer incubation, or may be so slight even after an hour's incubation as to leave one in doubt. It is well to shake the tubes after twenty minutes, as many of the specimens will show definite clouding or precipitate either at once or no further incubation, thus checking the ring reading. Although the extract may be opalescent, unless this is marked, it does not obscure the ring reaction. Should the extract be so opalescent as to obscure the ring, shake the tubes after incubation and add saline. The dilution may make visible the otherwise obscured precipitate.

METHODS FOR THE ISOLATION AND IDENTIFICATION OF STREPTOCOCCUS HEMOLYTICUS.[1]

In order to recommend a method practical for use on a large scale under the conditions obtaining in the army camps and cantonments, the committee has been compelled to adopt methods that are simple, even at some expense of thoroughness and accuracy.

[1] Adopted by the Medical Department of the United States Army.

The method recommended involves the isolation of the hemolytic streptococcus from blood agar plates into bouillon and the use of this bouillon culture for various tests.

COLLECTION OF PATHOLOGICAL MATERIAL.—Cultures from the tonsils and pharynx should be taken with ordinary sterile cotton swabs. After swabbing the throat, the swab is replaced in the sterile test-tube and sent promptly to the laboratory. Pus, if scant in amount, may also be collected on swabs. Considerable amounts of pus, pleural exudate, or sputum should be collected in sterile bottles or tubes (without antiseptic), taken promptly to the laboratory, and there kept cold until cultures are made.

BLOOD AGAR PLATES.[1]—*Inoculation.*—Swabs should be moistened with a drop or more of sterile salt solution unless they are obviously quite moist. Sputum should be washed as in the isolation of the pneumococcus and a kernel selected for culturing. A Gram stain of the sputum, swab, or exudate will often help to determine the amount of material that should be used for inoculation of the plate.

It is preferable to study both surface and deep colonies, hence material such as pleural exudates, pus and material from autopsies should be suitably diluted, inoculated into fluid blood agar (at 45° to 50° C.) and poured into Petri dishes. A small bit of the material may also be streaked out on the same or a different plate if desired.

For routine examination of throat swabs in large numbers, surface inoculation of blood agar plates is sufficient. It is recommended that one swab only be inoculated on each plate.

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[1] If streptococci are being sought in material in which they may be quite scarce, a preliminary growth in serum bouillon, dextrose blood bouillon, or cooked meat medium will serve to enhance their number. If, on the other hand, information is desired concerning the relative numbers of streptococci and other organisms present in the original material, plates should be made directly without preliminary "enrichment."

A convenient method of inoculation is to touch a spot near the edge of the plate with the moist swab and then, with a platinum loop, to spread the material from this spot back and forth across the surface of the medium with a view to securing greater dilution at points farthest from the spot touched by the swab. A cut into the agar by means of the edge of the loop for a short distance soon after it has left the inoculated spot will give some opportunity for growth in the depths of the agar.

Incubation.—Blood agar plates should be incubated at 37° C. in an inverted position for eighteen to twenty-four hours before final observations are made. The atmosphere of the incubator should be kept humid by the exposure of a large surface of water in shallow pans on the floor of the incubator.

Study of Plates.—Isolated colonies with well-defined colorless zones of hemolysis should be sought. There should be no pigmented (greenish or brownish) corpuscles visible under the low power of the microscope remaining next to or under the colony. The approximate number of these colonies (expressed as a percentage) in comparison with all other colonies should be noted.

BOUILLON CULTURE.—*Inoculation.*—A typical isolated hemolytic colony should be transferred to a tube of bouillon.

Incubation.—After incubation over-night or until there is a good amount of visible growth, the bouillon culture should be used as follows:

Study.—1. A Gram stain should be made and the film examined to determine the morphology of the organism isolated. The points to be noted are length of the chains, size, shape and arrangement of the cocci in the chains, and any striking peculiarities.

2. A stock culture should be made from the same colony if it is desired to keep the strain in cultivation or if there is a possibility that the strain may be used for further study.

3. 0.5 c.c. of the bouillon culture should be mixed with 0.5 c.c. of a 5 per cent. suspension of washed rabbit blood corpuscles in physiological salt solution and incubated in a water bath at 37° C. for two hours. Markedly hemolytic pathogenic streptococci of human origin produce laking of blood under these conditions.

4. To about 1 c.c. of bouillon culture add one-fifth volume of sterile ox bile. Observe for one hour at room or incubator temperature. Under certain conditions pneumococci on blood agar may cause some hemolysis, but solubility in bile serves to distinguish them from streptococci.

The above procedures should be regarded as essential. The following additional tests are advised for more detailed study of cultures when time and conditions permit.

5. The bouillon culture should be replated, after proper dilution, into fluid blood agar, so that deep colonies as well as surface colonies may be studied. This also serves as a check upon the purity of the culture.

6. The fermentation reactions toward lactose, mannite, salicin, inulin, raffinose and saccharose should be determined, the first three or four substances mentioned being of most importance. For this purpose sugar-free broth containing 5 per cent. or more of sterile serum, 1 per cent. of the test substance and 1 per cent. of Andrade indicator (0.5 per cent. aqueous acid fuchsin 100 c.c., normal NaOH 16 c.c.) may be employed. It is advisable to prepare a concentrated solution of the test substance in distilled water, and to add the required amount of this to the sterile bouillon before use. Final readings should be made after five days' incubation at 37° C.

7. A tube of milk should be inoculated and incubated for seven days. During this time it should be noted whether coagulation occurs promptly, slowly, or not at all, and if it has not occurred at the end of seven days the tube should be immersed in a bath of boiling water for ten minutes to determine whether or not coagulation occurs under these conditions.

8. Inoculation of rabbits intravenously with not more than 1 c.c. of the fresh bouillon culture and inoculation of mice intra-abdominally with smaller much amounts should be made.

NOTES AND DETAILS.—For the study of the hemolytic activity of the streptococci it is important that no sugar should be added to any of the media used, as it is well known that sugar in small amounts may inhibit the hemolytic activity. The small amount of muscle sugar present in the meat used for making the media, however, is not sufficient to produce this effect, hence it is not necessary that the meat be fermented before use in order to remove the muscle sugar present.

BLOOD AGAR PLATES.—*Agar.*—Standard beef infusion agar of the following composition should be used:

	Per liter of medium.
Aqueous extractives of	500 grams of meat
Agar-agar	20 grams
Peptone	10 grams
Salt	5 grams

Final titratable acidity (after sterilization) should be between neutral and 0.5 per cent. normal acid to phenolphthalein.

Kind of Blood.—Sterile defibrinated rabbit, horse or human blood may be used. The sharpest results are probably obtained with horse blood, but most hemolytic pathogenic streptococci are readily recognized on any of the kinds of blood mentioned.

Mixture of Blood and Agar.—The agar medium should be melted and cooled to 45° to 50° C., at which temperature 5 to 10 per cent. of blood is added and thoroughly mixed with the agar.

Pouring of Plates.—A Petri dish 9 cm. in diameter should

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receive 12 to 15 c.c. of blood agar, *i. e.*, sufficient to make a layer 2 to 3 mm. thick.

Caution.—Sterile defibrinated blood may be kept in the refrigerator for several days but should not be used if it has begun to lake or if the sedimented corpuscles have become viscid so that it is difficult to resuspend them by shaking.

Poured blood agar plates should not be kept on hand "ready for use" longer than twenty-four to forty-eight hours under ordinary conditions. If the surface of the medium becomes even slightly dry, streptococci will not grow readily on it. If kept too long in the refrigerator, the moisture collecting on the surface of the medium or on the under surface of the lid is likely to cause confluence of colonies or to encourage the overgrowth of "surface spreaders."

Bouillon Culture.—The bouillon should be standard meat infusion bouillon of similar composition to that used in making the agar described above. No dextrose should be added. No serum or ascitic fluid should be added if the culture is to be employed for the bile solubility test. The titratable acidity should be not above 0.5 per cent. normal acid to phenolphthalein.

The bouillon culture should be used for the various tests only when it is quite fresh, not after it is two, three or more days old. For later tests and experiments fresh bouillon cultures should be made directly from the stock culture.

Stock Cultures.—Streptococci remain viable for many weeks or months (1) on blood agar slants, (2) on standard agar slants to the surface of which a few drops of blood have been added, and (3) in bouillon containing a small percentage of blood.

Dextrose should not be added to any of the stock culture media. Before use the media described above should be incubated for forty-eight hours after blood has been added to test sterility. When slants are used the condensation fluid as well as the surface of the slant should be inoculated. After

inoculation the culture should be incubated not longer than twelve to fifteen hours and then kept cold.

SOLUBILITY IN BILE.—Fresh undiluted ox bile may be autoclaved, filtered through paper and again autoclaved. It is then ready for use as directed above.

SHIPMENT OF CULTURES.—Cultures on the slant media described above may be shipped, but for this purpose most of the condensation fluid should be pipetted off to prevent wetting of cotton plugs during shipment. It is also well to insert a cork stopper into the tube after the cotton plug has been burned off and pushed in.

STANDARD TECHNIQUE OF MENINGOCOCCUS CARRIER DETECTION.[1]

CULTURING.—(a) Whenever possible the cultures should be taken in a small room in the regimental infirmary. The floor of this room should be washed with an abundance of soap and water one hour before the men enter for culture, and should be wet during the process of culturing.

(b) Only *a few men* at a time should be admitted to the culture room.

(c) Windows and doors should be closed all the time.

(d) Cultures should not be made within an hour after meals.

(e) No men should be examined on the same day on which they are sprayed.

METHOD OF SWABBING.—(a) The swab used should be:
1. A naked wire, 25 cm. long, with a small absorbent cotton pledget on one end, well covering the end of the wire, and a ring handle on the other. The wire should be flexible, such as stove-pipe wire or hay-baling wire, of about 18-gauge. The swabs may be sterilized in glass or paper containers in groups of 5 to 20. The last 1 to 2 cm. of the pledget end of

[1] Adopted by the Medical Departments of the United States Army and Navy and the United States Public Health Service.

the swab is bent to an angle of about 30 to 40 degrees. This swab is simple and has proved most satisfactory, and can be at once discarded.

2. West Tube.—This gives satisfactory cultures, but is cumbersome, and not strictly necessary.

3. Straight unprotected nasal swab. This is useful in the case of individuals with highly irritable throats, but is not recommended as a routine.

(b) The subject should be seated facing the light. Tongue depressors are to be used when necessary. The swab should be passed behind the soft palate while the subject is phonating. The swab, having passed up behind the palate, is introduced successively into each posterior naris, and then is drawn across the posterior wall of the nasopharynx. The swab is then withdrawn, taking care not to touch the throat surfaces or the tongue; this is best accomplished during phonation.

The success of carrier search depends largely on the care with which the swabbing is done, hence the man taking the cultures should be able to execute these directions skilfully. He should be either a nose and throat specialist, the bacteriologist himself or directly under the latter's supervision.

METHOD OF INOCULATING PLATES.—(a) The mucus charged swab should be applied over a limited area at the periphery of the plate. From this the spread is made by a wire loop passed by a series of radial strokes, each starting from the infected point. If there is very little mucus it is often possible to smear directly from the end of the swab.

(b) A single person per 10 cm. plate is preferable. More than two cultures on one plate should never be made.

(c) The plate must be inoculated while warm and kept warm until replaced in the incubator. To accomplish this, sterile plates, already warmed by storage in the incubator, should be packed in a device insulated against the loss of heat, such as a fireless cooker, for transportation.

Care should be taken to keep the plates warm during the inoculation process. It is advisable to carry a plate seeded with known culture of meningococcus to act as control.

CULTURE MEDIUM.—(a) Standard nutrient, 2 per cent. agar. (Beef infusion or Liebig's beef extract, 0.5 per cent. NaCl, 1 per cent.; peptone, Fairchild's or Difco; reaction plus 0.5 to phenolphthalein.) Dextrose is not necessary. A convenient method of storage is in 200 to 300 c.c. quantities, in flasks of sufficient size to permit of the addition and mixing of the following:

1. Defibrinated blood—human, horse, sheep, goat or rabbit—about 1 c.c. to 10 c.c. of agar.

2. Laked Blood. (Blood, 1 part; distilled water, 3 parts; of this mixture add 1 c.c. to 10 c.c. of agar.) Both these blood preparations should be stored ready for use in the icebox. They are good as long as they remain uncontaminated, and the whole blood unhemolyzed. They should be added to the agar only when the latter is at a temperature of 45° to 50° C.

(b) Starch agar is suitable for stock cultures and for mailing cultures.

(c) Plates should be incubated, inverted, over night before use, to insure sterility.

EXAMINATION OF INOCULATED PLATES.—(a) Plates should be inverted when placed in the incubator.

(b) Plates will be ready for examination after twelve to eighteen hours' incubation at 37.5° C.

(c) Discard all plates that are crowded and do not show discreet well-separated colonies.

(d) The meningococcus colony on the whole blood medium does not produce green coloration or hemolysis. It tends to be somewhat larger than the streptococcus and pneumococcus. The colonies are moist, elevated, outlines are ill defined and on moderately opaque blood agar have a faintly bluish tint. The colonies are not usually opaque, a characteristic which distinguishes them from the staphylococcus.

On transparent blood medium and with transmitted light under lens magnification the colony may be nearly clear, but often shows a very faint smoky gray-blue quality. This characteristic is intensified by passing the finger between the light and the colony to shade it. Further lens effect may be seen by moving the plate so that the colony passes across some distant obstruction to the light as a string stretched across the window. The colony is never granular in young cultures.

The colony should be confirmed by smear and Gram stain.

TRANSPLANTATION.—The suspected (ringed) colonies are transferred to warmed moist blood agar slants. They must have been incubated to ensure sterility. It is best to keep the tubes continuously in a warm water-bath until finally placed in the incubator. If transfers are made under these conditions there will be enough growth for identification in about eight hours.

Two essentials to success in growing meningococci are moist media, kept constantly from the time of inoculation at body temperature.

IDENTIFICATION.—(*a*) Microscopic. Smear preparations are made in the usual way stained by Gram. The presence of a few Gram-positive or other contaminating organisms does not make the culture unsuitable for agglutination.

(*b*) Agglutination. All Gram-negative micrococci are subjected to agglutination in the following way:

The serum dilutions are first set up and the bacterial emulsion is made directly in them. To make this emulsion a loopful of the suspected culture is scraped off, and the loaded wire is passed well down the tube almost to the level of the fluid. The bacterial mass is then rubbed off against the wall of the tube and mixed with the fluid. In the case of the meningococcus a smooth emulsion is usually rapidly produced. Many of the other organisms are much less readily emulsified.

An emulsion must be free of clumps for proper agglutination tests.

With the polyvalent agglutinating serum supplied by the Army Medical School, Washington, D. C., or the U. S. A. Laboratory No. 1, France, the Naval Medical School, Washington, D. C., or the Hygienic Laboratory, Washington, D.C., agglutinations should be made in a 1 to 100 dilution.

The quantity in each tube should be 1 c.c. The tubes are then incubated at 55° for twelve to eighteen hours, with necessary precautions against evaporation.

Controls. Each culture should be run in parallel with a normal horse serum control at 1 to 50. A known meningococcus culture should be run as a positive control with each set.

READING OF AGGLUTINATIONS.—The tubes which have been clarified are then shaken gently, when the clumps can be clearly detected by the naked eye or hand lens.

The organisms which are agglutinated by the polyvalent serum dilutions at 1 to 100 are to be regarded as meningococci. Organisms which agglutinate in both polyvalent and normal horse sera are to be thrown out.

A slight opalescence in the supernatant fluid due to contaminating organisms, if there is otherwise distinct evidence of agglutination, need not lead to the discard of the culture as negative.

TYPING.—The meningococcus isolated by agglutination with polyvalent serum should be typed as soon as possible. If this procedure cannot be carried out on the spot, the cultures, or heated suspensions of the cultures in saline with 0.5 per cent. phenol, should be sent to the nearest Department laboratory.

Diagnostic type sera should be obtained from the Army Medical School, Washington, D. C., or from Central Medical Department Laboratory, A. E. F., France, Naval Medical School, Washington, D. C., or the Hygienic Laboratory, Washington, D. C.

CULTURES.—*From Spinal Fluid.*—Fresh spinal fluid is to

be divided into two portions. One part is centrifuged and the sediment smeared, examined microscopically and planted on blood agar slants or plates and also in serum-dextrose-broth. The other uncentrifuged portion of the fluid is added directly to serum-dextrose-broth.

The examination of cultures is made at twenty-four-hour intervals for five days. It should be noted that often in the absence of colonies on the surface of the slant there may be a luxuriant growth in the water of condensation.

From Blood.—Meningitis suspects, like all other febrile cases, should be cultured as promptly as possible. About 5 to 10 c.c. of blood, drawn from a vein by a syringe, should be planted in 100 c.c. of warm dextrose broth. The cultures should be examined at twenty-four-hour intervals over a period of not less than six to eight days. To get the culture going it is necessary to transplant each day for the first five days.

TYPING OF ORGANISMS.—Organisms recovered from spinal fluid or blood should be typed and compared with the organism isolated from the patient's nasopharynx.

Inagglutinable strains are sometimes encountered. To establish further the nature of these organisms they should be grown on dextrose, maltose and saccharose serum litmus-agar.

INDICATIONS FOR CULTURING.—On the appearance of a case of cerebrospinal fever, all contacts should be cultured for the detection of carriers as soon as possible. By contacts is meant those intimately associated with the patient—that is, all those in the same tent or squad room with him, as well as his close associates at mess or elsewhere.

Experience has shown the inadvisability of attempting to culture larger groups than this.

In military service, those who give positive cultures should be held in the detention camp until they have had three successive negative cultures at five-day intervals. If a second

case appears in the same company within a week of the first case the whole organization should be swabbed.

DISPOSAL OF CARRIERS.—The ordinary carrier usually clears up in a week or two.

Those who carry longer than this constitute the chronic carriers, and usually give large numbers of colonies or even pure cultures; these are probably the important cases from a public health point of view.

Such carriers should not be recommended for furlough to their homes nor discharged from the Service without authority from the Surgeon-General.

RAPID METHOD FOR THE IDENTIFICATION AND ISOLATION OF MENINGOCOCCI FROM THE NASOPHARYNX. (Olitsky.) *Method.*—To 1 per cent. glucose broth (made from veal infusion and having an acidity of from 0.5 to 0.7 + phenolphthalein) is added 5 per cent. of unheated, sterile, clear normal horse serum. This medium is then distributed in small tubes (from 8 to 10 mm. diameter and 9 cm. length), 1 c.c. being placed in each tube.

Suspicious colonies on the plate cultures are fished and seeded, a colony to each tube.

These small tubes are then incubated twelve hours (or overnight, if more convenient). At the expiration of this time they will show the distinctive characters of the organism in question, and at this early period "negatives" may be determined.

The bacteria that complicate the isolation and identification of the meningococcus on plate culture are the *Micrococcus flavus, crassus, pharyngis-siccus* and *catarrhalis*; the *B. influenzæ*, and an unclassified Gram-positive bacillus; and occasionally, in the hands of beginners, the staphylococci and streptococci.

Owing to the presence of normal horse serum in the medium, practically a normal horse serum control is at hand, so that the *M. flavus, crassus* and *pharyngis-siccus* and the unclassi-

fied Gram-positive bacillus will show firm agglutination. While the bacillus culture may show slight turbidity over an agglutinated sediment, the diplococci cultures show clear supernatant fluid over agglutinated masses of those organisms. As hemoglobin is absent, *B. influenzæ* fails to grow. *M. catarrhalis* grows with a dense turbidity, and often shows a pellicle on the surface. The Gram-positive staphylococci grow also with a dense turbidity, and show agglutinated masses in the sediment and often a surface pellicle as well. The streptococci grow with clear or turbid supernatant fluid, but show an agglutinated sediment.

The meningococci, on the other hand, grow in a characteristic manner. The fluid becomes faintly turbid, and a slight sediment forms; but, and this is all important, the sediment emulsifies uniformly when the tube is shaken.

The cultures in the fluid medium are divided into two sets by simple inspection. One set is readily excluded from being meningococci on the basis of the characters described above. The other set, which exhibits the qualities of the growth of the meningococcus, is regarded as suspicious, and to each of the tubes is added o.1 c.c. of a 1 to 10 dilution in 0.85 per cent. saline solution of a high-titer polyvalent anti-meningococcic serum. The tubes are then incubated in a water-bath (not in an incubator) at from 37° to 38° C. for two hours.

The reading of the tubes is definite and distinct. Those containing meningococci exhibit distinct agglutination; those containing other organisms remain unchanged. The readings are checked by means of films stained by Gram's method. All the tubes recorded as positive will show agglutinated masses of Gram-negative diplococci of typical meningococcus morphology.

From the tubes containing the agglutinated meningococci, transplants on solid mediums may be prepared for further identification or for stock cultures. The last procedure is not necessary in order to detect or exclude the suspected meningococcus carriers.

B. DIPHTHERIÆ.

SWAB TUBES.—Swabs are made by winding absorbent cotton tightly about one end of wooden sticks or pieces of wire six inches in length. These are inserted into small tubes, which are then plugged with cotton and the whole sterilized in the hot-air sterilizer, after which labels are affixed.

Medium.—The medium should be Loeffler's, preferably made at a "base" laboratory. The preparation is described elsewhere.

TAKING OF THROAT CULTURES, ETC. (See page 14).—No unnecessary loss of time should occur between taking of culture and its incubation, since at room temperature other organisms may overgrow the diphtheria bacilli.

After twelve to eighteen hours' incubation, smears are made on slides. In taking the material for smears a generous sweep should be made with the platinum loop, especially over the most suggestive areas of growth. This material is then spread on the slide with the aid of a drop of water, a large smear being desirable. After drying and fixing by heat, stain with Loeffler's methylene blue (page 24) for five minutes. This stain is satisfactory in the hands of most workers. If in doubt the application of the Neisser stain (page 25) may be of help.

It must be remembered that the above-described method, if positive, only demonstrates the presence of bacilli having the morphology of diphtheria bacilli. When a typical membranous inflammation exists, actual tests have shown that the bacilli are almost always true diphtheria bacilli. In normal throats, and more commonly in non-diphtheritic (without membrane) inflammations, or after subsidence of diphtheritic inflammations, there are encountered non-pathogenic bacilli morphologically identical or similar to the diphtheria bacillus. The morphological demonstration, therefore, of diphtheria-like bacilli in cultures from suspected carriers from the

inflamed throats of exanthemata patients or convalescent diphtheria cases is not conclusive evidence that true diphtheria bacilli are present. For practical purposes, however, they should be considered such and for quarantine purposes the cultures repeated until, as will occur in the majority, these bacilli disappear. If positive cultures continue a complete identification should be carried out, to release from quarantine those harboring harmless diphtheria-like bacilli. This determination is spoken of as the "virulence test," as the final identification of a suspected diphtheria bacillus rests on the demonstration that it produces a toxin which is neutralized by known diphtheria antitoxin.

Virulence Test.—The first step in the test is the isolation of a pure culture of the bacillus. A number of plates are poured, using ascitic infusion agar. After hardening, the surface is streaked across with the growth from the Loeffler tube, selecting colonies which, in color and size, resemble those of the diphtheria bacillus and also with a general mixture, preferably from the drier portions of the serum medium. After incubation for sixteen hours at 37° C. the growth developing along the line of streak should be examined with the microscope, using a low-power objective, preferably a No. 2. Diphtheria colonies are most likely to be found at the edges of the streak. These are fished by inserting a straight wire between the objective and the agar, the path of the wire and the actual touching of the correct colony being observed with the microscope. Care must be taken that the wire does not touch anything but the colony. The growth is transferred to ascitic infusion broth or Loeffler's medium, depending on the subsequent method of test selected.

If very few bacilli are present in the original culture, it is well to inoculate several tubes of ascitic broth. As the diphtheria bacillus will grow mostly on the surface of the broth, this portion can be used to inoculate plates with success when direct plates fail.

After incubation the pure cultures are examined by smear to determine whether diphtheria-like bacilli are present. If the examiner has some experience he can exclude some cultures at once because of their atypical morphology, especially the short bipolar staining *B. hofmanni*. If in doubt. inoculation of glucose media can be resorted to, as these atypical types usually fail to produce acid in glucose media, whereas *B. diphtheriæ* does.

After determining that the cultures are pure and morphologically typical, animal inoculation is resorted to. Two methods are available, the subcutaneous and the intracutaneous. In the former method, 1 c.c. of the ascitic broth culture (incubated forty-eight hours) is injected subcutaneously into a guinea-pig about 250 grams in weight. A second guinea-pig is injected with the same amount of culture to which had been added 50 to 100 units of diphtheria antitoxin. If the first pig dies within two or three days, and on postmortem shows typical engorgement of the suprarenals, and the second pig lives, the bacillus is a true toxin-producing diphtheria bacillus, otherwise it is not.

The intracutaneous method requires some experience in recognizing the skin lesions. Its advantage is that four to six cultures can be tested with only two pigs. The abdomens of the pigs are denuded of hair by pulling it out. This is done by grasping an area of hair between thumb and finger and exerting a jerk-like pull. Diphtheria antitoxin, about 500 units, is administered to the control pig by intraperitoneal injection. The growth from a Loeffler's slant is rubbed up in 20 c.c. of salt solution and 0.15 c.c. injected intracutaneously into both pigs. The culture used must not be over twenty-four hours old. If the bacillus is a true diphtheria bacillus it will develop toxin, which will produce in the non-immunized pigs a definite local inflammatory lesion after twenty-four hours, which goes on to superficial necrosis in

forty-eight to seventy-two hours. The pig receiving anti-toxin will show no lesion.

Although a very few true diphtheria have been encountered which produce too little toxin to be demonstrable by these methods, they are so infrequent that for practical purposes they can be ignored. If the virulence test is negative the case may be discharged from quarantine.

SCHICK REACTION.—The Schick reaction provides a means of determining the presence of antitoxin in the blood, and hence serves as a measure of immunity to infection with the diphtheria bacillus. The reaction depends upon the fact that if an amount of diphtheria toxin, arbitrarily determined by experiment in the work of Schick and others, is intracutaneously injected into an individual containing sufficient antitoxin in his blood, the toxin is neutralized and produces no reaction. If, however, this minimum protective amount of antitoxin is not present there will be a sufficient excess of toxin to produce a local reaction.

When a case of diphtheria occurs in a ward or among people who are in close contact with each other, if Schick reactions can be done it is not necessary to give prophylactic injections of diphtheria antitoxin to those who have negative Schick reactions.

The usefulness of the reaction depends entirely upon the fact that the proper dose of toxin for such test has been worked out on a large number of cases. The amount of toxin for the test used at present in this country is $\frac{1}{50}$ of a M. L. D. for a guinea-pig of 250 grams. This is so diluted that the amount is contained in 0.2 c.c. volume. Freshly made dilutions should be employed. Diluted toxin deteriorates more rapidly than undiluted toxin, the deterioration being more rapid the greater the dilution.

Such dilutions are made as follows: Of the toxin furnished by the health department or other laboratory, make a small amount in stock solution so that 1 c.c. shall contain 10

M. L. D. Keep this in dark glass bottles on ice, and, in making it, use 0.25 per cent. phenol or cresol in salt solution as diluent. Then:

1.0 c.c. of stock	=	10.0 M. L. D.
0.1 c.c. of this	=	1.0 M. L. D.
0.1 c.c. of this added to 9.9 c.c. salt solution gives		
1.0 c.c.	=	0.10 M. L. D.
or 0.1 c.c.	=	0.01 M. L. D.
or 0.2 c.c.	=	0.02 M. L. D. or $\frac{1}{50}$

This amount is injected with a very fine needle intracutaneously on the flexor surface of the forearm. In making an intracutaneous injection it is necessary to be sure to inject into the skin and not below it. A good way to make sure that this is occurring is to insert the needle with the oval opening upward for an eighth of an inch or more and inject when the oval opening of the needle is just visible through the upper layer of the skin. A little swelling should result from the injection.

A control injection should be made by injecting in another place the same amount of toxin, heated to 75° C. for five minutes.

A positive reaction consists of a red papule for twenty-four hours, which increases in size and depth until the third or fourth day, and when at its height, shows a circumscribed red infiltrated area from $\frac{3}{4}$ to 2 cm. in diameter.

In a negative reaction the little wheal at the time of injection is rapidly absorbed and no inflammatory symptoms appear.

A pseudoreaction appears early, within six hours or less, and may simulate a true reaction. This is controlled by the heated toxin injection. Qualitatively it is difficult to describe a difference between the true and pseudoreaction, except its early appearance and more urticarial nature. However, an

individual in whom the heated toxin injection and the active toxin injection are alike in severity and time of appearance, etc., may be regarded as showing a pseudoreaction.

An individual may have both a pseudo- and a true reaction, in which case there is a definite difference between the site of the injection of the unheated toxin and that in which the heated toxin has been injected. The latter appears earlier, is less severe and disappears sooner, leaving none of the brownish color and scaling which is present in the true reaction.

Toxin for a Schick reaction is put up by the New York Department of Health and supplied by the War Department. The toxin is contained in a capillary tube, which when washed out into the 10 c.c. of sterile saline contained in the outfit gives the proper dilution for use. Any toxin can be used for the test if the M. L. D. is known and it is properly diluted. Freshly prepared toxins deteriorate rapidly in their M. L. D. content, so that it is preferable to obtain an "aged" toxin, that is, one which has deteriorated to a nearly stable M. L. D. content.

BACILLUS OF TETANUS (B. TETANI).

The scanty growth of tetanus bacillus in the animal body and its usual association with other bacteria render its isolation in pure culture difficult. The simplest and most successful method consists in inoculating the suspected material, pus or tissue from the infected wound, into a fermentation tube of glucose bouillon to which a piece of fresh sterile tissue has been added and the medium layered with sterile albolene or paraffin oil. Incubate at 37° C. twenty-four to forty-eight hours. If typical drumstick spore-bearing bacilli or free spores are present, heat the culture for one-half hour at 80° C. The growth of other spore-forming bacteria may render isolation difficult. The presence of tetanus bacilli under these circumstances, however, may be demonstrated by reinoculation of the heated culture into fresh bouillon.

This culture should be injected (1 to 2 c.c.) subcutaneously into the thigh of a guinea-pig to elicit the typical symptoms of tetanus. First signs of paralysis will be evident in the region of infection.

TUBERCLE BACILLUS.

I. STAINING METHOD FOR TUBERCLE BACILLUS.—Tubercle bacilli are not readily stained by the ordinary anilin dyes. The stain can be intensified by the use of mordants (anilin water or phenol), with a long exposure in the cold or the application of heat.

The best method for general purposes is the Ziehl-Neelson method, as follows:

1. After drying and fixing slides, immerse in Ziehl carbol-fuchsin (see page 25) overnight in the cold, or flood slide completely and heat gently—steam, not boil—for five minutes.

2. Wash with acid-alcohol, 3 per cent. hydrochloric in 95 per cent. alcohol (made by adding 64 c.c. concentrated hydrochloric acid, specific gravity 1.2 to 1000 c.c. alcohol).

2a. Or wash with 5 per cent. nitric acid or 5 to 20 per cent. sulphuric acid and then with 80 to 95 per cent. alcohol.

3. Wash in water. If any red remains, repeat 2.

4. Counterstain one minute with Loeffler's methylene blue (see page 24).

5. Wash in water, blot and dry.

Tubercle bacilli retain the fuchsin, other bacteria the blue.

II. DETECTION OF TUBERCLE BACILLI.—A. *Sputum.*—Collect in a clean, sterile receptacle. The most convenient container is a low, wide-wouthed, 1- to 2-ounce bottle that can be easily boiled. Sputum should come from the deeper air passages; avoid mouth and nasopharyngeal secretions. Have the patient expectorate immediately the sputum is coughed up. The most favorable time is just after rising.

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1. *Direct Smears.*—Pour the sputum into Petri dishes and select for examination the yellowish particles of pus. If present the bacilli are almost invariably to be found in the pus or minute purulent particles, even though the mucus contains none. It is a good working rule that the more pus the more frequently are bacilli likely to be found. Repeated negative examinations of a constantly purulent sputum is presumptive evidence against tuberculosis and in favor of bronchitis, bronchiectasis, abscess, etc. Spread a small purulent kernel of sputum in a thin film on a clean glass slide. Dry in the air, fix by passing the slide several times through the flame and stain with carbol fuchsin (Ziehl-Neelson).

2. *Concentration Method.*—If after painstaking search of two direct smears no tubercle are found, concentrate the sputum by:

(*a*) *Antiformin Method.*—1. To 20 c.c. of sputum add 65 c.c. sterile distilled water and 15 c.c. antiformin. This can be purchased or prepared fresh as follows:

Solution A.—Sodium carbonate . . . . 15 grams
Chlorinated lime . . . . 8 grams
Distilled water . . . . 100 c.c.
Solution B.—Sodium hydrate . . . . 15 grams
Distilled water . . . . 100 c.c.

Take equal parts of solutions *A* and *B*.

2. Shake frequently and allow the antiformin to act one or two hours at incubator or room temperature until sputum is homogenized.

3. Collect sediment by centrifugalizing. Discard supernatant fluid, add salt solution and again centrifuge. Repeat washing of sediment several times.

4. Spread sediment on slide, air dry, fix and stain.

Caution.—Dead tubercle bacilli stain as well as living forms. All receptacles used in collecting sputum, urine or other

specimens should therefore be cleansed previous to use with concentrated sulphuric or nitric acid or in hot acid-bichromate solution. Use only clean, new slides. Use only freshly distilled water, as non-pathogenic, acid-fast bacilli are found in distilled water that has been allowed to stand for considerable time in an unclean container.

(*b*) *Feces.*—Select purulent or mucous particles and make film preparations. If no tubercle bacilli are found, dilute feces with three volumes of water, mix thoroughly and strain through several thicknesses of gauze or cheese-cloth. Saturate the filtrate with dry sodium chloride and allow to stand one-half hour. Skim off the film from the surface, dilute this material with distilled water and add antiformin to 15 per cent. concentration. Treat sediment as in the case of sputum.

A diagnosis of enteric tuberculosis should not be made on the presence of bacilli alone. The abdominal symptoms and signs, the number of bacilli and the character of the feces—particularly their consistence and the presence of pus, mucus and blood—should all be given proper weight. It should be remembered that the tubercle bacilli, if found, may have been swallowed in infected sputum. All cases showing positive findings in feces should be controlled with a sputum examination. Care should be taken not to confuse acid-fast particles, spores, etc., with tubercle bacilli.

(*c*) *Urine.*— 1. *Collection.* — Twenty-four-hour specimen should be collected by voiding, after thorough cleansing, into a sterile 2-liter bottle.

2. *Sedimentation.*—The bacilli will be carried down by the settling of the formed elements in the urine, therefore allow bottle to stand in cool place (ice chest), preferably tilted so that all sediment collects in as small a space at the bottom as possible.

3. *Preparation.*—The sediment is pipetted off and centrifuged at high speed. Volumes as large as possible, at least 15 c.c., should be used and two such specimens prepared.

Success or failure depends largely on *fixing* this centrifugated sediment on slide. The best fixative is blood serum which can be collected and kept in sealed, fine capillary tubes two inches long. Spread film of fixative over slide and then the centrifugalized sediment. Dry in air, fix in flame, stain by Ziehl-Neelson method. Bacilli are often found only after prolonged and painstaking search. If no tubercle bacilli are found, animal inoculation with the sediment is carried out. (See below.)

(*d*) *Spinal Fluid.*—In tuberculous meningitis the cerebrospinal fluid is rarely turbid. It is, however, not so limpid as normal fluid. It always forms a fine coagulum or clot on standing, and in the fibrin network of this bacilli may be found when nowhere else demonstrable. It is important therefore to collect coagulum and handle it properly.

1. *Preparation of Specimen.*—Take a small short conical glass. Insert $\frac{3}{4}$ to $\frac{7}{8}$ No. 1 cover-slip so that it is suspended on sides of conical glass, a slight distance from the bottom. Pour in spinal fluid immediately after its withdrawal. Allow it to stand overnight. The entire coagulum will be deposited on the cover-slip. Carefully pipette off all fluid and remove cover-slip with fine forceps. Spread coagulum over cover-slip. Fix in flame and stain (Ziehl-Neelson). If after prolonged and careful search no tubercle bacilli are found, centrifugalize whole fluid, break clot with antiformin and inject sediment into guinea-pigs.

(*e*) *Pus.*—Pus obtained from localized tuberculous infections, such as cold abscesses, sinuses, broken-down glands, the middle ear, etc., very often do not contain tubercle bacilli in sufficient numbers to make possible their demonstration by direct smear. When no bacilli are found the antiformin method of concentration may be used and animal inoculation employed.

(*f*) *Blood.*—While there is no doubt that tubercle bacilli may appear in the blood stream in miliary tuberculosis and

in the agonal stage of pulmonary disease, their presence, as a rule, is so infrequent that a search for tubercle bacilli in the blood for clinical purposes is not recommended.

III. METHODS OF CULTIVATION.—1. *Media.*—For the *isolation and first growth* of tubercle bacilli the following egg media are best: Dorsett egg medium, Lubenau's glycerin-egg medium and Petroff's gentian-violet egg medium. (See page 120.) For *routine cultivation,* however, Loeffler's coagulated blood serum (page 121), with the addition of 3 to 5 per cent. glycerin and glycerinated agar, are to be preferred. The serum and agar media are not good for isolation.

The material containing tubercle bacilli should be rubbed thoroughly over the surface of the medium with a sterile platinum spade or loop, the cotton plugs impregnated with melted paraffin and the tubes incubated at 37° C. As a rule, incubation for ten days to three weeks is necessary before growth appears.

2. *Direct Cultivation from Sputum, etc.*—Take equal parts of sputum and 30 per cent. solution of antiformin in water, mix in a sterile tube and allow to stand at room temperature one hour. Centrifuge at high speed, decant supernatant fluid, resuspend the sediment in sterile distilled water, again centrifuge and repeat the washing process three times. Streak the washed sediment over the surface of Dorsett or Lubenau egg media.

Petroff's Method.—Equal parts of sputum and 3 per cent. sodium hydroxide are shaken and incubated at 37° C. for fifteen to thirty minutes, the time depending on the consistency of the sputum. The mixture is neutralized to litmus with hydrochloric acid and centrifugalized. The sediment is inoculated into the medium described above. Pure cultures are obtained in a large proportion of cases.

Petroff's method can be applied to feces, in which the problem is made more difficult by the presence of many spore-formers which resist sodium hydroxide. Feces are collected

and diluted with three volumes of water and then filtered through several thicknesses of gauze. The filtrate is saturated with sodium chloride and left for half an hour. The floating film of bacteria is collected in a wide-mouthed bottle and an equal volume of normal sodium hydroxide is added. This is shaken and left in the incubator for three hours, shaking every half-hour. It is then neutralized to litmus with normal hydrochloric, centrifugalized and the sediment planted.

IV. ANIMAL INOCULATION.—The centrifuged sediment from urine, spinal fluid, etc., or the sediment from antiforminized material, is emulsified in sterile 0.85 per cent. salt solution and injected into the groin of guinea-pigs. *Always use guinea-pigs* except when differentiating types of tubercle bacilli. These animals are highly refractory to colon, streptococcus, staphylococcus, pneumococcus, etc. If tumor develops one to three or four days after inoculation, this is always non-tuberculous and usually subsides. *Always use at least two pigs*, since one may die from secondary infection or the tubercle bacilli may be so few as to miss infection in one. *Always inoculate subcutaneously, not intraperitoneally.* The former route is more likely to yield tuberculous infection with the same number of bacilli and less likely to produce septic infection from secondary bacteria. Inoculate into the loose tissue of the groin, well out and away from the midline, avoiding the mammary gland. If the material cannot be readily passed through a needle (never go over No. 18 gauge) introduce it into a small pocket cut under the skin.

If the suspected material contains tubercle bacilli the inguinal glands usually begin to swell in from twelve to twenty days, never before eight days, and only rarely at eight to twelve. Tumor on the first three days means a non-tuberculous process and usually subsides, and the tuberculous lesions develop in their own time later.

To hasten the diagnosis the intradermic test may be

applied. For inoculation purposes use only guinea-pigs that have been tested intradermically and found non-tuberculous. Animals of the lighter colors are preferred. From fourteen days on after inoculation with suspected material repeat the intradermic tests. Positive reaction means tuberculous infection. The intradermic reaction is considered positive when at the point of injection there occurs an area at least 10 x 10 mm. of redness and necrosis, with induration after twenty-four or forty-eight hours or both. The intradermic test (on guinea-pigs only—too high dosage for man) is made by injecting into the layers of the skin in a shaved area on the side of the animal 0.2 c.c. of 1 to 10 solution of Old Tuberculin (O. T. previously tested for potency). The injection is best made on the side of the guinea-pig, as the skin of the back is too thick, that of the belly too thin.

BACILLUS OF ANTHRAX (B. ANTHRACIS).

BACTERIOLOGICAL DIAGNOSIS.—The source of the material will depend on the type of infection, internal; intestinal or pulmonary, external; malignant pustule or edema. Meningeal types of disease are also encountered. Direct smears may reveal the typical bacilli. Spores are present only if free oxygen is available. If the bacilli are present and secondary organisms are not numerous no difficulty will be encountered in obtaining discrete more or less typical medusa head colonies by streaking the material over agar plates. If few in number or if other organisms are numerous, broth should be inoculated and part of the material injected subcutaneously or intraperitoneally into mice. If the latter succumb from anthrax a septicemia develops and the bacilli are easily cultivated from the heart's blood. If other means fail the broth culture should be heated to 60° C. for twenty minutes to kill the associated bacteria, and cultures again attempted as well as the injection of mice. Some difficulty may be encountered in the later stages of the disease in isolating the bacilli from

local lesions. Shortly before death a septicemia develops and blood cultures should be made if previous examinations have been negative.

For final identification it suffices if the bacillus is a Gram-positive, square end, chain-producing, spore-bearing, non-motile bacillus and is virulent for rabbits, causing a fatal septicemia on intravenous injection.

Should the examination of shaving brushes, etc., be requested the bristles should be ground in a mortar with broth. Half of the broth should then be heated to 60° C. for twenty minutes. The heated and unheated broth suspensions are then plated, added to broth and injected into mice, the subsequent steps being as above.

GAS GANGRENE AND THE DEMONSTRATION OF ANAËROBIC BACILLI IN WOUNDS.

GAS GANGRENE.—This is essentially a disease of injured or devitalized muscle tissue and is caused by an infection of the tissues with certain anaërobic bacilli. Among the anaërobes of war wounds the following are important: *B. welchii, Vibrion septique, B. sporogenes, B. edematiens, B. bellonensis* and *B. histolyticus.* The relative etiological importance of these organisms or particular combinations of them is still a debated question. It is exceptional to find a case of gas gangrene from which only one of these anaërobic bacilli can be isolated, mixed infection, with two or more, being the rule. In addition, secondary infection, with aërobic organisms, occurs, especially in wounds of long standing. Among these are *Streptococcus hemolyticus, Staphylococcus* and *B. pyocyaneus.*

There are three theories concerning the manner in which these organisms bring about their pathological effects. The oldest of these theories ascribes the local edema and necrosis and the systemic intoxication to the acid by-products of the

infecting bacilli. A later theory considers that the local lesion is occasioned by the accumulation of gas in the focal area, which mechanically interferes with the blood supply, thus causing cell death from lack of nutrition. According to this view the systemic reaction is due to absorption of the toxic products from the decomposing tissue. The third explanation may be called the specific toxin theory. This conception of the disease attributes both the local lesions and the constitutional symptoms to specific extracellular toxins, elaborated by the infecting organisms during growth. While it is possible that the acid by-products of the bacilli and the gases generated in the tissue may aid in the destruction of tissues and the intoxication of the patient, experimental data seem to justify the conclusion that these factors are less important than the specific toxins produced by the infecting organisms.

THE DEMONSTRATION OF ANAËROBIC BACILLI IN WOUNDS.[1] —The method outlined is adapted for field work and serves for preliminary isolation of the anaërobes present rather than for their final identification.

I. *Media.*—*Litmus milk* covered with paraffin.

Meat Medium.—To eight ounces of bullock's heart, freed from fat and finely minced, add the same amount of tap water and cook slowly. Add normal caustic soda solution until medium is alkaline to litmus. Tube, cover with paraffin and autoclave.

Veillon Tubes.—0.2 per cent. dextrose agar containing 0.2 per cent. sodium nitrate is sterilized in deep tubes used for agar shake cultures. The nitrate inhibits formation of gas and thus facilitates observation of colony structure. All media should be heated and cooled just before inoculation to drive out air and facilitate anaërobic conditions.

[1] Taken from Report No. 1 of the Anaërobe Committee of the Medical Research Committee of Great Britain, April, 1918.

II. *Material for Examination.*—1. Serous exudates from wounds.

2. Infected muscle tissue.

3. Fluid from hemorrhagic blisters.

4. Blood cultures.

III. *Microscopic Examination.*—1. *Film and Hanging-drop Preparations.*—Stain film preparations of fresh wound material by Gram's method and examine hanging-drop preparations of infected fluids for the presence of motile organisms. Gram-positive bacilli of any kind in films are to be looked upon as indicating the presence of anaërobes, but do not necessarily warrant a diagnosis of gas gangrene. Motile organisms found under these conditions are *B. sporogenes* (Metchnikoff) and *Vibrion septique.* The presence of large non-motile bacilli is indicative of *B. welchii.*

2. *Morphology.*—*B. welchii*, stout bacillus of varying length, ends rounded, non-sporulating in the presence of fermentable sugars, not infrequently showing capsule. Bacilli are generally single but often are seen in short chains.

B. sporogenes, sporulating bacillus, variable in length, more slender than *B. welchii;* spores are oval, central or subterminal in position, occasionally absent in very recent wounds.

Vibrion septique corresponds roughly in size to *B. sporogenes.* Certain morphological variations are characteristic: (*a*) club-shaped forms; (*b*) small oval types deeply and uniformly stained, (*c*) "citron" or "navicular" forms, irregular in size and staining, one or both poles stained deeply and the rest of body granular.

The "citron" or "navicular" forms are diagnostic of infection with *Vibrion septique.*

Oval End-sporing Organisms.—These are slender bacilli having a terminal spore at one extremity.

IV. *Cultural Methods.*—Small pieces of tissue, fluid from blisters or wound, should be inoculated into tubes of litmus

milk and meat medium. Blood cultures should be made, using the same media. Incubate at 37° C.

1. *Litmus Milk.*—(*a*) Acid clot torn by gas, the so-called "stormy fermentation," indicates the presence of *B. welchii.* Examination of film from positive milk tube (stormy clot) will show stout Gram-positive bacilli of the morphology of *B. welchii.* (See page 172.) If this reaction does not occur after twenty-four to forty-eight hours it may be assumed that *B. welchii* is not present.

As soon as characteristic clot has appeared in original milk culture, often within eight hours at 37° C., inoculate from this into second paraffined milk tube. Heat the latter at 60° C. for thirty to forty-five minutes. This will kill off the majority of cocci and other non-sporing aërobes. After heating, incubate this second milk tube twelve to twenty-four hours at 37° C. The appearance of "stormy fermentation" in this culture suffices for diagnosis of *B. welchii,* which is further confirmed by inoculating a dilution series of shake agar tubes and observing the growth of characteristic opaque lenticular colony.

(*b*) *Alternative Method.*—*B. welchii* does not form spores in milk, so that if a portion of the milk culture after three days' incubation be heated to 80° C. for twenty minutes, *B. welchii* together with cocci and other non-sporing bacilli will in most cases be eliminated from the mixture. This heated material when reinoculated into milk will yield growth of only such anaërobes as form spores in milk, most important of which are *B. sporogenes, Vibrion septique* and *B. tertius.*

B. sporogenes in milk causes precipitation of casein in fine shreds or soft friable coagulum without gas formation. The casein is rapidly digested with separation of slightly yellow turbid whey.

B. tertius.—This saccharolytic end-sporing type causes acid clot and gas after two to four days.

Vibrion septique gives rise to slow formation of gas and

Name.	Motility.	Spores.	Colony in agar shake.	Milk.	Meat.
B. welchii	—	Spores formed only on inspissated serum and in sugar-free media rich in protein	Opaque lenticular colony	Stormy fermentation; very rapid clotting with evolution of gas; clot torn with gas	Gas; pink color, sharp butyric acid odor; no blackening
Vibrion septique	+	Spores readily in all media; spores central or subterminal	Semitransparent with fern-like branching	Acid and clot; some gas may be formed; slow reaction 3 to 6 days	Gas; pink color; which fades later; rancid odor; no blackening
B. œdematiens	— or very slight	Spores formed, but not readily	Irregular woolly and transparent	Acid after 4 to 6 days; very slow clotting	Gas; no blackening; pink color at first, then bleached
Saccharolytic, oval and sporing type, B. tertius	+ or very slight	Spores oval, and always terminal	Opaque and irregular	Acid and finally clot	Gas; no blackening
Non-saccharolytic oval end-sporing types	+	Spores readily in mixed culture, much less readily when pure	Lenticular colony	No change in medium	Little gas; no blackening
B. sporogenes	+	Forms spores readily; central or subterminal	Opaque woolly colonies	Precipitation of casein which is later digested	Blackened; gas, very putrid odor
B. tetani	+	Spores, terminal and round	Branching and flocculent	No change in medium	No blackening

FREQUENTLY FOUND IN WOUNDS

Inspissated serum.	Fermentation in Saccharose.	Salicin.	Animal reaction.	Remarks.
No digestion; spores formed; filaments and involution types	+	−	Pathogenic for guinea-pigs and pigeons; less so for rabbits and mice. Spores not formed; no long filaments on surface of liver.	Sugars fermented—glucose, levulose, maltose, lactose, galactose, saccharose, and starch. Variable in glycerin and inulin according to type.
No digestion; variations in morphology-citrons, club forms, etc., may be developed	−	+	Pathogenic for guinea-pigs, pigeons, rabbits and mice: long threads formed on peritoneal surface of liver: citrons and club forms are characteristic	Animal passage very valuable as a means of separation and identification. Sugars fermented—glucose, levulose, galactose, maltose, lactose, salicin.
No digestion	−	−	Pathogenic for guinea-pigs produces colorless gelatinous edema. Does not usually form spores in animal body, no long filaments	Very resistant to heat. Rods often curved. Sugars fermented—glucose, levulose, maltose.
No digestion	+	+	Not pathogenic for laboratory animals	Sugars fermented—glucose, levulose, galactose, saccharose, maltose, lactose, salicin, mannite.
No digestion	−	−	Not pathogenic for laboratory animals	No sugars fermented
Liquefaction	−	−	Not pathogenic for laboratory animals	Sugars fermented—glucose, maltose
Liquefaction absent or slight	−	−	Inoculation produces tetanus	No sugars fermented

clot appearance not characteristic and not diagnostic. (This milk tube can be inoculated into a series of agar shakes and the various colonies dissected out and planted into meat medium for further study.)

2. *Meat Medium.*—Inoculate meat tube with fresh material and incubate twelve to twenty-four hours. Culture will contain all the anaërobes from the specimen and many aërobic organisms. *B. sporogenes* and *Vibrion septique* will under these cultural conditions show spores. Heat cultures to 80° C. for fifteen to twenty minutes. This will kill most of the cocci and aërobic bacilli and *B. welchii* in the majority of instances. The organisms likely to survive this heating are *B. sporogenes, Vibrion septique* and the oval end-sporing organisms. These last can be recognized by their characteristic appearance.

Reinoculate heated material into second tube of meat medium and incubate twenty-four to forty-eight hours at 37° C. The second meat tube inoculated with heated material may show blackening of the medium due to the development of iron sulphide; this together with digestion of the meat, putrefactive odor and the demonstration of motile organisms of characteristic morphology establishes a diagnosis of *B. sporogenes.* This culture should now be seeded in a dilution series of agar shakes (Veillon tubes) and these examined after twenty-four to forty-eight hours' incubation for the large, opaque, woolly colony characteristic of *B. sporogenes. Vibrion septique,* if present and not completely overgrown by *B. sporogenes* in the meat tube, will appear in the agar cultures as small semitransparent colonies, with a very faint nucleus from which arise fern-like outgrowths. Colonies of *Vibrion septique* are not sufficiently characteristic to be diagnostic.

Single colonies should be fished; stock cultures should be preserved for final purification and their fermentation reaction tested on salicin and saccharose. *B. sporogenes* does not

ferment either of these carbohydrates, *Vibrion septique* ferments salicin but not saccharose and the saccharolytic end-sporing type *B. tertius* ferments both.

V. *Animal Inoculation.*—If mice or guinea-pigs are available the separation of *Vibrion septique* from other anaërobes can be expedited by animal inoculation. The intramuscular injection of meat culture, or material obtained directly from the wound in quantities of 1 c.c. for a guinea-pig or 0.25 to 0.5 c.c. for a mouse, will, if *Vibrion septique* be present, cause death, with characteristic appearances, in twenty-four hours. These are a bloodstained edema with intense red coloration of the affected muscles. Make a culture in paraffined meat medium from the heart's blood of the infected animal; this will often yield a pure culture of *Vibrion septique.* The muscle at the site of injection will probably show a number of different bacteria, among them the characteristic "citron" and club forms of "*Vibrion septique.*" The presence of long snake-like filaments in film preparations from the peritoneal surface of the liver is diagnostic in *Vibrion septique* infection.

BACILLI OF THE TYPHOID, PARATYPHOID AND DYSENTERY GROUP.

Preparation of Special Media for Isolation from Feces and Urine.

STOCK EXTRACT AGAR.—Beef extract 3 grams, peptone 10 grams, sodium chloride 5 grams, water 1000 c.c. Dissolve by heating; add 15 grams of agar and dissolve by heating in the autoclave at 15 pounds' pressure for thirty minutes or by boiling over the free flame, making up the loss due to evaporation. Adjust reaction by the addition of alkali to 0.6 per cent. to 0.7 per cent. acid to phenolphthalein (hot titration) or to pH 7.4. If the Andrade indicator (see below)

is to be used the reaction may be set directly to the neutral point of this indicator. Add 1 per cent. of this indicator to a measured volume of the medium and determine how much normal soda solution is required to bring the medium to a point where it is pink when hot but colorless when cold. Add the appropriate amount of soda to the bulk of the medium, retest and readjust if necessary. Cool agar and add one egg per liter, heat to clear, filter and mix all of filtrate thoroughly to ensure a uniform product, fill into flasks or bottles in 100 c.c. amounts or multiples, and sterilize.

The Andrade indicator mentioned above is prepared by adding 16 c.c. of a normal sodium hydroxide solution to 100 c.c. of a 0.5 per cent. solution of *acid* fuchsin. The alkali changes the red of the fuchsin to orange or yellow. Should a tested normal soda solution not be available an approximate normal solution by weight (4 per cent.) may be used, adding the solution until the orange to yellow shade is obtained. Some time should elapse between additions, as the change of color proceeds gradually.

BRILLIANT GREEN PLATING MEDIUM (KRUMWIEDE).—To each 100 c.c. of melted stock agar add Andrade indicator 1 c.c., lactose 1 per cent. and glucose 0.1 per cent.[1] Mix and finally add the appropriate amount of brilliant green as determined by preliminary test; mix again and pour plates, six from each 100 c.c.

The brilliant green is added from a stock 0.1 per cent. solution in distilled water, the dye being dissolved by the aid of heat. This solution keeps for about a month. The optimal amount must be determined for each batch of agar. Plates are poured, using three dilutions of the dye, namely,

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[1] Most conveniently added from stock solution in distilled water of 20 per cent. lactose and 2 per cent. glucose, 5 c.c. to 100 c.c. of medium giving the approximate concentration desired. To prepare the double sugar solution add carbohydrates to sterile water and heat in Arnold for twenty to thirty minutes.

1 to 200,000, 1 to 330,000 and 1 to 500,000, or expressed in terms of dye solution per 100 c.c., the amounts are 0.5, 0.3 and 0.2 c.c. For standardization of medium, plates are inoculated with equal amounts of material, spreading it by means of a spatulum. Plates of a control medium such as Endo should be employed. The best material for standardization is positive stools from typhoid carriers or cases. If obtainable, several fecal specimens should be inoculated to compensate for the variability of the fecal flora, slight variations in resistance of different strains of B. typhosus and to average the variables due to technique. If only one positive stool is available this should be plated in duplicate or used to inoculate suspensions of two or three normal stools, which are then used for inoculation. The suspensions should be comparatively light so that the resulting plates will have discrete colonies, otherwise comparison by count or estimate will be difficult or impossible.

The stools should be freshly collected, as the persistent types in older stools tend to be the dye-resistant types and are therefore not satisfactory as a measure of the restraining action of the dye on the average fecal flora. If positive stools are not available, suspensions of fresh normal stools may be inoculated with broth cultures of recently isolated typhoid strains and the plates inoculated. The addition of a loopful or 0.1 c.c. of an eighteen-hour-broth culture to 10 c.c. of a stool suspension of moderate density will give a well-balanced mixture of B. typhosus and fecal bacteria. Pure cultures of B. coli are not satisfactory to determine the restraining action of the dye. The degree of restraint of the average fecal flora is the fact wanted.

After incubation the plates are inspected, and, based on the results, an approximation made of the dilutions to be used. The results may indicate that two of the dilutions as tested may be used, or that intermediate quantities should be used. The dilutions are chosen to obtain: (1) the greatest

amount of dye which has little effect on the number and size of the typhoid colonies, but which shows a marked restraint of the fecal flora; (2) a greater amount which shows a moderate reduction in the number and size of the typhoid colonies and usually a still greater reduction of the fecal flora. Slightly greater concentrations of dye are used in practice than would seem to be indicated by the standardization results, as the inoculation will be heavier, thus reducing the activity of the dye to some extent. With most batches of media and with Bäyer, Höchst or Grübler dyes the two optimum dilutions average 0.2 to 0.3 c.c. respectively of a 0.1 per cent. solution of the dye to 100 c.c. of agar. Other brands may give equally good differential action but more dye will usually be required. If the increased amount, however, leads to too deep a color the typical characteristics of the typhoid colony will be obscured.

Two concentrations are advised to compensate for the following variables: (1) differences in resistance of strains of B. typhosus, (2) variable amount of reduction of activity due to material inoculated and (3) variations in resistance of fecal flora of different stools. As B. paratyphosus is more resistant to the dye, greater concentrations may be used. If 0.2 to 0.3 c.c. is found satisfactory for B. typhosus, 0.35 to 0.5 c.c. may be employed if B. paratyphosus is the only type sought for.

Colony Characteristics.—On the medium after eighteen hours' incubation the typhoid colonies will be found to be very characteristic. They are of good size (1 to 1.5 mm.); the glucose not only enhances their growth but is also the main cause of their characteristic appearance. Viewed through the plate against a dark background, the light passing obliquely through the agar, they have a peculiar striated, flaky appearance. With artificial light and a hand lens, under the same conditions they have the appearance of a coarse wool fabric. They may take on a faint mauve tinge from fer-

mentation of the trace of glucose, which accentuates somewhat their characteristic appearance. The larger colonies may resemble the paratyphoid "B" type. Colonies of this organism tend to be larger, heavier and more opaque. They are often tinted a delicate green, and the markings are less distinct, though still evident at the periphery. The paratyphoid "A" colonies resemble the typhoid colony more nearly than that of the paratyphoid "B," but an extremely flattened, slightly tinted colony whose edges melt into the agar is frequently observed. The markings of the colonies of all three organisms are usually more prominent on the stronger dye plates. The only organisms which simulate the typhoid colony are certain partially restrained colon types which may develop on weak dye plates. They present exaggerated markings, with distinct cross-bar striations, and they are usually completely excluded on the stronger dye plates. Some of the dye-resistant "intermediates" develop paratyphoid-like colonies.

With no known change in the mode of preparation an occasional batch of agar will develop very large typhoid colonies. These luxuriant colonies tend to be much less characteristic in their markings, and the tendency to the development of this type should be noted when standardization tests are made, so that such colonies will not be overlooked in using the medium subsequently in routine work.

The colonies of the fecal types which develop take on a moderate pink color if they ferment lactose, but the color change is not marked. Even without a sharp color change they are so different from the typhoid or paratyphoid colony that with a little practice the eye passes over them without hesitation. Neutral red, 0.25 c.c. of a 1 per cent. solution, may be employed in place of the Andrade indicator to give a deeper color to the colonies of the lactose fermenting types. In this case the medium must be somewhat more acid (pH 7.0 to 7.2), being roughly adjusted to the turning point of this indicator, which is pH 6.8.

Endo (Kendall Modification).—Add 1 gram of lactose to 100 c.c. of stock agar, melt, during which process the lactose is dissolved and sterilized. Then add 1 c.c. of decolorized fuchsin prepared as follows: To 10 c.c. of a freshly prepared 10 per cent. watery solution of sodium sulphite add 1 c.c. of a saturated alcoholic solution of fuchsin and heat in the Arnold for twenty minutes. Pour plates.

METHYLENE BLUE; EOSIN (HOLT-HARRIS AND TEAGUE).— To 100 c.c. of melted stock agar add 0.5 per cent. lactose and 0.5 per cent. saccharose and 2 c.c. of a 2 per cent. solution of yellowish eosin, mix and add 2 c.c. of a 0.5 per cent. solution of methylene blue, mix and pour plates. The dye solutions are made in distilled water and kept in the dark. The protective colloidal action of the agar prevents the combination and precipitation of the two dyes. When acid is produced this protective action is destroyed, so that the fermenting types produce colored colonies. This color change is impeded if the colonies are very numerous.

Caution.—Plates for inoculation of feces must be dry. Either dry in incubator with covers slightly open or use porous earthenware tops to absorb the water of condensation.

RUSSELL DOUBLE SUGAR MEDIUM.—To stock agar add sufficient (about 5 per cent. by volume) sterile 5 per cent. solution of purified litmus to give a distinct purple color, also lactose 1 per cent. and glucose 0.1 per cent. (preferably by sterile solution—see above). If necessary, adjust reaction until neutral or very slightly alkaline to the litmus. Tube, preferably from a sterile covered funnel into sterile tubes, heat in Arnold for fifteen minutes and then slant in a position to give a long butt, and cool. Incubate for sterility. If mixed under non-sterile conditions, heat for fifteen minutes in the Arnold for three successive days. Modifications: (*a*) Substitution of 1 per cent. Andrade indicator for litmus gives sharper color changes and changes are visible with artificial light; (*b*) further addition of 1 per cent. saccharose will

exclude many types which, having a low avidity for lactose, would with the two sugars give a reaction like paratyphoid bacilli; (*c*) use 1 per cent. Andrade indicator, mix and tube aseptically, cool to 50° to 60° C. (to prevent flocculation of the peptone by the lead salt), and to each tube add sufficient sterile 0.25 per cent. aqueous solution of basic lead acetate to give a concentration of 0.05 per cent. Mix, slant and allow to set. This addition gives further cultural differences (see below) between the types. The Russell medium and its modifications are inoculated by streaking the surface of the slant and by stab into the butt. Each batch of Russell medium should be tested with known types to determine its suitability.

ENDO MEDIUM.—Into a container put 1 liter of tap water, marking the level of the fluid. Add 30 grams of thread agar, 10 grams of peptone, 5 grams of NaCl, 5 grams of beef extract. Cook until dissolved—it is best to autoclave thirty minutes—15 pounds; filter through sterile gauze or cotton. If necessary clear with egg. For this purpose, for each liter beat up the white of one egg with 10 c.c. of warm water until the egg is well mixed. Add this to agar cooled to 55° C., mix thoroughly, heat for thirty minutes or autoclave and filter through cotton.

This stock agar is kept on hand in quarter-liter flasks or bottles. Agar is standardized just before use and reaction adjusted to 0.2 per cent. acid to phenolphthalein. Before use, fuchsin and sodium sulphite are added. A filtered, saturated solution of basic fuchsin in 95 per cent. alcohol is kept on hand. A 10 per cent. solution of dry sodium sulphite crystals in sterile water is freshly made.

Teague has shown that a 10 per cent. solution of crystalline sodium sulphite can be heated for twenty minutes at 15 pounds' pressure with practically no change, and that the 10 per cent. sodium sulphite solution covered with a layer of liquid petrolatum about 1 cm. thick and sterilized in the

autoclave can be kept at room temperature for three weeks and probably much longer with but very slight change.

A 1.8 c.c. of fuchsin solution is added per liter to the agar. After this has been done the sodium sulphite solution is added gradually until the hot agar is almost decolorized— usually about 25 c.c. to the liter. A pale rose color should be present in the hot agar, which fades to a very faint pink on cooling; 10 grams of lactose is dissolved in a little water, filtered and added to each liter.

Various fuchsin solutions may differ and the absolute quantities given above may not be exactly the proper balance in separate lots. These are approximate, however, and the proper balance can easily be attained by a little preliminary testing in which sodium sulphite solution is added to small quantities of fuchsin solution in a test-tube.

The finished product is poured into large sterile Petri dishes. The cover is left off until the agar is hard. Smears are made on these plates.

It is helpful to lay a piece of filter paper into the lid of the Petri plate in order to absorb liquid evaporating from the agar in the incubator. If there is not enough filter paper for this, plate should be placed upside down in the incubator.

After incubation of Endo's medium the smaller colorless colonies of typhoid bacilli may be fished. Further identification is by transplantation and agglutination.

COLLECTION OF FECES.—Feces may be collected by transferring to wide-mouthed bottles, by means of a wooden tongue depressor, or by transferring a small mass to a tube, using the regular swab tubes. Sufficient feces should be collected so that the mass will not dry in transit. In case of dysentery, select blood-stained mucus. Specimens should reach the laboratory quickly after delivery to avoid dying or overgrowth of the pathogenic bacilli. Label each specimen with name, rank, company and regiment and state whether or not from kitchen personnel and the type or types the specimen is to be examined for.

PLATING OF MATERIAL.—If typhoid or paratyphoid bacilli are to be searched for, use Endo, brilliant green agar or methylene blue-eosin media; if dysentery bacilli, use Kendall modification of Endo. (*Caution.*—Dysentery bacilli are sensitive to dye and are easily inhibited, especially the Shiga type. The Endo medium must be freshly prepared, as any appreciable return of the fuchsin color will result in failure of growth of the Shiga types.) If the brilliant green medium is to be used, dilute fluid feces or emulsify solid feces in peptone water or broth to a density corresponding to one part of solid feces to 15 of diluent. Allow the suspensions to stand fifteen to thirty minutes to allow sedimentation of the particles and then inoculate the plates from the surface of the suspension. For each specimen duplicate sets of plates are employed, each set consisting of a weak and a strong brilliant green agar and an Endo or methylene blue-eosin plate. The fecal suspension is inoculated directly on the brilliant green plates, one loop being placed on each of the first set and two loops on each of the other two plates. The material is then spread, using a bent platinum, nichrome wire or glass rod as a spreader, rubbing out the material on the weak dye plate, then on the strong dye plate, and passing in turn to the Endo or methylene blue-eosin plate, the material adhering to the wire sufficing to inoculate this plate. The second series is then streaked in the same fashion.

If the relatively non-restraining media, such as Endo or methylene blue-eosin only are used, discrete colonies may be obtained in two ways: (*a*) inoculate a plate with a loop of the suspension, streak this over the plate and then streak an uninoculated plate with the material adhering to the spreader; (*b*) dilute original suspension 1 to 5, 1 to 50 and 1 to 500 and transfer a loop of each of these dilutions as well as the original suspension to an individual plate and streak the plates with the spreader, proceeding from the dilute to the less dilute. In the case of acute dysentery, select frag-

ments of bloody mucus, rinse free of fecal material, inoculate a plate and streak several plates in succession.

Examination of the Plates.—Colonies having the appearance of those of the typhoid-paratyphoid-dysentery types are searched for and fished to the Russell medium or one of its modifications. If only one type is being sought for, four to six fishings usually suffice. If it is not known which type is likely to be present, more numerous fishings should be made. After incubation a tentative decision as to type can be made from the appearance of the tubes, the reactions being as follows:

	Russell double sugar, modifications without lead acetate, Andrade indicator.	Further changes, if lead acetate is also added.
B. typhosus	Slant unchanged, butt acid.	Usually browning along stab.
B. paratyphosus "A"	Slant unchanged, butt acid and gas.	No browning.
B. paratyphosus "B"	Slant unchanged, butt[1] acid and gas.	Browning along stab.
B. dysenteriæ.	Slant unchanged,[2] butt acid.	No browning.
B. coli and members of colon group.	Slant acid, butt acid and gas.	

Final Identification.—This is done by setting up macroscopic agglutinations with the appropriate serum or sera depending on the reaction or reactions obtained with the Russell tubes. The specific titer of the serum employed must be known as well as its range in group or common agglutinins. If not known, the serum should be controlled by setting up parallel series of agglutinations with a known culture of the type homologous to the serum and with several closely allied types of bacilli. Although freshly isolated cultures may not agglutinate as well as the stock culture used to

[1] With litmus as indicator, butt decolorized and gas.

[2] With the triple sugar modification, saccharose fermenting dysentery types may or may not change the color of the slant, depending on their avidity for saccharose.

determine the specific titer of the serum, agglutination must occur in dilutions beyond the range of group action before a diagnosis of type can be made. Prepare a series of four to six dilutions ranging from the upper level of group action to the limit of the specific action of the serum. Place 1 c.c. of each in a series of tubes and 1 c.c. of saline in another tube for a control. With a small loop take some of the growth from the Russell tube, emulsify at the upper edge of the saline and shake tube, repeating this, until a faint turbidity results; do the same with the highest serum dilution, then with the other tubes in the order of dilutions, refilling the loop as necessary until the series is complete. Care must be taken to obtain a uniform turbidity in the series of tubes. Incubate at 37° C. for two hours (water-bath preferable) and read at once or after allowing to stand for a short period at room temperature or in refrigerator overnight to allow the clumped bacilli to settle. For general purposes, a typical Russell result plus a positive agglutination is sufficient for identification of the type of bacillus. In the case of B. dysenteriæ, should no type sera be available, a polyvalent serum may be used and a presumptive differentiation based on fermentative results.

Further cultural data which may be used to verify the diagnosis of type are given in the table (see below).

SLIDE AGGLUTINATION.—The macroscopic slide agglutination may be employed for a tentative identification of the suspected colonies. Highly potent sera are necessary which have been tested to determine the dilution applicable, so that an undue number of false reactions will not occur, because of the action of normal or group agglutinins on allied types. The dilution applicable will range from 1 to 50 to 1 to 200, depending on these factors. These very low dilutions are necessary to elicit a prompt or almost immediate reaction. Delay results in drying up of the drops. To carry out the test, place a drop of saline (control) on a slide and

a drop of the diluted serum, typhoid or paratyphoid, etc., depending on type to be searched for. Sweep up the suspected colony with a small platinum loop, rub sufficient growth into the saline to give a grayish clouding, then rub in likewise the drop of dilute serum. A positive result is shown by visible clumping in the serum drop. Spontaneous agglutination is controlled with the saline drop. The applicability of this method will depend on the variety of bacillus sought for and the number of sufficiently potent type sera available. With sufficient experience and a thorough knowledge of the serum used a negative result can be considered conclusive. Verification of positive results is carried out by fishing to the Russell medium and carrying out the tube agglutination as given above.

EXAMINATION OF URINE FOR B. TYPHOSUS OR B. PARATYPHOSUS.—Sample should be collected under aseptic precautions. Inoculate plates (brilliant green or Endo) directly with urine, preferably with sediment obtained by centrifuging at high speed. The bacilli, if few in number, can be enriched by adding 1 volume of broth to 2 volumes of urine; shake, incubate eighteen to twenty-four hours and inoculate plates, preferably brilliant green. As organisms other than B. typhosus may have developed, inoculate plates with a loop of dilutions of the culture 1 to 100, 1 to 10,000 and 1 to 1,000,000 as well as with the undiluted.

BLOOD CULTURES, TYPHOID AND PARATYPHOID FEVER.—(a) Draw 10 to 20 c.c. of blood and inoculate flasks of broth and melted agar for poured plates according to the regular blood culture routine; or (b) add blood to sterile ox bile in the proportion of 1 to 3. If broth is used, the dilution of the blood must be about 1 to 50, or more, otherwise clotting will occur which may lead to a negative result. Coagulation is inhibited by the bile and the smaller culture volume is a great advantage. If growth occurs, subcultures are made on Russell tubes for tentative differentiation and agglutination

CULTURAL REACTIONS.

	Fermentation of						Indol.	Motility.	Lead acetate, Browning.	Gelatin liquefaction.
	Dextrose.	Mannite.	Maltose.	Lactose.	Saccharose.	Xylose.				
B. dysenteriæ (Shiga)	+	−	−	−	−	−	−	−	−	−
B. dysenteriæ (Hiss and Russell Y.)	+	+	−	−	−	−	+	−	−	−
B. dysenteriæ (Flexner)	+	+	+[1]	−	−[2]	−	+	−	+	−
B. dysenteriæ (Strong[3])	+	+	−?	−	+	−	+	−	+	−
B. typhosus	+	+	+	−	−	≠	−	+	+[4]	−
B. paratyphosus A	(+)	(+)	(+)	−	−	(+)	−	+	−	−
B. paratyphosus B	(+)	(+)	(+)	−	−	(+)	−	+	+	−
B. enteritidis[5]	(+)	(+)	(+)	−	−	(+)	−	+	+	−
B. cholera-suis[5]	(+)	(+)	(+)	−	−	−	−	+	−	−
B. coli (communis)	(+)	(+)	(+)	(+)	−	?	+	+	?	−
B. coli (communior)	(+)	(+)	(+)	(+)	(+)	?	+	+	?	−

Symbols: Fermentation: − not fermented. + acid production. ≠ variable acid production. (+) acid and gas produced.

[1] Maltose very easily splits in sterilization; use maltose negative, glucose positive type to control medium.

[2] Usually with freshly isolated strains, subject to variation.

[3] Rare type.

[4] Subject to exception, positive results proportionate to suitability of medium.

[5] Encountered in acute enteritis due to food infection.

carried out as under feces. Strains from blood may be very resistant to agglutination, this characteristic tending to disappear after several transfers on artificial culture media.

WIDAL REACTION (DREYER METHOD).—The following method, a slight modification of the Dreyer technique, gives uniform and comparable values because a standardized bacterial suspension is used. This is an advantage in determining the agglutinin rise of the blood in response to vaccination and in diagnosing the existence of enteric fevers. This is especially the case in attempting a diagnosis of suspected enteric infection among those previously vaccinated. In these instances, although a positive Widal exists, owing to previous inoculation, quantitative fluctuations of the agglutinin content, indicative of active infection, can be determined by this method.

The method consists simply of macroscopic agglutination tests on varying dilutions of serum, using a killed culture of B. typhosus or B. paratyphosus A or B, the agglutinability of which has been determined by titration against a given standard procurable from the Army Medical School.

The technique for the test is quite simple. Sufficient blood is taken in the capsule to yield 0.3 c.c. of serum. This amount is withdrawn in a pipette and transferred to the first tube. This tube has previously received 2.7 c.c. of saline solution, while the following nine tubes have each received 1.5 c.c. of saline solution. Of this first 1 to 10 dilution of serum, 1.5 c.c. are carried into the second tube, mixed thoroughly, and 1.5 c.c. of this 1 to 20 dilution is carried forward. This procedure is carried out through the series of ten tubes, the last 1.5 c.c. being discarded. Beginning in the weaker dilution, the diluted serums are each divided into three tubes, each tube containing 0.5 c.c. of the serum dilution. The tube rack then contains three series of ten tubes containing serum dilutions ranging from 1 to 10 to 1 to 5120.

To each tube in the first series, 0.75 c.c. of the standardized suspension of B. typhosus is added; to each tube of the second series, 0.75 c.c. of the standardized suspension of B. paratyphosus A is added; and to each tube of the third series a like amount of the suspension of B. paratyphosus B is added. The tubes are then thoroughly shaken.

The tubes are then placed in a water-bath at from 50° to 55° C. for two hours; are removed and cooled for fifteen minutes and are then read. The highest dilution showing agglutination, without sedimentation visible to the naked eye, gives the reading. The dilution of the serum in this tube, divided by the factor of agglutinability of the culture used, gives a final reading expressed in the number of agglutinin units per cubic centimeter of the serum. (It must be kept in mind that the addition of the suspension has increased the dilution of the serum one and a half times, the first tube representing a dilution of 1 to 25, the last a dilution of 1 to 12,800.) These readings are comparable with each other, whenever the same technique has been used, and a standardized agglutinable suspension has been used.

To make up a supply of the standardized suspension of B. typhosus or other types, the procedure is as follows: B. typhosus is subcultured daily in broth, for about ten days, to increase its agglutinability and reduce its auto-agglutinability. Finally, it is planted in broth in partly filled flasks and incubated twenty-four hours. At the end of that time 0.1 per cent. formalin (40 per cent. formaldehyde solution) is added. It is placed in an ice box for four or five days and shaken repeatedly. The then sterilized culture is ready to be standardized for (1) opacity and (2) agglutinability.

To standardize for opacity, two series of fifteen tubes each (tubes of equal size and of clear glass) are set up, and varying dilutions of standard suspension in one series, and the suspension to be standardized in the other are made in accordance with the accompanying schedule.

DILUTIONS.

Cultures:
0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.2 1.4 1.6 1.8 2.0
Physiological sodium chloride solution:
1.9 1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0 0.8 0.6 0.4 0.2 0.0

A tube from one series, chosen at random, is matched for opacity with the tubes in the other series. The dilution of each culture in these tubes is noted. This procedure is repeated six times and the average taken. The necessary dilution of the new culture is thus determined, the dilution to be made with physiological sodium chloride solution, to which has been added 0.1 per cent. formalin. It is then bottled and kept in the ice box.

To standardize for agglutinability an immune serum is procured. A rabbit immune serum of known agglutinin unit content is to be preferred, though human serum, taken soon after inoculation, serves the purpose. The agglutinating serum is set up in two parallel series, in varying dilutions, the variations not to be excessive. To one series the standardized suspension is added, to the other the suspension to be standardized. These tubes are then shaken, incubated at 55° C. for two hours and read for the highest dilutions, showing agglutination visible to the naked eye. That dilution of the standardized suspension is to the dilution of the new suspension as the known factor of the standardized suspension is to x (the factor of the new suspension).

Example.—If the dilution in which the standardized suspension is agglutinated is 1 to 6400 while that of the new suspension is 1 to 3200 and the factor of the standardized suspension is 1.0, then

$$\frac{6400}{3200} = \frac{1}{x} \text{ or } X = \frac{3200}{6400} = \tfrac{1}{2}$$

The factor of the new bacterial suspension is therefore one-half.

This method is, of course, similarly applicable to the B. paratyphosus A and B. It is also applicable to the three strains of B. dysenteriæ.

Readings should always be made under constant lighting conditions. Bacterial count may be substituted for standardization by opacity; 0.1 per cent. formaldehyde solution kills bacteria, but not fungi. If work is to be done intermittently it is best to bottle the culture in small bottles for preservation, since contamination by fungi is common. For routine work, large bottles, from which the daily amount is removed under aseptic conditions, yield the best results. Under proper storage conditions (a dark ice chest) the factor of agglutinability does not change in six months, and probably continues the same for a much longer period of time.

In an emergency when standardized bacterial suspension is not available, add to an 18-hour broth culture an equal quantity of physiological salt solution containing 0.2 per cent. formalin and carry out the test as with standardized bacterial suspension. Sufficient of this suspension can be made to compare results over a short period of time and to leave a residuum which can be used for comparison with the standardized suspension when available.

CHOLERA.

BACTERIOLOGICAL EXAMINATION.—The methods differ somewhat as to whether one has a suspected case or carrier. Two characteristics make the isolation of the vibrio easy. First the cholera vibrio in peptone water grows most abundantly at or near the surface, forming a narrow dense zone of growth at this point, so that even in mixed cultures from feces this zone will show many vibrios or even nearly a pure culture. Second, the vibrio will grow on alkali—albuminate agar media, so alkaline that the growth of the majority of fecal bacteria is inhibited.

EXAMINATION OF SUSPECTED CASE.—Smears should be made from the feces and stained with carbol fuchsin diluted 1 to 10. If large numbers of typical slightly bent rods are present, a tentative diagnosis can be made at once, the probable correctness of which will vary directly with the proportion of vibrios present. When numerous vibrios are present they are easily isolated by streaking plates of plain agar, the vibrio colony having a very typical transparency, with opalescent sheen. Agar slants are used in fishing the colonies. When fewer in number, peptone water should also be inoculated for initial enrichment and the surface growth inoculated on agar after eight to twelve hours. Alkaline albuminate media are more satisfactory for plating, especially when the vibrios are relatively few in number because of the restraint of the associated bacteria. The alkaline blood agar of Dieudonné has certain disadvantages which are overcome in the alkaline egg medium of Krumwiede. The modification of Goldberger gives especially luxuriant growth. To 5 parts of glucose extract agar (agar 3 per cent.) add 1 part of an alkaline egg mixture prepared as follows: Make an egg-water mixture of equal volumes of egg and water and add to this mixture an equal volume of 6.5 per cent. solution of anhydrous sodium carbonate; mix and steam in the Arnold for twenty minutes. After mixing the agar-and-egg solution the plates are poured and allowed to stand open, but protected from dust, until the water of condensation has evaporated. On this medium, after twelve to eighteen hours' incubation, the vibrio colonies, with transmitted light, appear to be deep in the agar and have a peculiar hazy appearance, due to a halo about the colony.

If no vibrio colonies are present on the plates and if no vibrios are present in smears from the surface of the peptone water a negative diagnosis can be returned. If in doubt the surface from the peptone water should be transferred to a second peptone tube. After incubation this second enrich-

ment should show almost a pure culture of cholera vibrios when examined in smear, had they been present in the stool.

EXAMINATION OF SUSPECTED CARRIERS.—As the number of vibrios is usually small, direct smears of the feces are of little or no value. Direct plates using alkaline egg medium may be made as described above and peptone water should also be inoculated. These are examined and handled as described above. The number of vibrios in some cases may be so small that direct plates fail and the primary peptone culture may not give a definite picture. For this reason a secondary peptone culture should always be inoculated from the surface of the primary tube. Unless there is extreme urgency, routine examination of carriers is best carried out by inoculating peptone water only, incubating six to ten hours, subinoculate to a second peptone tube, incubate eight to twelve hours and examine in smear. A negative smear is conclusive and the positives can be reported tentatively to hold the case. Plating and final identification can then be carried out and reported.

Because of the occurrence in feces of cholera-like vibrios, some culturally identical with the cholera vibrios, agglutinating serum is essential for final identification. If the serum available is sufficiently potent, preliminary identification can be done by the slide method. In any case final identification should be done with the macroscopic tube method. Follow out the step as described under "Typhoid."

In the absence of serum a considerable number of cholera-like vibrios can be excluded by cultural means. Some do not enrich in mixed culture as does the true cholera vibrio on successive subinoculation from peptone water to peptone water. After obtaining pure cultures, some will be found not to produce acid from glucose, and many do not give the cholera-red reaction. This reaction is the same as the indol reaction, except that it is elicited by the addition of several drops of concentrated H_2SO_4 or 1 c.c. of a 10 per cent. solu-

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tion to the peptone culture, the nitrite solution not being used, as the vibrio in its growth reduces the nitrates of the medium to nitrites.

With some training the nearly pure surface growth from the smear-positive peptone tubes can be employed for agglutination, using the hanging-drop method. This is the method of choice when the examinations mount to the hundreds or even thousands per day.

Two very essential facts must be remembered in relation to cholera examinations: (1) stock culture strains may differ very markedly from freshly isolated strains, both morphologically and culturally, so that they may be very misleading if used as controls; (2) among those exposed to infection, but not developing the disease, as high as 10 per cent. may become "contact carriers" and serve as a source of further infection. Such carriers are also potential cases, as a vigorous cathartic, indulgence in alcohol, etc., may upset the prctective balance with the development of the acute disease.

ISOLATION OF CHOLERA-VIBRIO FROM WATER. — As the vibrios are usually few in number, large volumes of water should be used. Place 100 c.c. amounts in flasks and add to each 10 c.c. of a ten-fold strength peptone water, mix and incubate. Examine surface growth, subinoculate for secondary enrichment, etc., as above.

BACILLUS OF BUBONIC PLAGUE (B. PESTIS).

MEDIUM.—The medium of choice is 5 per cent. glycerin agar, neutral or slightly alkaline to litmus.

COLLECTION OF MATERIAL.—1. Aspirated contents of unopened buboes.

2. Purulent discharge from opened lesions.

3. Sputum in pneumonic plague.

4. Blood cultures in the latter stages of both types of disease, especially the pneumonic forms.

Microscopic and Cultural Examination.—1. Make smears of pus or sputum and stain by Gram and with methylene blue or dilute carbol fuchsin (1 to 10). The presence of typical ovoid, Gram-negative, polar-staining bacilli alone (bubo) or in great relative predominance (sputum) is positive presumptive evidence of infection with B. pestis.

2. If these typical bacilli are present alone or in great relative predominance, plate material directly on glycerin agar and incubate at 30° to 35° C. At the same time inoculate animal (see 3).

3. If material is grossly contaminated, resort to animal inoculation. Shave the abdomen of the guinea-pig without the use of soap. This results in fine scarification. Apply the infected material or a loopful or two of culture directly to the scarified area. The plague bacillus possesses such extraordinary invasive capacity that the mere application of virulent material to a small scarification results in the development of internal lesions with coincident septicemia. The animal may develop septicemia, with death in twenty-four hours, or may show lesions of the lymph nodes and viscera, developing septicemia more slowly. Pure cultures are readily obtained from heart's blood and organs. Caution: Organisms of this morphology are encountered in spontaneous infection in guinea-pigs. None of these have the invasiveness of plague bacillus, and some can be excluded because of cultural differences.

4. *Identification.*—The typical morphology taken in consideration with the clinical condition renders diagnosis almost certain. Full identification rests upon the isolation of an organism having the following characteristics: The bacillus is Gram-negative, non-motile, non-sporulating, non-gas-producing, typically ovoid polar staining, with a marked tendency to develop bizarre involution forms. This latter tendency is exaggerated by growth on 3 per cent. salt agar. On moist media the colony after twenty-four hours'

incubation is small, delicate and dewdrop-like in appearance. Broth is slightly clouded, flocculi are deposited along the sides and at the bottom of the tube. The positive "cutaneous test" (3) added to the above characteristics is sufficient for identification.

DIAGNOSIS OF PLAGUE INFECTED RATS.—Lesions are important and often so striking that diagnosis can be made from macroscopic appearance. Subcutaneous vessels are engorged and the tissues a diffuse pink. Superficial lymph nodes are markedly enlarged, injected and frequently surrounded by edema and hemorrhagic areas. Contents may be firm, less often are purulent. Peritoneal and pleural surfaces are injected; the cavities contain excess of fluid. The spleen is enlarged and soft; the liver mottled with small punctate hemorrhages and yellowish areas of necrosis. Parasitic cysts are common in the livers of rats and the smaller types of these cysts should not be confused with the plague lesion. Smears and cultures from lesions and blood confirm diagnosis. Animals submitted for diagnosis are frequently decomposed and the overgrowth of contaminating bacteria makes difficult the isolation of B. pestis in pure culture. In these instances the plague bacillus can be isolated by using the method of animal inoculation described in 3.

CAUTION IN CARE AND HANDLING OF PLAGUE INFECTED ANIMALS.—In handling animals with suspected plague infection or inoculated with plague suspected material, protect the skin of the hands and arms by wearing long-sleeved gowns and rubber gloves.

Inoculated animals should be kept in glass jars, the upper portion of the inner surface of which should be greased with vaselin and covered with a fine gauze to prevent the escape of infected fleas.

BACILLUS OF GLANDERS (B. MALLEI).

MEDIA.—B. mallei grows very poorly or not at all on the usual laboratory media. Meat infusion agar containing 5 per cent. glycerin, with a reaction of 2.5 per cent. acid to phenolphthalein, yields a fair growth. This medium can be enriched by the addition of 5 per cent. volume of potato juice prepared as follows: Grate white potatoes, add 1 liter of water for each pound, soak overnight, press through cheese-cloth, add 1 egg per liter, autoclave to clear, filter through cotton and sterilize in autoclave.

MATERIAL FOR EXAMINATION.—(Directions for collecting and forwarding specimens for the diagnosis of glanders, see page 18.)

1. Material from unopened gland or nodule obtained by aspiration or incision.
2. Discharge from sinuses.
3. Nasal secretion.
4. Sputum.
5. Blood cultures.

MICROSCOPIC AND CULTURAL METHODS.—Film preparations from infected discharge stained by Gram's method may show very few organisms. B. mallei is a Gram-negative small organism showing considerable variation in form and staining. Direct isolation on glycerin potato agar should be attempted only with material from freshly opened lesions. Upon this medium in twenty-four to forty-eight hours, small, whitish, transparent colonies develop, increasing in size upon further incubation. The discharge from sinuses from the nose or sputum should be inoculated intraperitoneally into male guinea-pigs to elicit the Straus reaction and to obtain pure culture if the reaction occurs. A positive reaction is evidenced by tumefaction and descent of the testicles. This usually occurs within two to five days. When the reaction is at its height the pig may be killed and cultures made from any softened or necrotic areas of the testicle as

well as from the heart's blood and spleen. A negative result is not conclusive and the pig may die from septicemia, due to the associated bacteria injected. Blood cultures are likely to be positive in the more acute types of disease.

IDENTIFICATION. — *Pure Cultures.* — Pure cultures, when obtained, are identified on the basis of their morphological and cultural characteristics. To be considered B. mallei, the bacillus should be Gram-negative, stain with some difficulty and show considerable variation in form and staining. It should be non-motile and non-sporulating, should grow poorly or not at all on ordinary media and only slowly on its optimum medium. On sterilized potato slants B. mallei develops slowly, the growth after twenty-four to thirty-six hours being moist, transparent and yellow; later the color deepens and finally becomes reddish brown, and about the growth the potato takes on a greenish-yellow tint.

SEROLOGICAL TESTS.—As cultures vary widely in their agglutinability, many being very resistant, the agglutination test is limited to special laboratories, where selected cultures and control sera are available. The complement-fixation test likewise requires special facilities.

B. INFLUENZÆ (PFEIFFER'S BACILLUS).

MEDIUM.—The medium of choice is meat infusion agar having a reaction of o.3 to o.5 acid to phenolphthalein and containing o.5 c.c. of sterile defibrinated blood per 10 c.c. of medium. Rabbit, horse or human blood may be used; the last of these is best when obtainable.

COLLECTION OF MATERIAL.—1. Sputum: in respiratory infection. (See pp. 13 and 132.)

2. Cerebrospinal fluid, pleural fluid, etc. (See p. 15.)

3. Purulent exudates from lung, brain, etc., at autopsy.

4. Posterior nasopharynx by method of swabbing described for meningococcus carrier detection. (See p. 149.)

MICROSCOPIC AND CULTURAL EXAMINATIONS.—1. Make smears of sputum or purulent exudate. Stain by Gram, using as a counterstain 10 per cent. aqueous solution of safranin or diluted carbol fuchsin. The influenza bacillus is Gram-negative, very small, showing many polar staining organisms, and often great variation in morphology from coccoid to bacillary forms.

2. Sputum should be washed as described for the isolation of pneumococcus. A small kernel, preferably a mucous plug, should be spread back and forth across the surface of a blood agar plate. The following method may also be used. Place a drop of defibrinated or citrated blood in center of plain agar plate. Mix a small loopful of the material to be inoculated with the drop of blood and then streak this radially across plates. This has the advantage that the medium is transparent and the typical colony can be searched for under the microscope. The colony is small, colorless, translucent and homogeneous.

In making cultures from cerebrospinal fluid and purulent exudates, providing there is much precipitated pus present, one drop of the material should be spread upon the medium. If there is no purulent precipitate, 0.2 c.c. of the turbid fluid should be used for inoculation, since a loopful in such instances may yield no growth. For cultures made at autopsy from the lung, several loopfuls of the exudate should be used. In cases of respiratory infection in which no sputum can be obtained, cultures may be made by swabbing the posterior nasopharynx, using the technic described under Meningococcus, page 149.

Cultures should be incubated eighteen to twenty hours at 37° C. With the use of a hand lens, search for minute colorless, round, discrete colonies. (On moist medium the organism on first isolation may show many bizarre forms, but on second or third transfer they assume the typical small poled type.) Colonies should be subcultured on blood agar slants

on which the colorless, discrete, never confluent growth is characteristic.

IDENTIFICATION.—The bacillus of influenza is extremely small, polar staining and Gram-negative. The colony is minute, colorless, translucent and discrete, showing neither hemolysis or pigmentation of the medium. B. influenzæ grows only on media containing blood, the presence of hemoglobin being essential. This fact is of differential value. In the final identification transfers to plain agar should be made. The first subculture on a blood-free medium may show faint growth due to a small amount of blood carried over from the original blood-agar culture. Second and third transfers should show no growth on plain agar if the organism isolated is to be identified as B. influenza.

Recently a medium described by Park and Williams has given excellent results. It is made by adding hemolyzed erythrocytes, preferably from the horse, to the agar in a flask while the latter is still hot (90° C. or more).

A horse may be bled into a flask containing sterile sodium citrate solution in such amounts that the final concentration will not be less than $1\frac{1}{2}$ per cent. The supernatant fluid is decanted, distilled water is added to the erythrocytes and the laked blood in turn added to hot agar. Tubes and Petri dishes are then filled. The mixture should be incubated overnight and contaminated culture media discarded. The optimum reaction of the agar is between $\frac{2}{10}$ and $\frac{8}{10}$ plus. Involution forms on this medium are frequent, but need not lead to confusion.

RABIES.

In a Case of Dog Bite See that the Wound is Cauterized with Fuming Nitric Acid.—Do not Kill the Dog.—Except the actual cautery, other agents than nitric acid are valueless.

Keep the dog under observation of a veterinarian if possible; should he show or develop definite symptoms of rabies, he

may be killed and the brain examined for Negri bodies as a confirmatory measure. Should he remain alive and well for fifteen days, rabies can be practically excluded; three weeks gives absolute certainty. Should the dog have been killed the brain examination must be done.

If the examination cannot be carried out at the place, the head should be removed, packed in ice and sawdust and forwarded. Care must be taken that it will reach its destination in proper condition. If decomposition sets in the examination will be made difficult or impossible, especially when the Negri bodies are not well developed.

METHOD OF EXAMINATION.—If the material is fresh, smears are made by pressing between a slide and a cover-slip a small thin section of the gray matter from (a) Ammon's horn, (b) cerebellum and (c) cerebral cortex, the material being spread along the slide by pressure on the cover-slip.

Without allowing to dry, the smears are fixed about ten seconds in neutralized methyl alcohol (0.25 gm. of sodium carbonate to 500 c.c. of alcohol), to each 40 c.c. of which are added 3 drops of a 1 per cent. solution of picric acid. The excess of fixative is removed by blotting with fine filter paper. The fixed smears are stained with a modified Van Gieson stain prepared as follows:

Saturated alcoholic fuchsin solution . 0.5 c.c.
Saturated alcoholic methylene blue solu-
   tion . . . . 10.0 c.c.
Distilled water . . . . 30.0 c.c.

The stain deteriorates rapidly at room temperature, but keeps well on ice.

The stain is poured over the smear, which is held over the flame until steam rises. Wash in tap water and blot. With this stain the Negri bodies appear magenta, the nerve cells blue and the red blood cells a yellow or salmon.

If Negri bodies are not found an emulsion in salt solution is made from good-sized portions of the brain and injected intracranially into guinea-pigs. This is done by making a short incision over the skull vault to the side of the longitudinal sinus and drilling through with a small awl or with the point of a knife. The material is then injected by syringe, the needle being cut off about $\frac{1}{4}$ inch above the connector. About 0.25 c.c. of the emulsion is injected. Three pigs are used. If the material is contaminated a suspension is made in undiluted sterile glycerin and preserved on ice for three days, after which proceed as above. The glycerin tends to kill off the associated bacteria. Filtration through a Berkefeld filter can also be resorted to. Guinea-pigs are used in preference to rabbits, as the incubation period is shorter and more regular.

Verification that the pigs have died of rabies rests on the symptoms developed and the examination of the brain for Negri bodies. The bodies, however, in guinea-pig brains average smaller than in dog brains. They are also more easily distorted in making smears.

Fixed material, preferably by Zenker, after embedding in paraffin, cutting sections 3 to 4 micra in thickness, removing paraffin, etc., are stained as follows: Place in Gram's solution to remove bichloride of mercury, wash in water, then in alcohol 95 per cent. to remove iodin, stain in 5 per cent. aqueous solution of eosin for twenty minutes, wash in water, stain in Loeffler's methylene blue for fifteen minutes, wash in water, place in saturated solution of colophonium (resin) in absolute alcohol until sections turn to pink color, place in absolute alcohol, xylene and mount in balsam. With this stain the Negri bodies are pink to magenta, the nerve cells blue and the red blood cells yellowish.

A negative search for Negri bodies is of value in direct proportion to the skill of the examiner. In about 1 per cent. of rabid dogs the bodies may be poorly developed or absent,

though even in these cases the very skilled observer will note distinctive degenerative changes.

DETECTION OF THE SPIROCHETA ICTEROHEMORRHAGIÆ.

A. IN MAN.—1. Dark-field illumination.

2. Examination of blood films: Stain by Wright or Fontana method.

3. Injection of the blood into the peritoneal cavity of a guinea-pig.

The earlier in the course of the disease the blood is obtained the better the chance of success. The organisms are not very numerous in the human blood and are somewhat difficult to stain. Guinea-pig inoculation with blood is a valuable procedure and should always be done unless the patient is first seen late in the disease. If the Spirocheta icterohemorrhagiæ are present in the inoculated blood the guinea-pig will usually sicken and die in about ten days. Postmortem examination shows a well-marked combination of jaundice and hemorrhage such as, so far as known, is not produced by any other infection.

In a light-skinned guinea-pig a distinct yellowish tinge, especially noticeable in the ears and about the genitals, is usually observed. On dividing the skin of the abdomen in a case of this disease the operator is at once struck with the widespread hemorrhages which lie beneath the skin and between the connective-tissue planes. They range from minute petechiæ up to massive effusions of blood, perhaps 1 cm. in diameter. The hemorrhages are especially well marked about the axillary and inguinal lymph nodes, and as the skin is reflected, hemorrhagic areas will be seen between the fascia covering the skeletal muscles. The skin is usually quite yellow and the abdominal muscles frequently show a yellowish tinge. On opening the body cavity the liver appears distinctly enlarged and of a brownish-yellow color. The spleen is not

enlarged. The intestines are stained yellow and hemorrhages into the intestinal walls are of frequent occurrence. Post-peritoneal hemorrhages are frequent and abundant, especially about the kidney and adrenal. This organ is frequently the seat of marked effusions of blood. In the thorax the lungs especially attract attention, being the seat of the most characteristic gross change observed in the guinea-pig. These consist of numerous sharply defined hemorrhagic foci. The description by the Japanese of the lungs as resembling the mottled wings of a butterfly is a very apt one. Histologically the liver and kidneys show the most characteristic changes. The liver shows an exudation of polymorphonuclear leukocytes about the bile ducts and widespread degenerative changes of the parenchyma. Many of the cells contain an abnormal amount of pigment while others show pronounced vacuolization and dispersion. The kidneys show an acute exudative nephritis, with hemorrhages throughout the cortex.

The tissues of the guinea-pig contain many spirochetes, which may be best demonstrated by staining portions of the liver by the older method of Levaditi, making sections and examining by the microscope. Dark-field examination of the liver pulp will also usually reveal them.

4. Microscopic examination of the urine: Examination—centrifugalized sediment by the dark-field, Wright or Fontana stain or India ink. The dark-field method is recommended. Urine contains spirochetes in a variety of conditions, and one must be entirely familiar with the morphology of the S. I. to hazard any diagnosis by a microscopic examination of the urine. Microscopic examination of the urine, however, has a special field in expert hands to determine whether a convalescent is excreting the spirochetes in his urine and is therefore a carrier.

5. Injection of urinary sediment into the peritoneal cavity of a guinea-pig: This method has frequently been followed by positive results and should be regularly practised.

6. Examination of tissues obtained at necropsy by the older method of Levaditi: Spirochetes may frequently be demonstrated in the viscera, especially in the kidneys. The cultivation of the organism is a matter of considerable difficulty. Noguchi's technic for the Treponema pallidum is the method used.

B. In Rodents.—Here, as in the detection of the disease in man, guinea-pig inoculation is the method of choice and reliability. The rats should preferably be taken alive, killed and the kidneys removed at once, with precautions not to contaminate them. The kidneys should then be emulsified and the emulsion injected into the peritoneal cavity of a guinea-pig, if possible using a guinea-pig for each rat. The guinea-pigs should then be observed for at least two weeks. If the Spirocheta icterohemorrhagiæ are present the pig will become ill, show some rather variable pyrexia, become slightly jaundiced, collapse and die in about ten days, and at post-mortem examination will show the marked picture of jaundice and hemorrhage referred to above. Spirochetes may be demonstrated in the tissues, as previously indicated.

BACTERIOLOGY OF ROPY BREAD (BACILLUS MESENTERICUS).

Infection of loaves of bread with Bacillus mesentericus occurs, producing the condition known as "ropy bread." Patches in the central part of the loaf become grayish in color, are sticky, glue-like and have a characteristically sour odor. The infected bread shows an acid reaction which may be demonstrated by applying an indicator, methyl red, to the cut surface of the loaf; a yellow color showing the reaction characteristic of this condition. From the patches the Bacillus mesentericus may be cultivated or shown in smears. It is a medium-sized Gram-positive bacillus, somewhat granular in appearance and containing a large oval spore. The organism grows rapidly at 37° C. On plain agar the colonies are dry, granular, grayish white in color and tend

to spread. It produces acid in dextrose, saccharose, lactose, mannite and inulin.

Litmus milk becomes acid and is coagulated in forty-eight to seventy-two hours, peptonization following quickly. In bouillon the growth is cloudy and a thick, dry, skin-like pellicle forms. To further identify the organism fresh loaves of bread may be inoculated and incubated at 37° C. for forty-eight hours. This organism is non-pathogenic, but its effect on bread is to make it undesirable for use.

The development of the organism can be prevented by the addition of 10 per cent. vinegar in the proportion of 1 pint to every 100 pounds of flour.

QUANTITATIVE ANALYTICAL METHODS.[1]

GENERAL METHODS.

USE OF THE BALANCE.[2]—See that the balance is perfectly level, as indicated by the plumb-bob or spirit-level; if it is not, make it so by turning the adjusting screws at the right and left front corners.

Always place the object to be weighed on the left-hand pan, and the weights on the right-hand pan. One reason for this is to equalize errors through possible inequalities in the length of the two arms of the beam. To obtain the true weight of a substance when extreme. accuracy is required, counterbalance it with sand or weights and then replace the object with weights; or weigh in one pan and then exchange the substance and weights, weigh again, and take the mean of the weights so obtained.

The beam and pans must always be supported before adding or removing weights, and the weights must be handled only with forceps.

To avoid errors in noting weights always count them twice: (1) by noting those missing from the box; (2) by noting the weights as they are taken from the pan and replaced in the box.

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[1] The preparation of the chapter on Quantitative Analytical Methods has been facilitated by free use of the recently published "Methods Employed at St. Luke's Hospital," which the authors, Drs. Wood, Vogel, and Famulener placed at the Department's disposal.

[2] A balance suitable for use in preparing volumetric standard solutions and for gravimetric analyses should have a capacity of from 100 to 200 grams with a sensibility of one-tenth of a milligram when fully loaded.

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All objects must be at room temperature when weighed. Warm objects cannot be weighed accurately as currents of air are caused which introduce an error.

Crucibles should be cooled in a desiccator when the precipitates weighed in them take up water from the air. If the crucible gains weight measurably during the weighing it should be reheated and weighed again very quickly. The weights to balance the crucible approximately are in this case placed on the pan before the crucible is removed from the desiccator.

A platinum crucible should remain in the desiccator ten to fifteen minutes and a porcelain crucible twenty to twenty-five minutes before weighing.

When, as in the case of barium sulphate, the precipitate is not hygroscopic a desiccator should not be used. Accurate results are more readily obtained when the crucible, both before and after the precipitate is in it, can be cooled in the open. The time required for cooling is about half as long as in a desiccator.

The supports of beam and pans must be lowered gently to avoid injury to the knife edges.

The balance case must never be left open or with the beam unsupported, and the rider must be removed from the beam. When not in use the balance should be protected from fumes and dust by the regular use of a rubber covering placed over the case.

Be careful to avoid spilling the substance to be weighed on the pans or on the floor of the balance case. If this happens, remove at once by dusting carefully with a camel's-hair brush.

Liquids must be weighed in closed weighing bottles, and solids in weighing bottles, watch-glasses, or aluminum pans.

From time to time determine the true zero point of the scale by noting the point at which the rider must be placed in order to make the empty pans balance.

The point of balance in weighing is always determined by

adjusting the weights until the pointer swings equal distances to right and left of zero on the scale. It is never determined by adjusting the loads until the pointer remains stationary when released.

VOLUMETRIC METHODS.— *Burettes.*— With colorless solutions read the bottom of the meniscus. With colored solutions such as permanganate read the top of the column of fluid. To avoid errors through parallax keep the eye at the level of the top of the column of fluid. Solutions should be within $10°$ of standard temperature engraved on the volumetric apparatus when they are measured.

Keep burettes covered with caps when not in use, and always use a funnel to fill the burette, but remove it before adjusting the level of the fluid.

As the absolute error of reading remains constant the percentage of error is greater the smaller the amount of solution used, and the quantity of liquid employed should therefore preferably not be less than 20 to 25 c.c.

Burettes and pipettes must be free from grease or the solution will not moisten the surface of the glass evenly and will collect in droplets. To clean burettes and pipettes, use a mixture of 100 c.c. concentrated sulphuric acid to which 1 gram of potassium bichromate has been added. Fill the burettes with the mixture, allow to stand for some time, and then wash carefully in water. Finish by rinsing with distilled water.

Burettes may be cleaned quickly by scrubbing them with a soapy burette brush.

The opening of the pipette should be small, so that from fifteen to thirty seconds will be required to empty the instrument.

The finger used in closing the upper end of the pipette must be sufficiently moist to be soft, but must not be wet, in order to control the flow of liquid properly.

If corrosive fluids are to be measured with a pipette, always slip a piece of absorbent cotton into the stem to

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avoid the possibility of getting fluid into the mouth. Never draw ammonia into a pipette with the mouth. Pipettes are ordinarily made to discharge the amount indicated by the graduation, and measuring flasks to contain it. In both instances the bottom of the meniscus is the point read.

CALIBRATION OF VOLUMETRIC APPARATUS.—All apparatus used for accurate work must be calibrated. Except for that checked by the Bureau of Standards no commercial apparatus is entirely reliable, errors exceeding 1 per cent. being frequent.

Flasks are calibrated by weighing into them the amount of water necessary to make the desired volume at the temperature of calibration. The following table shows the weights of water over the range of ordinary room temperature which fill a volume of 1 c.c. The figures are corrected for the weights of air displaced by the water and by the brass weights. The water should be weighed to 1 part per 1000, *i. e.*, the water held by a 10 c.c. flask is weighed to 0.010 gm., but a liter flask is sufficiently accurate if within 1 gm.

Temperature, C°.	Weight of 1 c.c. of water in gm.	Volume of 1 gm. of water in c.c.
15	0.9981	1.0019
16	0.9979	1.0021
17	0.9977	1.0023
18	0.9976	1.0024
19	0.9974	1.0026
20	0.9972	1.0028
21	0.9970	1.0030
22	0.9967	1.0033
23	0.9965	1.0035
24	0.9963	1.0037
25	0.9960	1.0040
26	0.9958	1.0042
27	0.9955	1.0045
28	0.9952	1.0048
29	0.9949	1.0051

Burettes are calibrated by allowing them to deliver distilled water, 2 c.c. at a time, into a bottle and weighing the water. The bottle should contain a layer of paraffin oil a few millimeters thick. This floats on top of the water and prevents loss by evaporation. It is not necessary, therefore, to stopper the bottle. The grams of water noted are multiplied by the volume of 1 gram at the temperature observed. If the results do not agree to within 0.05 c.c. (for a 25 to 50 c.c. burette) with the readings the corrections should be plotted on a sheet of coördinate paper, which is hung by the burette for reference.

The following figures for the first 10 c.c. of a burette serve as an example.

Burette reading. c.c.	Weight of water delivered at 22° C. gm.	Volume of water delivered (= wt. × 1.0033). c.c.	Correction to burette. c.c.
2	2.000	2.006	+0.01
4	4.002	4.008	+0.01
6	6.009	6.017	+0.02
8	8.020	8.050	+0.05
10	10.020	10.050	+0.05

The + signs indicate that each correction is to be *added* to the observed reading in order to give the actual volume of liquid delivered. Were the volumes of water delivered at any points less than indicated by the burette readings the corresponding corrections would be indicated by minus signs.

Pipettes are calibrated by filling to the mark with distilled water and discharging into a weighing bottle. The water delivered should be weighed to within 1 part per 1000. If the mark is not accurate a correct one should be made with a wax pencil, subsequently etched in (see below) and indicated by an arrow.

Pipettes may be calibrated for either *drainage* or *blow-out* delivery. For drainage the tip of the pipette is allowed to

touch the side of the receiving vessel as delivery is finished and a drop of liquid remains in the tip. For blow-out delivery this final drop is expelled. The expulsion is conveniently effected by closing the upper end of the pipette with the right forefinger and warming the bulb by gripping it with the left palm. The expansion of air in the bulb forces the last drop of water out of the tip. For all pipettes below 5 c.c. blow-out delivery should be used. Unless all of the pipettes in the laboratory are calibrated for either blow-out or drainage delivery each pipette must be etched "Blow-out" or "Drainage."

ETCHING GLASS APPARATUS.—To etch new calibration marks, corrections, etc., on glass apparatus, warm the apparatus by passing it through a flame a few times and paint it with a thin, even layer of melted paraffin. The layer may be kept even by rotating the vessel as it cools. The desired marks are then made through the paraffin with a sharp point. A sharp pencil serves very well. To make a mark around the stem of a pipette or neck of a flask the sharp point is fixed and the mark made by rotating the pipette or flask neck against it. The marks are then etched in by painting them with hydrofluoric acid. A brush mounted on a long handle should be used, and contact of the acid with the fingers avoided or necrosis may result later. After the acid has acted on the glass for a minute or two it is washed off under a tap. The vessel is then warmed until the paraffin melts, and the latter is wiped off with a towel. In case one desires to make the marks stand out more sharply they may be filled with the colored wax of a wax pencil or with black asphalt paint. The coloring substance is smeared on warm and then wiped off, only that caught in the etched lines remaining.

PYCNOMETERS.—The volume held by a pycnometer is determined by weighing the water which it contains and multiplying this weight in grams by the volume of 1 gram

of water at the observed temperature (*i. e.*, by 1.0028 if temperature is 20°). The density of any liquid is determined by weighing the pycnometer full of the liquid and dividing by the volume. The density is thus determined in absolute units, the density of water at 4° being taken as 1.

Urine specific gravities usually refer to water at the same temperature (rather than water at 4°) as unity. In this case the weight of urine held by the pycnometer is divided by the weight of water held at the same temperature. Such a specific gravity, if taken at 25°, for example, is indicated by D_{25}^{25}. If water at 4° is taken as the unit, as is done in tables of densities of acids, alcohol, etc., the density is indicated by D_4^{25}.

Any vessel into which a volume of liquid may be accurately measured may serve as a pycnometer. A pipette calibrated to *contain* 1, 2 or more cubic centimeters may be used.

INDICATORS.—According to the strength of their basic or acid properties, different indicators change color at greater or less hydrogen ion concentration. The table on pages 74 and 75 indicates the properties of some of the more useful indicators, and should be consulted in the selection and preparation of an indicator for any particular purpose.

The expression pH was introduced by Sörensen to indicate the negative power of the hydrogen ion concentration. Thus for $\frac{N}{10}$ hydrogen ion, pH = 1, for $\frac{N}{100}$ pH = 2, for $\frac{N}{1000}$ pH = 3, etc. At the neutral point pH = 7. Values of pH *greater than* 7 are on the *alkaline* side, values *less than* 7 on the *acid* side of neutrality.

Only indicators which change color well on the acid side, viz., at pH less than 5, can be used for titration of alkali in the presence of carbonic acid. Likewise for titration of ammonia the change must be on the acid side, although pH may be as high as 5 or 6. For titration of weak organic acids, on the other hand, the indicator must change at pH = 8 or more. At an end-point pH = 3 to 4, weak organic

TABLE OF INDICATORS.

Indicator. Chemical name.	Common name.	Color. Acid.	Color. Alkaline.	pH at which color changes.	Form in which indicator is prepared for use.	Special use in titration.
*Thymol-sulphon-phthalein (acid range)	Thymol blue	Red	Yellow	1.2–2.8	Water sol. Na salt 0.1 per cent.	
Dimethyl-amino-azo-benzene	.....	Red	Yellow	3–4	Alcohol sol. 0.5 per cent.	Titration of mineral acids in presence of organic acids (HCl in stomach contents).
Dimethyl-amino-azo-benzene-sulphonate	Methyl orange	Red	Yellow	3–5	0.1 per cent. sol. in 50 per cent. alcohol.	Titration of mineral acids in presence of carbonic acid.
*Tetra-brom-phenol-sulphon-phthalein	Brown phenol blue	Yellow	Blue	3.0–4.6	Water sol. Na salt 0.1 per cent.	Titration of mineral acids in presence of carbonic acid.
Diazo compound of benzidine and naphthionic acid	Congo	Blue	Red	4–5	Alcohol sol. 0.5 per cent.	Titration of weak bases (ammonia) with mineral acid.
*Ortho-carboxy-benzene-azo-dimethyl-aniline	Methyl red	Red	Yellow	4.8–6.4	Alcohol sol. 0.05 per cent.	Titration of weak bases (ammonia) with mineral acid.
Sodium-alizarin-sulphonate	Alizarin red	Red	Yellow	5–6	Water sol. Na salt 1.0 per cent.	Titration of weak bases (ammonia) with mineral acid.
*Di-brom-ortho-cresol-sulphon-phthalein	Brom-cresol purple	Yellow	Purple	5.2–6.8	Water sol. Na salt 0.1 per cent.	

	Litmus	Red	Blue	About 7	Paper	
*Di-brom-thymol-sulphon-phthalein	Brom-thymol blue	Yellow	Blue	6.0–7.6	Water sol. Na salt 0.1 per cent.	Titration of mineral acids with alkali, and of mineral acids with mineral alkali in presence of weak organic base (benzidine sulphate method).
*Phenol-sulphon-phthalein	Phenol red	Yellow	Red	6.8–8.4	Water sol. Na salt 0.05 per cent.	
*Ortho-cresol-sulphon-phthalein	Cresol red	Yellow	Red	7.2–8.8	Water sol. Na salt 0.05 per cent.	
Phenol-phthalein	.....	Color-less	Red	8–9	50 per cent. alcohol, 1.0 per cent. solution.	
*Thymol-sulphon-phthalein (alk. range)	Thymol blue	Yellow	Blue	8.0–9.6	Water sol. Na salt 0.1 per cent.	Titration of mineral acids with alkali, and of mineral acids with mineral alkali in presence of weak organic base (benzidine sulphate method).
*Ortho-cresol-phthalein	Cresol phthalein	Color-less	Red	8.2–9.8	Alcohol sol. 0.05 per cent.	

* Indicators starred are those recommended by Clark and Lubs for colorimetric determination of the p_H of biological fluids. These indicators are but little affected by protein. Journal of Bacteriology, 1917, vol. ii, p. 33.

acids exert but little influence on titration results, consequently dimethyl-amino-azo-benzene can be used to titrate HCl with approximate accuracy even in the presence of some acetic or lactic acid.

STANDARD SOLUTIONS.—As ordinarily employed the term "normal solution of an acid" is used to indicate a solution of such a concentration as to contain 1 gram of replaceable hydrogen per liter, *e. g.*, normal HCl solution contains 1 gram molecule of HCl (36.458 gm.) per liter. But normal H_2SO_4 contains only 0.5 gram molecule, because H_2SO_4 has two replaceable H atoms. Similarly the concentration of a normal solution of an alkali is such that the amount of the base present corresponds to the amount of acid in the normal acid solution, *e. g.*, normal NaOH contains 40.01 gm. of NaOH per liter, this being its molecular weight, but of sodium carbonate only one-half the molecular weight would be taken, as one molecule of this base is capable of replacing two atoms of acid hydrogen.

Since oxygen (atomic weight 16) is bivalent, oxidizing solutions are made on the basis of 8 grams of available oxygen per liter. For example in potassium permanganate ($KMnO_4$, molecular weight, 158.15) five atoms of oxygen of the eight contained in two molecules of the salt are available for oxidizing purposes, *i. e.*, 80 parts by weight; therefore, one-tenth of two gram molecules (2 x 158.15), or 31.63 grams, must be taken for the normal solution and 3.163 for the decinormal solution.

PREPARATION OF $\frac{N}{10}$ SULPHURIC ACID.—Take of Merck's anhydrous reagent sodium carbonate about 7 or 8 grams and ignite gently in a previously weighed platinum crucible, not allowing the heating to exceed a dull red in order to avoid the conversion of small amounts of carbonate into hydroxide, which may take place at high temperatures. The object of the heating is to dehydrate the salt completely and to decompose any bicarbonate which may be present.

Allow to cool in the desiccator, and on the balance quickly remove enough to leave exactly 5.3 grams in the crucible.

Dissolve this in hot distilled water, rinsing the crucible well. Allow the fluid to cool and then make up to exactly 1 liter.

Take 6.2 c.c. of chemically pure H_2SO_4 (sp. gr. 1.84), dilute with four or five volumes of distilled water and allow to cool.

Transfer to a 2-liter cylinder and add distilled water to the mark. Shake well and fill a 50 c.c. burette with the acid and another with the sodium carbonate solution, in each case rinsing out the burette first with some of the solution.

Measure 50 c.c. of the carbonate into a beaker, add a few drops of methyl orange, and titrate with the acid until a pink tinge is noticeable and the addition of a drop of alkali restores the neutral color. Repeat until duplicates are obtained differing by not more than 0.1 c.c.

The acid will be found to be too strong and the amount of water for dilution is poured into the cylinder. The amount of water is calculated as follows: for example,

49.5 c.c. of acid neutralizes 50 c.c. of the alkali; then

$$C = \frac{N.d}{n} \qquad C = \frac{1900 \times .5}{49.5} \qquad C = 19.2$$

C = Number of cubic centimeters of water to be added.
N = C.c. of solution remaining.
d = Difference between number of cubic centimeters theoretically required and number of cubic centimeters actually used in titration.
n = Number of cubic centimeters used in titration.

Repeat the titration and correction until the two solutions are adjusted so as to balance evenly. If the acid is too weak it is simple to make it a little too strong again by adding a drop or two of concentrated acid and then diluting to the required degree.

PREPARATION OF $\frac{N}{10}$ HYDROCHLORIC ACID.—HULETT AND BONNER.—This extremely accurate method depends on the fact that when hydrochloric acid solution is distilled at

760 mm. pressure the concentration of HCl in the undistilled portion approaches 20.24 per cent. When this is reached further distillation yields a distillate also containing HCl of this concentration. To prepare stock HCl solution for standards, add to concentrated HCl (sp. gr. 1.2) an equal volume of water and bring to a density at 25° of 1.096 (see "Pycnometers") by addition of more water or concentrated HCl. Distill off three-quarters of this mixture. The remaining one-quarter has within 1 part in 10,000 the following composition:

Barometric pressure at distillation.	Per cent. HCl.	Grams of solution to make 1 liter of N/10 HCl.
770	20.218	18.04
760	20.242	18.02
750	20.266	18.00
740	20.290	17.97
730	20.314	17.95

PREPARATION OF $\frac{N}{10}$ SODIUM HYDROXIDE.—Dissolve 100 grams of c. p. sodium hydroxide in 100 c.c. of water and let the solution stand. Sodium carbonate is insoluble in such a concentrated NaOH solution, and whatever carbonate is present settles to the bottom as sediment. For each liter of $\frac{N}{10}$ sodium hydroxide remove with a graduated pipette 5.7 c.c. of the clear solution and dilute to 1000 c.c. Standardize against $\frac{N}{10}$ acid as described on the preceding page.

SPECIAL DETERMINATIONS.

DETERMINATION OF THE NITROGEN PARTITION IN THE URINE.—The chief nitrogenous constituents of the urine are urea, uric acid, ammonia, and creatinin. Under normal conditions these together make up about 95 per cent. of the total nitrogen of the urine, the balance being composed of small amounts of creatin, amino-acids, hippuric acid, allantoin, purine bases, pigments, etc. As carried out for clinical purposes a complete nitrogen partition includes the deter-

mination of the total N and of the percentages of the total N in the forms of urea, uric acid, ammonia, and creatinin. Ordinarily the factors of greatest clinical interest are the total N, the urea N, and the ammonia N.

Accurately collected twenty-four-hour specimens are essential for quantitative determinations on the urine. As a preservative about 50 c.c. of toluol per liter of urine may be used, and the receptacle should be kept in a cold place. *The toluol and urine should be shaken together*, so that the urine is saturated with toluol. *No other antiseptic than toluol should be used* for routine, as most of the others in common use interfere with some of the determinations described in this pamphlet. The calculations ordinarily may be simplified by diluting the entire specimen with water to the nearest round number of cubic centimeters, as 1000, 1500, etc.

TOTAL NITROGEN DETERMINATION IN URINE.—KJELDAHL METHOD.—*Principle.*—By digestion with concentrated sulphuric acid all nitrogenous constituents in the organs, fluids, or excretions of the body are converted into ammonia and the organic bodies decomposed. Potassium sulphate facilitates the reaction by raising the boiling-point of the acid, and copper sulphate accelerates it catalytically. The ammonia is bound by the acid and forms ammonium sulphate. This is later liberated by the addition of alkali and is distilled into a measured amount of decinormal acid. The amount of acid so neutralized may then be determined by titration of the rest with decinormal alkali.

Reagents.—
Concentrated sulphuric acid.
Potassium sulphate.
$CuSO_4.5H_2O$ (powder).
Concentrated NaOH, specific gravity 1.50.
$\frac{N}{10}$ H_2SO_4.
$\frac{N}{10}$ NaOH.
Alizarin red or methyl red.

Pumice or small pieces of porous porcelain.

Technic.—Measure 5 c.c. of urine with a bulb pipette into a 500 c.c. Kjeldahl flask. Add 20 c.c. of concentrated H_2SO_4, 10 gm. of K_2SO_4 measured approximately in a marked tube, and about 0.2 gm. of copper sulphate. Heat over a wire gauze until the yellow color has disappeared, then for about one-half hour longer. Cool, redissolve the syrupy residue in 250 to 300 c.c. of water and add 2 or 3 drops of alizarin red, also a piece of porous porcelain or a spoonful of talcum powder to make the solution boil without bumping. When the solution, heated by mixture of H_2SO_4 and water, has cooled again, run 50 c.c. of the concentrated sodium hydroxide down the side of the flask and allow it quietly to form a layer on the bottom. Do not get alkali on the mouth of the flask or it will be difficult to make the rubber stopper stick. The flask is now connected with the distilling apparatus. The lower end of the condenser is previously connected with a calcium chloride or other bulb tube about eight inches long, the lower end of which dips into 50 c.c. of $\frac{N}{10}$ H_2SO_4 or HCl in a 500 c.c. flask or bottle. All connections being made, the alkali in the bottom of the flask is mixed with the acid by a rotary motion and the distilling flame is turned on. The heat of neutralization is so great that the flame requires but a few minutes to raise the solution to the boiling-point. Boiling is continued until the drops falling from the condenser are no longer alkaline to litmus. In practice, boiling is usually continued until the solution is so concentrated that it begins to bump, at which point the distillation of ammonia is always complete. The excess acid in the distillate is titrated back with $\frac{N}{10}$ NaOH.

Protective goggles should invariably be worn while the analyst is doing Kjeldahl analyses, as the flasks sometimes break and scatter alkaline liquid. Each c.c. of $\frac{N}{10}$ acid neutralized by the ammonia indicates 1.4 mg. of nitrogen.

UREA DETERMINATION IN URINE.—1. BENEDICT.—*Principle.*—In the presence of potassium bisulphate and in the

absence of water urea is hydrolyzed at 160° C. almost quantitatively into ammonia and carbon dioxide in about an hour. Addition of zinc sulphate makes the decomposition of the urea complete. Under these conditions uric acid and creatinin are not hydrolyzed. The potassium bisulphate, having a replaceable H atom, retains the ammonia, which is later liberated by the addition of alkali and distilled into a known amount of standard acid.

Reagents.—

Potassium bisulphate.

Zinc sulphate.

Powdered talcum.

10 per cent. NaOH.

$\frac{N}{10}$ H_2SO_4.

$\frac{N}{10}$ NaOH.

Alizarin red or methyl red.

Technic.—Place 5 c.c. of urine (which must not contain sugar) in a wide test-tube (preferably provided with an ampulla near the top), add 3 grams of potassium bisulphate, 1.5 grams of zinc sulphate, and a small quantity of talcum. Evaporate almost to dryness, either cautiously over a flame or by immersion in a bath of sulphuric acid or paraffin at about 130° C. If the mixture foams badly add a fragment of paraffin the size of a pea. After dryness has been reached, heat the tube in the bath at 162° to 165° C. for one hour. The tube is removed and the contents washed into a 500 c.c. Kjeldahl distillation flask and diluted to about 300 c.c. Add a little talcum or pumice and 20 c.c. of 10 per cent. NaOH solution. Distil into 50 c.c. of $\frac{N}{10}$ H_2SO_4 and titrate as in the Kjeldahl determination (*q. v.*).

Result.—Each cubic centimeter of $\frac{N}{10}$ NH_3 equals 0.0014 gram of N, or 3 mg. of urea. Before calculating the amount of urea N and urea the N of the preformed ammonia must be determined and deducted.

2. MARSHALL. — VAN SLYKE. — CULLEN. — *Principle.* — Urease, an enzyme obtained from various legumes, converts

urea into ammonium carbonate. It acts at room temperature and is most active in a perfectly neutral solution. It decomposes nothing but urea and is not interfered with by the presence of glucose. The urea in the urine is converted into ammonium carbonate by the enzyme, the ammonia in the latter is then liberated by the addition of alkali, and is aspirated into a measured amount of standard acid. The ammonium carbonate formed would by its alkaline reaction retard the action of the enzyme, but the development of alkalinity is prevented by the presence of potassium acid phosphate (KH_2PO_4). An excess of this, however, interferes with the action of enzyme, so that it is advisable to use the minimum amount of phosphate that will keep the reaction sufficiently near the neutral point.

Reagents.—

Soy bean or Jack bean urease.[1]

[1] Either in tablets of 0.1 gm. each, containing also 5 per cent. of dipotassium phosphate to aid in preserving the activity of the solution, or in solution freshly made as follows: solid enzyme, one part by weight in ten of water. First mix to a paste with a little water, then add the rest of the water in portions, forming a cloudy solution.

Standardization of Enzyme.—Urease as supplied by the manufacturers varies somewhat in activity, and fresh supplies must always be tested in the following way:

Test solution:

Dibasic potassium phosphate (K_2HPO_4) . . .	43 grams
Diacid potassium phosphate (KH_2PO_4) . . .	34 grams
Urea	60 grams
Water to make	1000 c.c.

Place 5 c.c. of this solution in the tube of the aëration apparatus and bring to exactly 20° C. in a water bath. Add 1 c.c. of 10 per cent. enzyme solution also at 20° C. and allow to stand at this temperature for exactly fifteen minutes. Add 6 or 7 grams of potassium carbonate and aërate as usual into 30 c.c. of N/10 acid. The result is expressed in the number of cubic centimeters of N/10 acid neutralized. This should be at least 8.

Neutralizing phosphate solution containing 5 grams of KH_2PO_4 and 1 gram of Na_2HPO_4 per liter.

$\frac{N}{50}$ H_2SO_4.

$\frac{N}{50}$ NaOH.

Potassium carbonate, dry or in solution, containing 90 grams to 100 c.c. water.

Caprylic alcohol.[1]

Alizarin or methyl red.

Technic. — One-half c.c. of urine[2] is measured into the bottom of tube *A* (see Fig. 1). Exactly 5 c.c. of the neutralizing phosphate solution is then run in from a burette and 1 c.c. of a 10 per cent. solution of urease is added. The solutions in the tube are well mixed, 2 drops of caprylic alcohol to prevent subsequent foaming is added, and the stopper bearing the aërating tubes shown in the figure is put into place. Twenty minutes at a room temperature of 15° or fifteen minutes at 20° or above are allowed for complete decomposition of urea. No harm is done if the solutions are allowed to stand longer, but *the time must not be cut shorter unless more enzyme is used.* While the enzyme is acting one measures

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[1] An efficient antifoaming mixture which is much cheaper than caprylic alcohol may be made as follows:

Diphenyl oxide . . . . 60 c.c.
Amyl alcohol . . . . 40 c.c.

Caprylic alcohol may be made by mixing castor oil with an equal volume of concentrated sodium hydroxide solution, letting the mixture stand overnight, and distilling from an oil bath, the temperature of which is raised gradually to 250°. A liter of castor oil yields 200 c.c. of caprylic alcohol.

[2] An Ostwald pipette is used, the stem of which is a heavy walled capillary tube of only 1 mm. bore. The pipette, which should deliver in about twenty seconds, is calibrated by weight for blow-out delivery, and permits measurement with an accuracy of 0.001 c.c. The pipette is allowed to deliver with its tip against the lower part of the test-tube wall until the bulb is emptied; the remainder of the contents is then blown out.

25 c.c. of $\frac{N}{50}$ hydrochloric or sulphuric acid into tube *B* and connects the tubes as shown in the figure. After the time for complete decomposition of urea has elapsed the air current is passed for a half minute in order to sweep over into
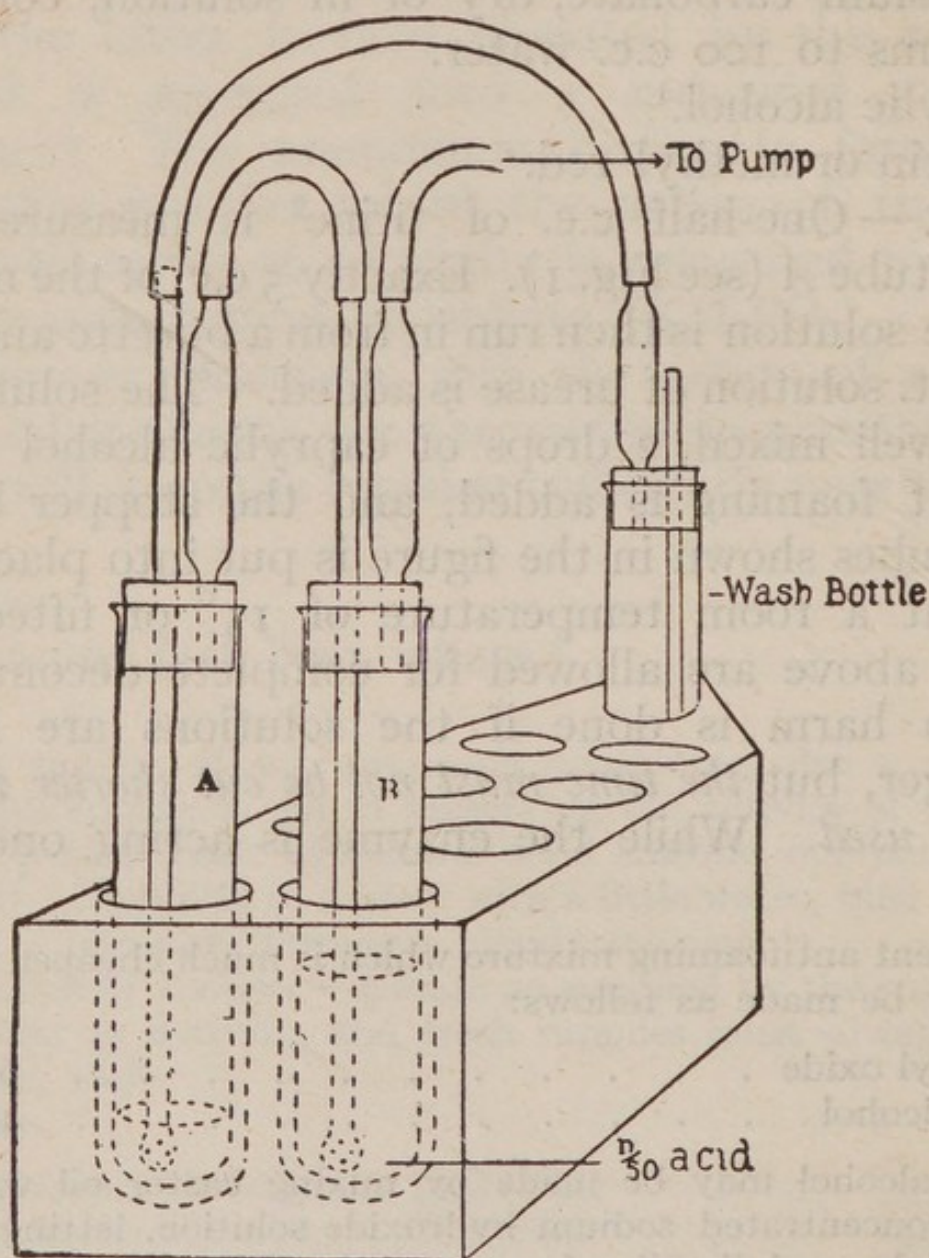

FIG. 1.—Apparatus for determining urea content by means of urease.

B a small amount of ammonia which has escaped into the air space of *A* during the decomposition. *A* is now opened and 4 or 5 grams of potassium carbonate, measured roughly from a spoon, or 10 c.c. of saturated potassium carbonate solution is poured in (in order to assure most rapid

removal of ammonia by air current it is necessary to have the solution at least half-saturated with carbonate). The air current is now passed through the tubes until all the ammonia has been driven over into the acid in *B*. The time required for this depends on the speed of the air current. With a rapid pump or house vacuum it is possible to aërate completely in five minutes while a slow pump may require an hour. The time required for complete aëration is determined for the particular vacuum used by trial, and a safe margin allowed in the determinations. When the aëration is finished the excess acid in *B* is titrated with $\frac{N}{50}$ NaOH.

The operations are concisely summarized in the following diagrammatic form:

1. Measure into *A*
$$\begin{cases} \text{0.5 c.c. urine.} \\ \text{5.0 c.c. phosphate solution.} \\ \text{1.0 c.c. 10 per cent. urease.} \\ \text{2 drops caprylic alcohol.} \end{cases}$$

Place stopper as shown in Fig. 1 and let stand fifteen minutes.

2. Meanwhile measure into *B*
$$\begin{cases} \text{25 c.c. } \frac{N}{50} \text{ acid.} \\ \text{1 drop 1 per cent. sodium alizarin} \\ \quad \text{sulphonate indicator or methyl} \\ \quad \text{red.} \\ \text{1 drop caprylic alcohol.} \end{cases}$$

3. After fifteen minutes' standing, aërate one-half minute. Then open *A* and add 4 or 5 grams K_2CO_3 or 10 c.c. of saturated K_2CO_3 solution.

4. Aërate all NH_3 from *A* over into *B*.

5. Titrate excess acid in *B* with $\frac{N}{50}$ NaOH.

6. Calculate: $0.056 \times$ c.c. $\frac{N}{50}$ acid $=$ grams urea $+$ ammonia nitrogen per 100 c.c. urine.

Each cubic centimeter of $\frac{N}{50}$ NH_3 equals 0.00028 of N, and the urea nitrogen multiplied by the factor 2.14 gives the amount of urea.

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The urea nitrogen on an average diet is 10 to 20 grams per twenty-four hours, and equals about 85 to 90 per cent. of the total N.

AMMONIA IN URINE.—In order to determine the *ammonia nitrogen* alone one measures 5 c.c. of urine into *A*, adds the carbonate at once, and aërates as described above. The acid neutralized is multiplied in this case by the factor 0.0056, to give grams of ammonia nitrogen per 100 c.c. urine. No extra time is required for the ammonia determination performed in connection with urea estimation, as one merely aërates the extra pair of tubes in series with the same air current used for the ammonia + urea determination. As a matter of fact, one can conveniently run as many as eight pairs of tubes on the same air current, taking the precaution at the end of the aëration to disconnect the series in the middle first in order to prevent back suction.

In normal urine the average amount of ammonia nitrogen is about 0.7 gram per twenty-four hours, or 3 to 6 per cent. of the total nitrogen. In terms of $\frac{N}{10}$ acid which the ammonia can neutralize the usual excretion is 400 to 600 c.c.

Points to be Noted in Determination of Ammonia by Aëration (in Ammonia, Urea or Micro-Kjeldahl Estimation).—A slow current of air should be used during the first two minutes, as the ammonia may otherwise be driven off so rapidly at the start that some escapes absorption in the receiver. Afterward one may use as rapid a current as the apparatus will stand.

In order to drive off all the ammonia in an apparatus set up exactly as described above, 75 liters of air is sufficient. The length of time required to complete the aëration depends on the rate at which this volume of air is drawn through.

In order to assure complete absorption of ammonia with a current as rapid as 5 liters of air per minute the column of acid in the receiving tube must be at least 50 mm. high. The use of a wider receiver with less depth of acid is not permissible.

In order to drive off all the ammonia with 75 liters of air, the solution from which it is driven must contain at least 1 gram of potassium carbonate for each 2 c.c. of solution. More does not accelerate the aëration, but less retards it.

The inlet tube for air in A must always reach to the bottom of the solution.

Standard alkali solutions increase in titratable alkali on standing in contact with glass, as they dissolve more alkali from it. The effect is most marked with dilute solutions, such as $\frac{N}{50}$ and $\frac{N}{100}$ NaOH. These should be kept in paraffin-lined bottles, and portions which stand more than a day in burettes should be discarded.

APPROXIMATE TITRATION OF AMMONIA (AND AMINO-ACIDS) WITH FORMALDEHYDE (MALFATTI-SOERENSEN).—*Principle.*—Formaldehyde condenses in neutral solution with ammonium salts, forming hexamethylene tetramine and setting free the acid which had been combined with the ammonia.

$$4NH_4Cl + 6CH_2O = N_4(CH_2)_6 + 6H_2O + 4HCl.$$
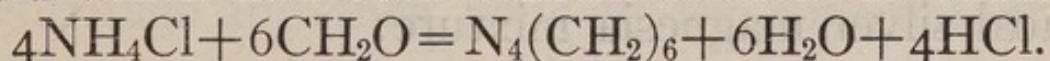
In the presence of a sufficient excess of formaldehyde the reaction is quantitative. The result is that addition of a neutral formaldehyde solution to a neutral ammonium salt solution sets free an amount of acid equivalent to the NH_4 present. The acid thus freed is titrated as a measure of the ammonia.

Amines and amino-acids also condense with formaldehyde, and are consequently titrated like ammonia. As small amounts of these substances are present in urine, the results of the titration are only approximate for ammonia, but are sufficiently accurate to show pathological increases. The chief merit of this method is its simplicity, which makes an approximate urinary ammonia determinable with no other apparatus than a burette.

Reagents.—Saturated solution of potassium oxalate. Forty per cent. solution of commercial formalin. To each solution add N NaOH to a faint pink coloration.

Technique.—To 25 c.c. of urine add 15 c.c. of oxalate solution and 0.5 c.c. of 1 per cent. phenolphthalein solution. Titrate to a faint pink with 0.1 N NaOH. Then add 5 c.c. of the formalin and again titrate to the same color. Each cubic centimeter of 0.1 N NaOH used in the *second* titration represents 1 c.c. of 0.1 N ammonia, equivalent to 1.7 mg. of ammonia or 1.4 mg. of ammonia nitrogen.

URIC ACID DETERMINATION IN URINE.—FOLIN COLORIMETRIC METHOD.—*Principle.*—The uric acid is precipitated by the use of ammoniacal silver solution, the supernatant fluid is discarded, and the precipitate is redissolved with potassium cyanide. The solution is then treated with the phosphotungstic reagent, which gives a blue color proportional in depth to the amount of uric acid present; this is compared in the colorimeter with the color produced by a standard solution of uric acid under similar conditions.

Reagents.—

Ammoniacal silver magnesia mixture:

3 per cent. silver lactate	70 c.c.
Magnesia mixture[1]	30 c.c.

5 per cent. potassium cyanide solution.
Uric acid reagent:

Sodium tungstate	100 grams
Phosphoric acid, 85 per cent.	80 c.c.
Water	750 c.c.

Boil for one hour in a flask with a funnel in the neck, cool and dilute to 1 liter.

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[1] Magnesia mixture is made as follows:

Crystallized magnesium sulphate	17.5 grams
Ammonium chloride	35.0 grams
Concentrated ammonia	60.0 c.c.
Water to make	200.0 c.c.

20 per cent. sodium carbonate solution.
Uric acid standard solution:

Disodium hydrogen phosphate (crystals)	9 grams
Sodium dihydrogen phosphate (crystals)	1 gram
Hot water	200 to 300 c.c.

If not clear, filter and make up to about 500 c.c. While still hot pour into a liter flask containing 200 mg. of uric acid suspended in a little water. Shake until the uric acid is dissolved and allow to cool. Add exactly 1.4 c.c. of glacial acetic acid, shake, dilute to the mark, and add about 5 c.c. of chloroform to prevent the growth of moulds. Five cubic centimeters of this solution contain 1 mg. of uric acid.

Technic.— From 2 to 4 c.c. of urine, according to the amount of uric acid expected, is measured into a centrifuge tube, diluted to about 5 c.c. with water, and treated with 15 to 20 drops of ammoniacal silver solution. Mix thoroughly with a small stirring rod and allow to stand for ten minutes. Centrifuge and then pour off the supernatant fluid as completely as possible, inverting the tube over a piece of filter paper. Aspirate the ammonia vapors from the tube by suction with the filter pump and then add to the residue in the tube 2 drops of 5 per cent. potassium cyanide solution. Stir well, add 10 to 15 drops of water and stir again. Add 2 c.c. of uric acid reagent, stir and add 10 c.c. of 20 per cent. sodium carbonate solution. Allow to stand about a half-minute and wash quantitatively into a 25 or 50 c.c. volumetric flask. According to the depth of color as compared with the standard, dilute to 25 or 50 c.c. The standard is prepared simultaneously by treating 5 c.c. of the standard uric acid solution in a 50 c.c. volumetric flask with two drops of potassium cyanide solution, 2 c.c. of uric acid reagent, and 10 c.c. of 20 per cent. sodium carbonate solution. Dilute to 50 c.c. at the end of a half-minute and compare the two in the Duboscq colorimeter.

Result.—The calculation is made as follows:

$$X = \frac{S}{R} \times \frac{Rd}{Sd} \times \frac{Y}{W}$$

X = Mg. of uric acid in twenty-four-hour amount of urine.

S = Reading of standard.

R = Reading of unknown.

Rd = Dilution of unknown.

Sd = Dilution of standard.

Y = Twenty-four-hour volume of urine in cubic centimeters.

W = Amount of urine in c.c. taken for determination.

1 mg. of uric acid equals 0.333 mg. of N. The amount of uric acid usually present is 0.2 to 1.5 gm. in twenty-four hours, equivalent to 0.5 to 2.5 per cent. of the total N.

Titration Method.—*Principle.*—The uric acid is precipitated as ammonium urate, which is then decomposed by the addition of H_2SO_4, and the liberated uric acid is titrated with $\frac{N}{20}$ potassium permanganate solution. To remove the mucoid substances always present, which would interfere with the subsequent operations, the urine is first treated with a reagent containing ammonium sulphate and uranium acetate. The resulting precipitate of uranium phosphate carries down the mucoid body. Ammonia is then added to alkalinize the solution and cause the precipitation of the ammonium urate.

Reagents.—

Solution of uranium acetate is made as follows:

Uranium acetate	5 grams
Ammonium sulphate	500 grams
Acetic acid (10 per cent.)	60 c.c.
Water	650 c.c.

Strong ammonia.

Ammonium sulphate solution, 10 per cent.

$\frac{N}{20}$ potassium permanganate solution.

Strong sulphuric acid.

Technique.—Into a tall beaker or c linder measure 200 c.c. of urine and 50 c.c. of the uranium reagent. Allow to stand for one-half hour and then decant, siphon, or filter off the supernatant fluid. Measure 125 c.c. (equal to 100 c.c. of urine) of this into a beaker, add 5 c.c. of strong ammonia, and set aside until the following day. Filter off the precipitate and wash with 10 per cent. ammonium sulphate solution until the filtrate is nearly or quite free from chlorides. Remove filter from funnel, open, and wash the precipitate into a beaker with the ammonium sulphate solution. Add water to make 100 c.c. and dissolve the precipitate with 15 c.c. concentrated sulphuric acid. Titrate at once with the $\frac{N}{20}$ potassium permanganate solution. The temperature of the solution during the titration should be $60°$ to $65°$ C. This is approximated by heat evolved from mixture of sulphuric acid and water, so that if titration is performed at once, no additional heat is as a rule necessary. The end-reaction is the first pink coloration extending through the entire liquid from the addition of 2 drops of permanganate solution while stirring with a glass rod, and remaining for thirty seconds.

Result.—Each cubic centimeter of permanganate solution used corresponds to 3.75 mg. of uric acid; add 3 mg. as correction due to solubility of ammonium urate.

CREATININ DETERMINATION IN URINE.—FOLIN.—*Principle.*—On adding picric acid and sodium hydroxide to a solution containing creatinin a deep red color is produced. The intensity of this in the specimen of urine is compared with that of a standard solution of potassium bichromate. Sugar and albumin do not interfere, but acetone and diacetic acid, if present, must be removed by heating.

Reagents.—

  $\frac{N}{2}$ potassium bichromate solution (24.55 gm. per liter).
  Saturated picric acid solution (about 12 gm. per liter).
  10 per cent. NaOH.

Technique.—Ten cubic centimeters of urine is measured into a flask (with a mark at 500 c.c.), 15 c.c. of picric acid

solution and 5 c.c. of sodium hydroxide are added, and the mixture is allowed to stand for five minutes. Pour a little bichromate solution into the two cylinders of the Duboscq colorimeter and set the left-hand one at the 8 mm. mark. Then make several readings in order to accustom the eye to the colors. Now dilute the urine mixture to the 500 c.c. mark and rinse out and half fill one of the cylinders with it, wipe off the glass rod, and then make several readings immediately and take the average. The reading must be made within ten minutes.

If the urine contains more than 15 mg. or less than 5 mg. of creatinin, repeat the determination with a smaller or larger amount of urine, as outside of these limits the determination is less accurate.

Calculation.— $10 \times \dfrac{8.1}{\text{Reading}} = \text{mg. creatinin.}$

1 mg. creatinin = 0.000371 gm. N.

The normal excretion of creatinin is about 20 to 30 mg. per kilo of body weight, fat persons yielding less and thin persons more. On an average diet the creatinin nitrogen equals about 3 to 5 per cent. of the total nitrogen.

CREATIN DETERMINATION IN URINE.—FOLIN.—*Principle.* —On heating creatin with dilute mineral acids it is dehydrated and its anhydride creatinin is formed. At a temperature of 117° to 120° C. the conversion is complete in fifteen minutes. This temperature is reached when the pressure is 1 kilo per square centimeter, or 14 pounds per square inch.

Technic.—Place 20 c.c. of the urine in a 500 c.c. Erlenmeyer flask, add the same amount of normal hydrochloric acid, and heat in the autoclave for twenty to thirty minutes at 117° to 120° C. Cool and make the volume up to exactly 50 c.c. with distilled water, shake thoroughly, and measure off 25 c.c. of the mixture, corresponding to 10 c.c. of the original urine. Neutralize this with 10 c.c. of normal sodium

hydroxide solution and then determine the creatinin by the method described above. From the amount of creatinin so obtained deduct the amount of creatinin determined in the unheated urine. The difference will be the creatin content of the original urine in terms of creatinin. To obtain the amount of creatinin multiply this figure by the factor 1.16. The dark color produced by the heating usually causes no difficulty, owing to the dilution necessary in making the mixture for the colorimeter.

DETERMINATION OF CHLORIDES IN URINE.—VOLHARD.— *Principle.*—The chlorides in a definite amount of urine are precipitated by a standard solution of silver nitrate in the presence of an excess of free nitric acid. The precipitate of silver chloride is filtered off and the excess of silver remaining in solution is determined in the filtrate by titrating with a standard solution of potassium sulphocyanide, using a solution of iron alum as an indicator. As soon as the sulphocyanide has combined with all the silver to form white silver sulphocyanide the deep red ferric sulphocyanide is formed and indicates the end-reaction. In this way the amount of silver solution which combined with the chlorides is ascertained. Albumin need not be removed unless present in large amount.

Reagents.—

$AgNO_3$ solution, of which 1 c.c. equals 10 mgm. NaCl.[1]

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[1] The silver nitrate solution should contain 29.059 gm. of $AgNO_3$ per liter, and the solution of potassium sulphocyanide equivalent to this requires 16.62 gm. per liter. As the latter salt is hygroscopic the exact amount cannot be weighed accurately, so weigh out about 22 gm. and dissolve in 1200 c.c. of water. Pipette 10 c.c. of the silver solution into a porcelain dish, dilute with 100 c.c. of distilled water, acidify with nitric acid and add 5 c.c. of ammonioferric alum solution. Titrate with the sulphocyanide solution to the appearance of a permanent reddish color, and then dilute the sulphocyanide solution to the proper degree according to the principles given on page 219. To check the accuracy of the silver solution, dry chemically pure sodium chloride at 120° C. and weigh out exactly 0.150 gm. Dissolve in 100 c.c. of distilled water, add a few drops of 5 per cent. potassium chromate solution, and titrate with the silver solution until an orange tint appears. Exactly 15 c.c. of the silver solution should be necessary.

Potassium sulphocyanide solution, of which 1 c.c. equals 1 c.c. of the AgNO$_3$ solution.

Saturated solution of ammonioferric alum.

Concentrated nitric acid.

Technic. — Ten cubic centimeters of urine, accurately measured with a pipette, is placed in a flask with a mark at 100 c.c.; about 50 c.c. of water, 5 c.c. of nitric acid, and 20 c.c. of the silver solution are added. The nitric acid may be measured in a graduate, but the silver solution must be measured from a pipette or burette.

The mixture is well shaken and distilled water is added to the 100 c.c. mark. The fluid is filtered through a small dry filter. Fifty cubic centimeters of filtrate is mixed with 5 c.c. of the alum solution, and is titrated with the sulphocyanide solution to the appearance of the first reddish tinge.

Calculation.—Twenty minus the number of cubic centimeters of sulphocyanide solution used equals the number of cubic centimeters of silver solution required to precipitate the chlorides. Each cubic centimeter of silver solution equals 10 mgm. of NaCl; therefore, 20 minus twice the c.c. cyanide, $\times$ 10 = milligrams of NaCl in 10 c.c. of urine. The usual amount in twenty-four hours is 10 to 15 grams.

DETERMINATION OF SULPHATES IN URINE.—Sulphur occurs in the urine in the following forms:

1. Inorganic or preformed sulphates.

2. Ethereal or conjugated sulphates in which H$_2$SO$_4$ is combined with aromatic compounds.

3. Neutral or unoxidized sulphur.

The partition of the three forms is determined as follows: The urine must be freed from albumin (see p. 104).

Barium Chloride Method.—Principle.—The sulphates in the urine are precipitated by the addition of an excess of barium chloride solution. The precipitate of barium sulphate is filtered off, washed, dried, ignited, and weighed.

Reagents.—

  Dilute HCl (1 part concentrated HCl to 4 parts H$_2$O by volume).

  Barium chloride solution, 5 per cent.

Inorganic Sulphates. — *Folin.* — *Technic.* — Into an Erlenmeyer flask place about 100 c.c. of water, 10 c.c. of dilute hydrochloric acid, and 25 c.c. of urine. If the urine is dilute take 50 c.c. instead of 25, and a correspondingly smaller amount of water. Ten cubic centimeters of barium chloride solution is added drop by drop from a pipette having a short piece of rubber tubing slipped over its upper end and provided with a screw pinchcock. The urine must not be disturbed while the barium chloride is being added. At the end of an hour or later the mixture is shaken and filtered through a weighed Gooch crucible, as described below. The precipitate is washed with at least 200 c.c. of water. The crucible is then dried, ignited, cooled, and weighed.

$$1 \text{ gram BaSO}_4 = 0.3430 \text{ gram SO}_3.$$
$$1 \text{ gram BaSO}_4 = 0.4201 \text{ gram H}_2\text{SO}_4.$$
$$1 \text{ gram BaSO}_4 = 0.1374 \text{ gram S}.$$

It is customary to report results in terms of SO$_3$

Total Sulphates.—*Folin.*—*Principle.*—The ethereal sulphates are split by boiling with HCl, and the total sulphates resulting determined just as above.

Technic. — Twenty-five cubic centimeters of urine and 20 c.c. of dilute HCl (or 50 c.c. of urine and 4 c.c. of concentrated HCl) are gently boiled for twenty to thirty minutes in an Erlenmeyer flask, into which a funnel has been placed to reduce the loss of steam. The flask is cooled for two or three minutes in running water, and the contents are diluted with cold water to about 150 c.c. The sulphate is then precipitated and weighed as above.

*Ethereal Sulphates.—Folin.—*The amount of these may be obtained by subtracting the amount of inorganic sulphates from that of the total sulphates.

*Total Sulphur.—Benedict.—*Oxidize the sulphur with Benedict's sulphur reagent as described under the Benzidine Method. The solution obtained by dissolving the residue in the porcelain dish is washed quantitatively into a small Erlenmeyer flask, diluted with cold distilled water to 100 to 150 c.c., 10 c.c. of 5 per cent. barium chloride solution is added drop by drop, and the solution is allowed to stand for about an hour. It is then shaken up and filtered as usual through a weighed Gooch filter.

To make the Gooch crucible filter, pour a suspension of asbestos fiber in water into the crucible while strong suction is being applied, so that a firm feltwork (about 2 mm. thick) is formed. The asbestos is prepared by scraping the crude material with a knife and adding the fibers to a large bulk of 5 per cent. HCl in a cylinder. Air is blown through to separate the fibers thoroughly, and the mixture is allowed to settle for a few minutes. The upper portion of the fluid containing the finer fibers is decanted and kept separate from the lower.

In making the filter the coarse material is poured on first and a little of the fine afterward. The filter is then washed by drawing distilled water through in a slow stream, is dried at 120° C., ignited, and weighed.

In igniting the barium sulphate precipitates the flame must not be applied directly to the bottom of the crucible, or mechanical losses occur. The Gooch is placed inside an ordinary porcelain crucible, and the flame of the Bunsen burner is used first gently and finally with full force.

*Benzidine Method.—Principle.—*The sulphates are precipitated as an insoluble salt of benzidine (p-diaminodiphenyl, $NH_2.C_6H_4.C_6H_4.NH_2$), and this is then dissociated by the addition of a volumetric solution of sodium hydroxide.

Reagents.—

> Benzidine solution.[1]
> Dilute HCl (1 part concentrated HCl to 4 parts H_2O by volume).
> Water saturated with benzidine sulphate.[2]
> $\frac{N}{10}$ NaOH.
> Phenolphthalein.

Inorganic Sulphates. — *Technic.* — Into a 250 c.c. Erlenmeyer flask pipette 25 c.c. of urine and add dilute HCl until it is distinctly acid to Congo red paper (usually 1 to 2 c.c.). Add 100 c.c. of the benzidine solution and allow to stand for ten minutes. The precipitate is filtered off by suction, using a small Buchner funnel or a funnel with a perforated porcelain filter plate, or with a platinum filtering cone. Do not allow the precipitate to be sucked dry. Wash the precipitate with water saturated with benzidine sulphate until the filtrate gives no reaction with Congo red (10 to 20 c.c.). Transfer precipitate and paper back to the original flask with about 50 c.c. of water and titrate hot with $\frac{N}{10}$ NaOH and phenolphthalein until pink.

[1] Rub into a paste with about 10 c.c. of H_2O 4 grams of Kahlbaum's or Merck's benzidine. Transfer this paste with 500 c.c. of H_2O to a two-liter volumetric flask, add 5 c.c. of concentrated HCl (sp. gr., 1.19) and shake until dissolved. Finally dilute to 2000 c.c. 150 c.c. of this solution, which keeps indefinitely, precipitates 0.1 gm. of H_2SO_4.

[2] *Preparation of Benzidine Sulphate.*—18.4 gm. of p-benzidine is dissolved in 50 c.c. of alcohol, filtering if solution is not complete. 5.7 gm. of concentrated (95 per cent.) sulphuric acid (sp. gr., 1.84), measured in a graduated pipette and also dissolved in 50 c.c. of warm alcohol, is slowly added to the benzidine solution with constant stirring. When the precipitate has settled, test the supernatant alcohol with moistened blue litmus paper, adding a little more alcoholic acid if necessary to ensure a slight acidity. The grayish-white benzidine sulphate is then filtered on a Buchner funnel, using suction, washed with alcohol several times, once with ether, and dried on a water-bath. If the precipitate appears to be at all lumpy it is best to grind it thoroughly in a porcelain mortar before filtering and washing. Yield, 28 gm.

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Result.—Multiply the number of c.c. of $\frac{N}{10}$ alkali used by 0.004 to obtain the number of grams of SO₃ present in 25 c.c. of urine.

Total Sulphates.—*Principle.*—The ethereal sulphates are split by boiling with HCl and the total sulphates resulting are determined just as above.

Technic.—Twenty-five c.c. of urine and 20 c.c. of dilute HCl are gently boiled in an Erlenmeyer flask for fifteen to twenty minutes. Cool, neutralize with sodium hydroxide, then make acid to Congo red with dilute HCl and proceed as above.

Ethereal Sulphates.—The amount of these is obtained by subtracting the amount of the inorganic sulphate from that of the total sulphate.

Total Sulphur.—*Reagents.*—Those used above. Benedict's solution:

Crystallized copper nitrate . . . 200 grams
Sodium or potassium chlorate . . 50 grams
Distilled water to . . . . . . 1000 c.c.

Principle.—All of the sulphur present is oxidized by heating with a reagent composed of copper nitrate and potassium chlorate. The former on heating decomposes into two vigorous oxidizing agents: nitrogen dioxide and cupric oxide, the latter forming a stable compound with the oxidized sulphur. This is dissolved in dilute hydrochloric acid, and the sulphur precipitated with benzidine as above.

Technic.—Ten c.c. of urine is measured into a small (7 to 8 cm.) porcelain evaporating dish, and 5 c.c. of the reagent added. The contents of the dish are evaporated over a free flame, which is regulated to keep the solution just below the boiling point, so that there can be no loss through spattering. When dryness is reached the flame is raised slightly until the entire residue has blackened. The flame is then turned up in two stages to the full heat of the Bunsen burner and the

contents of the dish thus *heated to redness for ten minutes after the black residue* (which first fuses) *has become dry.* This heating is to decompose the last traces of nitrate and chlorate. The flame is then removed and the dish allowed to cool more or less completely, 10 to 20 c.c. of dilute (1 to 4) HCl is next added to the residue in the dish, which is then warmed gently until the contents have completely dissolved and a perfectly clear, sparkling solution is obtained. This dissolving of the residue requires scarcely two minutes. With the aid of a stirring rod the solution is washed into a small Erlenmeyer flask, is neutralized with sodium hydroxide, made acid to Congo red with dilute HCl, and then precipitated with benzidine as above.

The total sulphur per twenty-four hours usually varies from 1 to 3.5 gm. of SO_3. Of this the inorganic sulphates are about 85 to 90 per cent.; the ethereal sulphates, 8 to 10 per cent.; and the neutral sulphur about 5 per cent., but the sulphur partition is largely modified by changes in diet.

DETERMINATION OF PHOSPHATES IN URINE.—*Principle.*— The urine is first treated with a solution of sodium acetate and glacial acetic acid in order to convert any monoacid phosphate into diacid phosphate, and also to neutralize any nitric acid that may be formed during the subsequent titration, as this, if allowed to remain free, would cause partial solution of the precipitated uranyl phosphate. The titration is then performed with a standard solution of uranium nitrate, which gives with phosphates in acetic acid solution a yellowish white precipitate of uranyl phosphate (UO_2HPO_4). As indicator potassium ferrocyanide is used, which gives a brownish-red color in the presence of an excess of uranium solution. Sugar and albumin do not interfere with the method. If the urine is deeply bile stained, it should be acidified with HCl and decolorized by the addition of a few crystals of potassium permanganate.

Reagents.—

Uranium nitrate solution of which 20 c.c. equals 0.1 gm. P_2O_5.[1]

Acetic acid solution:

Sodium acetate	100 grams
Glacial acetic acid	30 grams
Water to	1000 c.c.

Powdered potassium ferrocyanide.

Technic.—Fifty c.c. of filtered urine is treated with 5 c.c. of the acetic acid mixture. The fluid is heated to boiling and titrated with the uranium solution until a precipitate ceases to form and a drop of the mixture when removed by means of a glass rod and brought into contact with the powdered potassium ferrocyanide on a porcelain test-tablet produces a brownish-red coloration.[2] The titration is repeated until accurately corresponding duplicates are obtained, and the result is calculated as follows:

$x = 0.01\ n$, n being the number of cubic centimeters of uranium solution used.

The percentage of P_2O_5 equals x.

The usual amount of P_2O_5 on an average diet is 1 to 5 gm. in twenty-four hours.

TITRATABLE ACIDITY DETERMINATION IN URINE.—FOLIN. —*Principle.*—Determination of the acidity of the urine by direct titration is not accurate owing to the occurrence of calcium and ammonium salts in the presence of monobasic phosphates. The addition of potassium oxalate, however, overcomes these difficulties by holding in solution the phos-

[1] The uranium nitrate solution may be standardized against a phosphate solution made by dissolving exactly 1.918 gm. of KH_2PO_4 in 500 c.c. water. 50 c.c. of this solution is equal to 0.1 gm. P_2O_5. The titration is carried out as described for urine.

[2] Or the end-point may be determined by adding to the urine several drops of a 10 per cent. solution of cochineal in 25 per cent. alcohol. The red color disappears as soon as excess of uranium is present.

phates and preventing dissociation of the ammonium compounds.

Technique.—To 25 c.c. of urine add 5 gm. of powdered neutral potassium oxalate[1] and 0.5 c.c. of 1 per cent. phenolphthalein. Shake for one to two minutes and titrate at once with $\frac{N}{10}$ sodium hydroxide until a distinct permanent pink is obtained, shaking the flask during the titration.

To neutralize a twenty-four-hour amount of urine from 550 to 650 c.c. of $\frac{N}{10}$ alkali is usually required.

HYDROGEN ION CONCENTRATION OF URINE.—HENDERSON AND PALMER.—*Principle.*—Standard solutions of known hydrogen ion concentration are prepared, suitable indicators chosen and compared with the unknown solutions treated with the proper indicator.

Reagents.—

1. $\frac{N}{5}$ sodium hydroxide.

2. $\frac{M}{5}$ monopotassium phosphate, 27.2 grams KH_2PO_4 per liter. The phosphate should be recrystallized if not certainly C. P.

3. $\frac{M}{5}$ sodium acetate, 27.34 grams $NaC_2H_3O_2.3H_2O$ per liter.

4. $\frac{N}{5}$ acetic acid, standardized by titration with phenolphthalein against the $\frac{N}{5}$ NaOH.

5. Two per cent. aqueous solution of sodium alizarin sulphonate.

6. Two per cent. aqueous solution neutral red.

7. One per cent. alcoholic solution phenolphthalein.

8. Fifteen per cent. alcoholic solution of paranitrophenol.

9. Saturated solution of methyl red, 50 per cent. alcohol.

10. Toluol.

Apparatus.—250 c.c. flasks of glass yielding little or no

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[1] If the potassium oxalate is not *neutral* a saturated solution should be made, neutralized with sodium hydroxide and 15 c.c. of this solution used for each titration.

alkali to water are preferable. Medium-sized test-tubes of good, clear glass.

The hydrogen ion concentrations depend on the ratios

$$\frac{CH_3COOH}{CH_3COONa} \quad \text{and} \quad \frac{KH_2PO_4}{KNaHPO_4}$$

and the mixture required for any desired pH may be calculated by the equations

$$pH = 4.7 - \log \frac{CH_3COOH}{CH_3COONa}$$

and

$$pH = 6.8 - \log \frac{KH_2PO_4}{KNaHPO_4}$$

These standard solutions are put up in suitable bottles and a few cubic centimeters of toluol poured over each to prevent the growth of yeasts and moulds.

In each of nine flasks is placed a 10 c.c. sample of each of the standard solutions, 3 to 11, the volume is made up to 250 c.c. with distilled water, and 5 drops of the alizarin added. Care is necessary to have the concentration of indicator exactly equal in all cases. Ten c.c. of urine is next introduced into another flask and distilled water and indicator are added. The color of the diluted urine solution is next matched with one of the standard series.

If the reaction as thus measured falls between Solutions 3 and 5 a similar comparison is made, using neutral red (5 drops) as an indicator. If the reaction is more alkaline than Solution 3, undiluted urine is matched in test-tubes against undiluted standard Solutions 1 and 2, using phenolphthalein (10 drops) as indicator. In case the reaction falls between the standard solutions, rough interpolation is made. The standard series of flasks containing the alizarin will keep, if corked, for three or four days in cool weather; in warm

STANDARD SOLUTIONS OF KNOWN HYDROGEN ION CONCENTRATION

No.	Solution.	pH.[1]
No. 1.	50.00 c.c. M/5 KH_2PO_4 + 49.4 c.c. N/5 NaOH made up to 500 c.c. with H_2O	8.7[2]
No. 2.	50.00 c.c. M/5 KH_2PO_4 + 46.8 c.c. N/5 NaOH made up to 500 c.c. with H_2O	8.0[2]
No. 3.	50.00 c.c. M/5 KH_2PO_4 + 39.5 c.c. N/5 NaOH made up to 500 c.c. with H_2O	7.4[2]
No. 4.	50.00 c.c. M/5 KH_2PO_4 + 29.63 c.c. N/5 NaOH made up to 500 c.c. with H_2O	7.0[3]
No. 5.	2.25 c.c. N/5 CH_3COOH + 230.0 c.c. N/5 CH_3COONa made up to 500 c.c. with H_2O	6.7[4]
No. 6.	5.75 c.c. N/5 CH_3COOH + 230.0 c.c. N/5 CH_3COONa made up to 500 c.c. with H_2O	6.3
No. 7.	11.50 c.c. N/5 CH_3COOH + 230.0 c.c. N/5 CH_3COONa made up to 500 c.c. with H_2O	6.0
No. 8.	23.00 c.c. N/5 CH_3COOH + 230.0 c.c. N/5 CH_3COONa made up to 500 c.c. with H_2O	5.7
No. 9.	57.50 c.c. N/5 CH_3COOH + 230.0 c.c. N/5 CH_3COONa made up to 500 c.c. with H_2O	5.3
No. 10.	115.00 c.c. N/5 CH_3COOH + 230.0 c.c. N/5 CH_3COONa made up to 500 c.c. with H_2O	5.0
No. 11.	230.00 c.c. N/5 CH_3COOH + 230.0 c.c. N/5 CH_3COONa made up to 500 c.c. with H_2O	4.7

1 For definition of pH see "Indicators," pages 215, 312.
2 Phenolphthalein.
3 Neutral red and sodium alizarin sulphate.
4 This and remainder of table sodium alizarin sulphate.

weather they should be made up fresh every other day. The solutions containing neutral red and phenolphthalein are made every time their use is required. Sodium alizarin sulphonate is unreliable if much albumin is present. This difficulty is overcome by using a 15 per cent. alcoholic solution of paranitrophenol (5 to 15 drops) in Solutions 5 to 9 and methyl red saturated solution in 50 per cent. alcohol (10 to 15 drops) in Solutions 9 to 11.

DETERMINATION OF GLUCOSE IN URINE.—BENEDICT.—*Principle.*—Through the substitution of sodium carbonate for the strong alkali in Fehling's solution, Benedict has made the solution more sensitive as a reagent for glucose. The reduced copper produced when the solution is boiled in the presence of glucose is precipitated not as the red suboxide (Cu_2O) but as the white cuprous sulphocyanate (CuSCN), which makes it easy to determine when the last trace of blue has been removed from the solution, showing complete reduction of all the copper.

Reagents.—

Benedict's quantitative solution.[1]
Sodium carbonate, crystallized.
Powdered pumice.

[1] Benedict's quantitative solution is made as follows:

Copper sulphate (pure crystallized)	18 grams
Sodium carbonate (crystallized)	200 grams
Sodium or potassium citrate	200 grams
Potassium sulphocyanate	125 grams
Five per cent. potassium ferrocyanide solution ..	5 c.c.
Distilled water to make a total volume of . .	1000 c.c.

With the aid of heat dissolve the carbonate, citrate and sulphocyanate in enough water to make about 800 c.c. of the mixture, and filter if necessary. Dissolve the copper sulphate separately in about 100 c.c. of water and pour the solution slowly into the other liquid, with constant stirring. Add the ferrocyanide solution, cool and dilute to exactly 1 liter. Of the various constituents, the copper salt only need be weighed with exactness. Twenty-five c.c. of the reagent is reduced by 50 mg. of glucose.

Technique.—Twenty-five c.c. of the reagent is measured with a pipette into a porcelain evaporating dish and 10 to 20 gm. of crystallized sodium carbonate or one-half the weight of anhydrous sodium carbonate is added, together with a little pumice stone or talcum. The mixture is heated over a free flame until the carbonate is dissolved and then the urine diluted 1 to 10 (unless the sugar content is expected to be very low) is run in from a burette, rather rapidly, until a chalk-white precipitate forms and the blue color of the mixture begins to lessen perceptibily, after which the solution from the burette must be run in a few drops at a time until the disappearance of the last trace of blue color, which marks the end-point. Toward the end of the titration the sugar solution must be added in portions of a drop or two, with an interval of about thirty seconds after each addition. During the entire titration the fluid must be kept boiling vigorously and the water lost by evaporation may be replaced from time to time.

Result.—If the urine was diluted 1 to 10 the percentage of sugar may be obtained as follows:

$$\frac{0.050}{X} \times 1000 = \text{percentage of sugar in undiluted urine,}$$

X being the number of cubic centimeters of diluted urine required to produce the end-reaction.

FREEING URINE FROM ALBUMIN AND KJELDAHL DE-TERMINATION OF THE ALBUMIN.—Pipette 100 c.c. of filtered urine into a beaker and add to it 10 per cent. acetic acid, drop by drop, with stirring, until a drop of the urine touched to blue litmus paper turns it instantly red. Do not add more acid than is necessary, for excess of acid may cause some of the albumin to stay in solution. Heat the urine slowly to the boiling-point and boil for two minutes. If the albumin does not coagulate well add a drop or two more of the acetic acid. Filter while still hot through a small nitrogen-free filter

paper. Wash thoroughly with hot water and then determine the nitrogen of the filter paper and its contents by the Kjeldahl method. The weight of albumin may be calculated by multiplying the nitrogen by 6.25. One c.c. of $\frac{N}{10}$ acid used in the Kjeldahl titration indicates 1.4 mg. of albumin nitrogen or 8.75 mg. of albumin.

When one desires merely to obtain an albumin-free filtrate for other determinations, and quantitative washing of the precipitate is necessary, the filtration of the albumin coagulated as above outlined may be facilitated by adding to the urine after coagulation an equal volume of alumina cream.[1]

This procedure may be used for the determination of albumin by difference. Twenty-five c.c. of urine is coagulated, cooled and diluted to 50 c.c. with alumina cream. It is filtered through a dry filter paper without washing, and 10 c.c. portions of the filtrate are taken for Kjeldahl determinations. The difference between the total nitrogen obtained before and after removal of the albumin indicates the amount of albumin nitrogen. The determination of albumin by difference in this manner enables one to avoid the occasionally troublesome washing of the albumin precipitate. The determination by difference is, however, subject to greater errors than the direct determination described above, and the direct method is to be preferred unless albumin is present in large amounts.

DETERMINATION OF BETA-HYDROXYBUTYRIC ACID, DIACETIC ACID AND ACETONE IN URINE AND BLOOD.—VAN SLYKE AND FITZ.—The methods are based on a combination of Shaffer's oxidation of hydroxybutyric acid to acetone and of Deniges's precipitation of acetone as a basic mercuric

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[1] The aluminum hydroxide for this purpose is made as follows: To a 1 per cent. solution of ammonium alum add a slight excess of 1 per cent. solution ammonium hydroxide at room temperature. Wash the precipitate by decantation until the wash water gives a very faint residue on evaporation (Tracy and Welker).

sulphate compound. Oxidation of hydroxybutyric acid and precipitation of the acetone are carried out simultaneously in the same solution, so that the technique is simplified to boiling the mixture for an hour and a half under a reflux condenser and weighing the precipitate which forms. The acetone and diacetic acid may be determined either with the β-hydroxybutyric acid or separately. Neither the size of sample nor mode of procedure have required variation for different urines; the same process may be used for the smallest significant amounts of acetone bodies and likewise for the largest that are encountered. The precipitate is crystalline and beautifully adapted to drying and accurate weighing; but when facilities for weighing are absent the precipitate may be redissolved in dilute hydrochloric acid and the mercury titrated with potassium iodide by the method of Personne.

Solutions Required.—

Twenty per cent. copper sulphate:

> 200 grams of $CuSO_4.5H_2O$ dissolved in water and made up to 1 liter.

Ten per cent. mercuric sulphate:

> 75 grams of C. P. red mercuric oxide dissolved in 1 liter of H_2SO_4 of 4 normal concentration.

Fifty volume per cent. sulphuric acid:

> 500 c.c. of sulphuric acid of 1.835 specific gravity, diluted to 1 liter with water. Concentration of H_2SO_4 should be 17 normal.

Ten per cent. calcium hydroxide suspension:

> Mix 100 grams of finely divided $Ca(OH)_2$ with 1 liter of water.

Five per cent. potassium dichromate:

> 50 grams of $K_2Cr_2O_7$ dissolved in water and made up to 1 liter.

REMOVAL OF GLUCOSE AND OTHER INTERFERING SUBSTANCES FROM URINE.—Place 25 c.c. of urine in a 250 c.c.

measuring flask. Add 100 c.c. of distilled water, 50 c.c. of 20 per cent. copper sulphate solution and mix. Then add 50 c.c. of 10 per cent. calcium hydroxide, shake, and test with litmus. If not alkaline add more calcium hydroxide. Dilute to mark and let stand at least one-half hour for glucose to precipitate. Filter through a dry, folded filter. This procedure will remove up to 8 per cent. of glucose. Urine containing more should be diluted enough to bring the glucose down to 8 per cent. The filtrate may be tested for glucose by boiling a little in the test-tube. A precipitate of yellow Cu_2O will be obtained if the removal has not been complete. A slight precipitate of white calcium salts always forms, but does not interfere with the detection of the yellow cuprous oxide. This procedure is depended upon to remove interfering substances other than glucose, and should not be omitted even when glucose is absent.

REMOVAL OF PROTEINS FROM BLOOD AND PLASMA.—Of whole blood 10 c.c. is measured into a 250 c.c. volumetric flask half-full of water; 20 c.c. of the mercuric sulphate solution is added, the flask filled to the mark and shaken. After fifteen minutes or longer the contents are filtered through a dry, folded filter.

For plasma the procedure is the same, except that only 8 c.c. is taken, in a 200 c.c. flask, with 15 c.c. of mercuric sulphate solution. Only 30 to 40 c.c. of water is added before the mercuric sulphate. After the latter is in, the solution is shaken gently for about a minute, until the precipitate coagulates in floccules. It is then diluted to the mark.

In the case of either whole blood or serum, 125 c.c. of filtrate, equivalent to 5 c.c. of the original sample is taken for analysis.

Smaller samples may be used; these are the amounts chosen when there is plenty of blood.

SIMULTANEOUS DETERMINATION OF TOTAL ACETONE BODIES (ACETONE, DIACETIC ACID, AND HYDROXYBU-

TYRIC ACID) OF URINE OR BLOOD IN ONE OPERATION.—
Place in a 500 c.c. Erlenmeyer flask 25 c.c. of urine filtrate
plus 100 c.c. of water or 125 c.c. of blood filtrate. Add
10 c.c. of 50 per cent. sulphuric acid and 35 c.c. of the
10 per cent. mercuric sulphate. Connect the flask with
a reflux condenser having a straight condensing tube of
8 to 10 mm. diameter, and heat to boiling. *After* boiling
has begun, add 5 c.c. of the 5 per cent. dichromate through
the condenser tube. Continue boiling gently one and one-
half hours. The precipitate which forms consists of the
mercury sulphate compound of the preformed acetone and
of the acetone which has been formed by oxidation of
the hydroxybutyric acid. The approximate composition
is $2HgSO_4.HgCrO_4.5HgO.2(CH_3)_2CO$, the $HgCrO_4$ giving
the compound a chromate yellow color. It should not stand
in contact with the mother-liquor longer than one hour,
however, as its composition may be appreciably changed by
the precipitation of impurities.

It is collected in a Gooch or alundum medium density
crucible, washed with 200 c.c. of cold water, and dried for
an hour at 110°. The crucible is cooled in room air (desiccator
undesirable) and weighed. Several precipitates may be col-
lected, one above the other, without cleaning the crucible.
In case facilities for drying and weighing are not convenient,
however, the precipitate may be dissolved and determined
by titration of its mercury, as described later.

Normal urines may yield as much as 15 mg. of precipitate.
Normal blood yields only 1 to 3 mg. In ketonuria amounts
from 20 to 500 mg. are obtained.

ACETONE AND DIACETIC ACID.—The acetone plus the
diacetic acid, which completely decomposes into acetone
and CO_2 on heating, are determined without the hydroxy-
butyric acid exactly as the total acetone bodies, except that
(1) no dichromate is added to oxidize the hydroxybutyric
acid and (2) the boiling must continue for not less than

thirty-five nor more than forty-five minutes. Boiling for more than forty-five minutes, even without dichromate, splits off a little acetone from hydroxybutyric acid. In this case the precipitate is white, being free from chromate, and of the approximate composition $3HgSO_4.5HgO.2(CH_3)_2CO$.

BETA-HYDROXYBUTYRIC ACID IN URINE.—The hydroxybutyric acid alone is determined exactly as total acetone bodies except that the preformed acetone and that from the diacetic acid are first boiled off. To do this the 25 c.c. of urine filtrate plus 100 c.c. of water is treated with 2 c.c. of 50 per cent. sulphuric acid and boiled in the open flask for ten minutes. The volume of solution left in the flask is measured in a cylinder. The solution is returned to the flask and the cylinder washed with enough water to replace that boiled off and restore the volume of the solution to 127 c.c. Then 8 c.c. of the 50 per cent. acid and 35 c.c. of mercuric sulphate are added. The flask is connected under the condenser and the determination is continued as above, dichromate being added after boiling has begun.

The amount of precipitate obtained from β-hydroxybutyric acid corresponds to 75 per cent. of the acetone that would be obtained if each molecule of hydroxybutyric yielded a molecule of acetone. The other 25 per cent. of the β-hydroxybutyric acid is oxidized to products other than acetone, such as acetic acid. The oxidation is complete in one and one-half hours, and the yield cannot be increased by boiling longer. As above described, the conditions are so constant that duplicates usually check within 1 per cent.

BETA-HYDROXYBUTYRIC ACID IN BLOOD.— The following procedure enables one to determine separately in a single sample of blood both the acetone plus diacetic acid and the β-acid. The acetone and diacetic are precipitated as above described, and the filtrate poured as completely as possible through the Gooch or alundum crucible into a dry receiving flask. Of this filtrate 160 c.c. is measured into another

Erlenmeyer flask and 10 c.c. of water is added. The mixture is heated to boiling under a reflux condenser, 5 c.c. of dichromate solution added, and the determination continued as described for "total acetone bodies."

The procedure for beta-hydroxybutyric acid followed in urine cannot be used in blood, because the excess mercury used in removing proteins previous to boiling off the acetone would partly precipitate the latter before it escaped.

TEST OF REAGENTS. — Blank determinations must be performed, in which 25 portions of water are treated with precipitants, etc., exactly as in analyses of urine. The reagents must give negative results for acetone bodies.

TITRATION OF THE PRECIPITATE.—Instead of weighing the precipitate one may wash the contents of the Gooch, including the asbestos, into a small beaker with as little water as possible, and add 15 c.c. of normal HCl. The mixture is then heated, and the precipitate quickly dissolves. In case an alundum crucible is used, it is set into the beaker of acid until the precipitate dissolves, and then washed with suction, the washings being added to the beaker.

Filtration by suction may, if desired, be dispensed with when titration is employed. The precipitate may be washed on an ordinary small quantitative filter paper, which is transferred with precipitate to the beaker with 15 c.c. of normal HCl, broken up with a rod, and heated to effect solution.

In order to obtain a good end-point in the subsequent titration it is necessary to reduce the acidity of the solution. For this purpose 6 or 7 c.c. of 3 M sodium acetate are added to the cooled solution of redissolved precipitate. Then 0.2 M KI is run in rapidly from a burette with constant stirring. If more than a small amount of mercury is present, a red precipitate of HgI_2 forms at once, and redissolves as soon as 2 or 3 c.c. of KI in excess of the amount required to form the soluble K_2HgI_4 have been added. If only a few

milligrams of mercury are present, the excess of KI may be added before the HgI_2 has had time to precipitate, so that the titrated solution remains clear. In this case not less than 5 c.c. of the 0.2 M KI are added, as it has been found that the final titration is not satisfactory if less is present. The excess of KI is titrated back by adding 0.05 M $HgCl_2$ from another burette until a permanent red precipitate forms. Since the reaction utilized is $HgCl_2 + 4KI = K_2HgI_4 + 2KCl$, 1 c.c. of 0.05 M $HgCl_2$ is equivalent in the titration to 1 c.c. of the 0.2 M KI.

In preparing the two standard solutions the 0.05 M $HgCl_2$ is standardized by the sulphide method, and the iodide is standardized by titration against it. Slightly more than the theoretical amount of iodide is actually required in the titration, so that an error would be introduced if the iodide solution were gravimetrically standardized and used for checking the mercury solution, instead of *vice versa*. In standardizing the mercuric chloride[1] the following procedure is convenient: 25 c.c. of 0.05 M $HgCl_2$ is measured with a calibrated pipette, is diluted to about 100 c.c., and H_2S is run in until the black precipitate flocculates and leaves a clear solution. The HgS, collected in a Gooch crucible and dried at 110°, should weigh 0.2908 gram if the solution is accurate.

Factors.—

    1 mg. of β-hydroxybutyric acid yields 8.45 mg. of precipitate.

    1 mg. of acetone yields 20 mg. of precipitate.

    1 c.c. of 0.2 M KI[2] is equivalent to 13 mg. of precipitate in titration of the latter.

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[1] The 0.05 M $HgCl_2$ contains 13.575 gm. $HgCl_2$ per liter; the 0.2 M KI contains 33.2 gm. per liter.

[2] "M" signifies the molecular weight in grams dissolved in 1 liter of water.

EQUIVALENTS OF ONE GRAM OF PRECIPITATE IN GRAMS
OF ACETONE BODIES CALCULATED AS ACETONE
PER LITER OF BLOOD OR URINE.

Determination.	Urine. (25 c.c. of filtrate, equal to 2.5 c.c. of urine, taken for analysis.)	Blood. (125 c.c. of filtrate, equal to 5 c.c. of blood, taken for analysis.)
Total acetone bodies[1] . . .	24.8	12.4
β-hydroxybutyric acid . . .	26.4	13.2 (14.0)[2]
Acetone plus aceto-acetic acid	20.0	10.0

In order to calculate the acetone bodies as *β-hydroxybutyric acid* rather than acetone, use the factors in the table multiplied by the ratio of the molecular weights

$$\frac{\beta\text{-acid}}{\text{acetone}} = \frac{104}{58} = 1.793.$$

In order to calculate the acetone bodies in terms of *molecular concentration*, use the factors in the table divided by 58.

PHENOLSULPHONEPHTHALEIN TEST FOR KIDNEY FUNCTION.—ROWNTREE AND GERAGHTY.—Twenty minutes before the examination is begun the patient receives 200 to 400 c.c. of water. The patient then empties the bladder and is given

[1] The "total acetone body" factors are calculated on the assumption that the molecular ratio (acetone plus diacetic acid): (β-hydroxybutyric acid) is 1 to 3. Because the hydroxybutyric yields on oxidation only 0.75 molecule of acetone, the "total acetone body" factor is absolutely accurate only when the above ratio is 1 to 3. But with the range of mixtures encountered in acetonuria, when the ratio is usually between 1 to 2 and 1 to 3, with extreme limits of 1 to 1 and 1 to 4; the use of the above approximate factors for "total acetone bodies" seldom involves a significant error.

[2] The factor in parentheses is the usual factor x $\frac{170}{160}$ and is for use in determination of β-hydroxybutyric acid in blood when the acetone is precipitated and the β-acid determined in 160 c.c. of the filtrate.

6 mg. of the phthalein intramuscularly in the deltoid
lumbar muscles.

Exactly one hour and ten minutes from the time of in
tion the patient passes water, and again at the end of t
hours and ten minutes from the time of injection. The t
specimens are kept separate. If a long period before exa
nation is to elapse, they are rendered acid with phosphc
acid.

The next step is to determine how much of the drug
been excreted during the two periods of collection. B
lots (*i. e.*, that of the first and second hours) are separat
rendered alkaline with enough caustic soda solution of 25
cent. strength to bring out a deep reddish-purple color. Ea
specimen so colored is then diluted separately to 1000
with water.

The amount of phthalein is then determined by the col
metric method. The Duboscq instrument or the Rownt
and Geraghty modification of the Hellige colorimeter may
used. If the Duboscq instrument is used, the standard sc
tion should be one-half strength; that is, 3 mg. to the li
alkalinized with 1 or 2 drops of a 25 per cent. solution NaC
The left-hand cup of the instrument is half-filled with t
solution and set at 10, or sightly above or below 10 if
zero point requires correction as described in the next pa
graph. The right cup is similarly filled with the solution
be tested, the color equalized, and the reading made, incl
ing the fraction indicated by the vernier.

The equality of the two Duboscq cups should be tes
by placing standard solution in both cups. If, with the ri
cup at 10, the reading of the left is 10.5, the left, contain
the standard, should be placed at 10.5 instead of 10 wl
analyses are made.

The calculation is: per cent. excretion $= 50 \times \frac{10}{R}$ where
is the reading of the right-hand cup containing the uri

the left being adjusted as described in the preceding paragraph.

If the Hellige colorimeter is used, the wedge-shaped cup is filled with a standard solution made up of 6 mg. to the liter. In this case, obviously, the percentage is read directly from the scale. If no colorimeter is available fairly accurate values may be obtained by comparing a sample in a test-tube with a series of tubes containing known percentage solutions of the dye.

Results.—Under normal renal conditions the time of appearance of the drug after injection is from five to eleven minutes. Hence, the addition of the "ten minutes" to the hour collection. At the end of the first hour 40 to 60 per cent. of the dye should have appeared and at the end of the second hour 20 to 25 per cent. more. Altogether, therefore, 60 to 85 per cent. of the total injected should have appeared at the end of the experiment.

DETERMINATION OF TOTAL NON-PROTEIN NITROGEN IN BLOOD.—GREENWALD PRECIPITATION, FOLIN AND DENIS MICRO-KJELDAHL.—*Principle.*—The protein substances of the blood are precipitated with trichloracetic acid. The nitrogen in an aliquot part of the filtrate is converted into ammonia by digestion with concentrated sulphuric acid, and the ammonia is then transferred to an acid solution by aëration. The amount of ammonia may be determined either by Nesslerization or by titration with $\frac{N}{100}$ alkali solution.

Reagents.—

    5 per cent. trichloracetic acid.
    Potassium sulphate.
    5 per cent. solution of copper sulphate.
    Kerosene.
    Saturated solution of sodium hydroxide.
    Caprylic alcohol.

Nessler's solution.[1]

Concentrated H_2SO_4.

$\frac{N}{100}$ H_2SO_4.

$\frac{N}{100}$ NaOH.

Alizarin sulphonate or methyl red.

Standard ammonium sulphate solution.[2]

Technic.—Five cubic centimeters of the blood obtained by puncture from a vein and kept from clotting by the use of a little finely powdered potassium oxalate (about 0.1 gram for 20 c.c. of blood) is allowed to flow into a 50 c.c. volumetric flask half-filled with a 5 per cent. solution of trichloracetic acid. The flask is filled to the mark with trichloracetic acid and is well shaken. At the end of one-half hour the fluid is filtered. The filtrate should now be quite colorless. Ten cubic centimeters of this (equivalent to 1 c.c. of blood) is pipetted into a Pyrex glass test-tube (about 200 x 20 mm.) and 0.5 gram of potassium sulphate, 2 drops of 5 per cent. copper sulphate solution, 1 c.c. of concentrated sulphuric acid, and 3 drops of kerosene are

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[1] Nessler's solution is made as follows:

Mercuric iodide . . . . 200 grams
Potassium iodide . . . . 100 grams
Potassium hydroxide . . . . 400 grams

Rub the red iodide to a smooth paste with water and transfer to a 2-liter flask. Grind the potassium iodide to a powder in the same mortar and add to the iodide in the flask, using about 800 c.c. of water. Dissolve the potassium hydroxide in about one liter of water, cool thoroughly, and then add with constant shaking to the mixture in the flask. Make up to volume. The solution usually becomes perfectly clear. Place in incubator at 37° to 40° C. overnight or until the yellowish-white precipitate which may settle out is thoroughly dissolved and only a small amount of dark brownish-red precipitate remains. The solution is then ready to be siphoned off and used.

[2] Standard ammonium sulphate solution. This solution, of which 5 c.c. contains 1 mg. of nitrogen, is made by dissolving either 0.944 gm. of ammonium sulphate or 0.0764 gm. of ammonium chloride of highest purity in 1000 c.c. of distilled water.

added. The mixture is heated over a microburner until the water has been driven off and until digestion is complete, as shown by the mixture becoming perfectly clear. It is boiled two minutes longer to ensure complete breaking down of the organic compounds, is allowed to cool, and then carefully diluted with about 6 c.c. of water. The ammonia is removed from this fluid by the addition of alkali and aëration. For this purpose the arrangement shown in Fig.1,p.226,is used. The ammonia caught by the acid solution of the receiving tube may be determined either by Nesslerization or by titration.

A. Nesslerization. Into a 100 c.c. cylinder put 5 c.c. of standard solution and 50 c.c. of distilled water. Dilute 10 c.c. of Nessler's solution with 50 c.c. of water and add 25 c.c. of this mixture to the standard, then making up the volume to 100 c.c. with water. Without delay add 8 to 10 c.c. of the diluted Nessler's solution to the unknown solution in a 100 c.c. cylinder and at once dilute to 50 or 100 c.c. according to the depth of color produced, using the dilution which comes nearest in depth to that of the standard. The standard solution is placed in one cup of the Duboscq colorimeter and the unknown is compared with it (note remarks on use of the Duboscq on p. 256).

Result.—

$$X = S \times \frac{Rd}{R}$$

X = mg. of non-protein nitrogen per 100 c.c. of blood.
S = reading of standard.
R = reading of unknown.
Rd = volume in c.c. to which the unknown is diluted.

B. If the ammonia is to be determined by titration the receiving tube of the aëration apparatus must contain 25 c.c. of $\frac{N}{100}$ hydrochloric acid. The amount of this left unneutralized by the ammonia formed in the blood solution is then determined by titration with $\frac{N}{100}$ sodium hydroxide solution, using alizarin or methyl red as indicator.

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Result.—Each cubic centimeter of $\frac{N}{100}$ ammonia is equivalent to 0.00014 gm. of nitrogen.

The non-protein nitrogen in health is usually from 30 to 40 mg. per 100 c.c. of blood.

DETERMINATION OF UREA IN BLOOD.—VAN SLYKE AND CULLEN.—*Principle.*—Same as in determination of urea in urine by urease.

Reagents.—

Urease,[1] 5 per cent. solution.

$\frac{N}{100}$ H_2SO_4.

$\frac{N}{100}$ NaOH.

Neutralizing phosphate solution containing 5 grams KH_2PO_4 and 1 gram Na_2HPO_4 per liter.

Alizarin or methyl red.

Nessler's solution.

Saturated solution of potassium carbonate (90 grams to 100 c.c. water).

Standard solution of ammonium sulphate (1 mg. of nitrogen to 5 c.c.).

Caprylic alcohol.

Technic.—Into a large test-tube introduce 3 c.c. of neutralizing phosphate solution, and 1 c.c. of 10 per cent. urease solution. Add 3 c.c. of blood drawn from a vein and prevented from coagulation by the addition of potassium oxalate (see Non-protein Nitrogen Determination). Add 4 or 5 drops of caprylic alcohol to prevent foaming and allow to stand for fifteen minutes. Then arrange the aëration apparatus as for urea determination in urine, add 10 c.c. of saturated potassium carbonate solution to the mixture and aërate as in determination of urea in urine. The nitrogen is determined by Nesslerization or by titration, as described under non-protein nitrogen.

[1] See Determination of Urea in Urine.

Result.—Nesslerization.

$$X = \frac{S}{R} \times \frac{Rd}{3}$$

X = mg. of urea nitrogen per 100 c.c. of blood.
S = reading of standard.
R = reading of unknown.
Rd = volume in c.c. to which unknown is diluted.

If the result is to be determined by titration, place in the receiving cylinder 15 c.c. of $\frac{N}{100}$ acid plus 10 c.c. of water and titrate back with $\frac{N}{100}$ alkali. Each cubic centimeter of acid neutralized by the ammonia formed is equivalent to 0.1 gm. of urea or 0.0466 gm. of urea nitrogen per liter of blood.

Theoretically, a second determination should be done without the use of urease in order to determine the ammonia alone in the blood so that this may be subtracted from the amount obtained from the tube containing the solution treated with urease. This fraction is, however, extremely small and ordinarily may be neglected.

The urea of the blood in health is ordinarily about 0.2 to 0.3 gm. per liter.

McLean Index.—When this is to be determined, the following directions must be observed:

The patient is to be given 150 to 250 c.c. of water to ensure a free flow of urine. One hour later the bladder is emptied, by catheter if necessary, and the time is noted to within one minute. About thirty-six minutes later 5 to 10 c.c. of blood is withdrawn and prevented from clotting by the use of potassium oxalate as usual. At the end of exactly seventy-two minutes from the time of voiding the bladder is again emptied and the entire specimen, taking care to avoid the least loss, is at once sent to the laboratory, together with the specimen of blood. The patient must take no food or drink during the seventy-two-minute period. The

patient's weight, taken on the day of the test, must be stated on the label of the blood specimen.

The formula for the index of urea excretion follows:

$$\text{Index of urea excretion (I)}^1 = \frac{D\sqrt{C} \times 8.96}{Wt \times Ur_2}$$

> D = Grams of urea excreted per twenty-four hours (calculated from the above seventy-two-minute period).
> C = Grams of urea per liter of urine.
> Ur = Grams of urea per liter of blood.
> Wt = Body weight of individual in kilograms.

Usually the urea index (I) is 100 to 200. Variations between 80 and 300 are not infrequently observed in normal individuals.

DETERMINATION OF CHLORIDES IN BLOOD PLASMA.— MCLEAN-VAN SLYKE-DONLEAVY.—*Principle.*—The chlorides and protein in the blood plasma are precipitated simultaneously by the addition of picrated silver nitrate. The precipitated AgCl and proteins are filtered off and the excess silver nitrate determined by titration with a solution of potassium iodide in the presence of nitrous acid and starch.

Reagents.—AgNO₃ solution (1 c.c. equivalent to 2 mg. NaCl).

Silver nitrate	5.812 grams
Picric acid	.750 grams
Nitric acid (sp. gr. 1.42) . . .	250.0 c.c.
Water to	1000.0 c.c.

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[1] The calculation of the value of the index formula is greatly simplified by the use of the slide rule provided with a special scale, sold by Keuffel & Esser Co., 127 Fulton Street, New York. Note that the calculations are made in grams of urea, not urea nitrogen.

Potassium iodide solution (5 c.c. equivalent to 1 c.c. of the silver nitrate).

Potassium iodide	1.2 grams
Water to	1000.0 C.C.

Starch solution:

Sodium citrate ($Na_3C_6H_5O_7 +$ $5\frac{1}{2}H_2O$)	446.0 grams
Soluble starch	2.5 grams
Sodium nitrite	20.0 grams
Water to	1000.0 C.C.

Dissolve the starch in 500 c.c. of warm water, add the salts, heat to the boiling point, and boil five minutes. While still hot, filter through cotton, wash the filter with hot water, and after cooling, make filtrate up to 1000 c.c. The solution becomes cloudy on standing but keeps indefinitely. The citrate is necessary in order to regulate the acidity for the end point, the nitrite to liberate the iodin from the iodide.

The potassium iodide solution is standardized against the silver solution by adding 5 c.c. of the starch solution to 5 c.c. of the silver solution and titrating with the iodide to the first appearance of *green* color. The solution of potassium iodide is then diluted so that 20 c.c. are exactly equivalent to 5 c.c. of the silver solution.

Technique.—Centrifuge 5 c.c. or more of oxalated blood and with an Ostwald pipette calibrated to *contain* (not deliver) measure 2 c.c. of the plasma into a 50 c.c. calibrated flask containing 20 c.c. of distilled water. Pipette 10 c.c. of the picrated silver nitrate into the flask. Shake, make to the mark with distilled water and allow to stand ten minutes.

The above directions provide sufficient filtrate for duplicate titrations. In case it is desirable to use only 1 c.c. of plasma the precipitation may be made in a 25 c.c. instead of a 50 c.c. flask with only 5 c.c. of the picrated silver nitrate solution.

Filter through a dry filter and obtain a clear yellow filtrate. To 20 c.c. of the filtrate add a volume of citrate-starch solution equal to the volume of silver solution present in the portion of the filtrate taken; *e. g.*, in this case 10 c.c. of silver solution were diluted to 50 c.c. and 20 c.c. of filtrate used, therefore, 4 c.c. of silver solution is present and 4 c.c. of citrate-starch solution must be added. Then titrate with the potassium iodide to the first appearance of green color. As the end point is approached it is advisable, in order to avoid overrunning it, to pause after the addition of each drop of iodide, because the green color requires several seconds to develop.

Calculation.—When 20 c.c. of filtrate equivalent to 0.8 c.c. of plasma are used for the titration:

$$NaCl \text{ per liter plasma} = 10 - \frac{c.c. KI}{2}.$$

DETERMINATION OF THE BICARBONATE CONTENT OF THE BLOOD PLASMA UNDER CONSTANT CARBON DIOXIDE TENSION. —VAN SLYKE AND CULLEN.—*Drawing Blood Sample.*—For at least an hour before the blood is drawn the subject should avoid vigorous muscular exertion, as this, presumably because of the lactic acid formed, lowers the bicarbonate of the blood. The blood is drawn from the arm vein directly into a centrifuge tube containing enough potassium oxalate to make about 0.5 per cent. of the weight of the blood.

It is essential that the blood be collected with minimum gain or loss of carbon dioxide, as HCl is transferred from plasma to cells by increase of free CO_2 in the former, and *vice versa*, with resultant change of not only free carbon dioxide, but also of bicarbonate in the plasma. Consequently, overaccumulation of carbon dioxide in the venous blood is avoided by using as little stasis as possible. When stasis is necessary the ligature is released as soon as the vein is entered and a few seconds allowed for the stag-

nant blood to flow out before the main sample is drawn. It is equally necessary to avoid loss of carbon dioxide while the plasma is still in contact with the corpuscles *in vitro*. In order to prevent such loss the blood may be drawn into a tube arranged as in Fig. 2. After the sample has been

Paraffin Oil—
Potassium Oxalate—
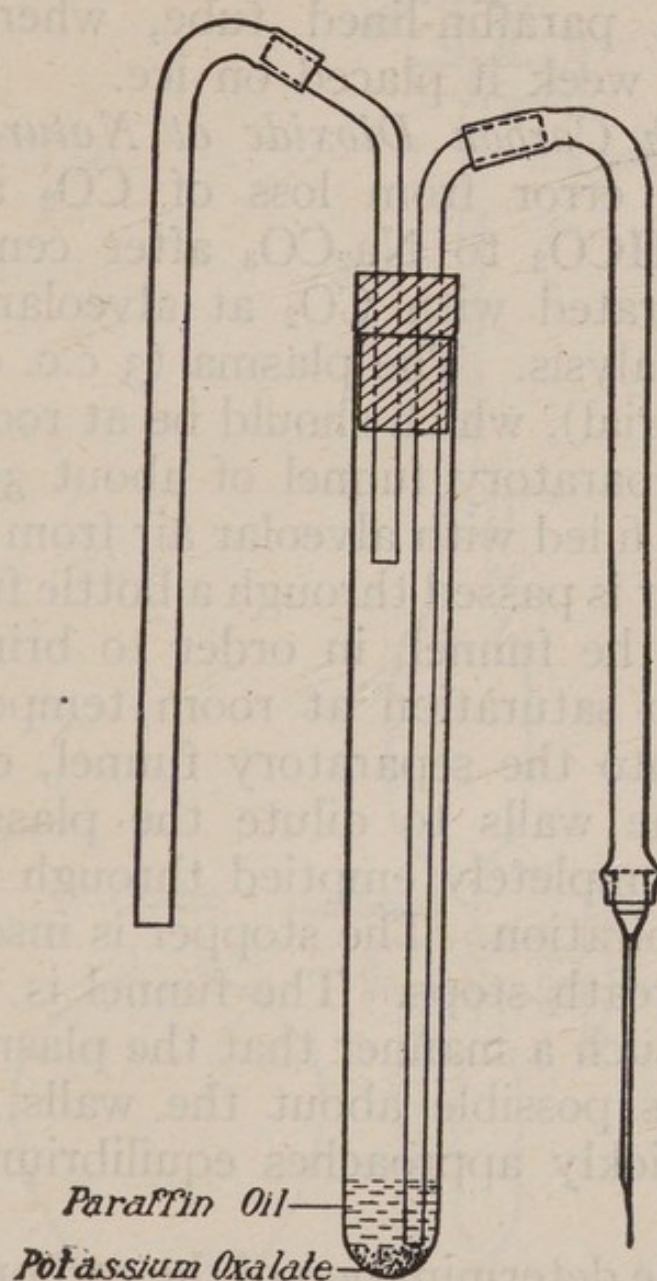
FIG. 2

lrawn the stopper is loosened and the blood stirred with the nlet tube in order to assure distribution of the oxalate. The tube should not be shaken or inverted. The blood is entrifuged in it within a half-hour. In place of the tube llustrated a syringe may be used if the blood is drawn with

minimum suction, free air space in the barrel is avoided, and the transfer to a centrifuge tube made with minimum exposure to air.

The clear plasma, being pipetted off, should, in case it is not convenient to determine its CO_2 capacity at once, be transferred to a paraffin-lined tube, where it will keep unchanged for a week if placed on ice.

Saturation with Carbon Dioxide at Natural Tension.—In order to correct error from loss of CO_2 and consequent reversion of $NaHCO_3$ to Na_2CO_3 after centrifugation, the plasma is resaturated with CO_2 at alveolar tension immediately before analysis. The plasma (3 c.c. or more if there is plenty of material), which should be at room temperature, is placed in a separatory funnel of about 300 c.c. capacity and the funnel is filled with alveolar air from the lungs of the operator. The air is passed through a bottle full of glass beads before it enters the funnel, in order to bring the moisture content down to saturation at room temperature. If one blows directly into the separatory funnel, enough moisture condenses on the walls to dilute the plasma appreciably. The lungs are completely emptied through the funnel by a quick, forced expiration. The stopper is inserted just before the stream of breath stops. The funnel is then rotated for two minutes in such a manner that the plasma is distributed as completely as possible about the walls, forming a thin layer, which quickly approaches equilibrium with the CO_2 in the air.

Analysis.—The determination of the carbon dioxide content of the saturated plasma is performed as follows: The CO_2 apparatus (Fig. 3) held in a strong clamp on a ringstand is completely filled with mercury, which should fill both capillaries above the upper stop-cock. The mercury levelling bulb is placed about on a level with the lower cock. The cup at the top of the apparatus is washed out thoroughly with dilute ammonia, followed by water, medicine droppers

being convenient for this purpose. One cubic centimeter
of the saturated plasma is introduced into the cup and

1mm. bore

1.25 cc
15
2
2.5

3mm. bore

50 cc
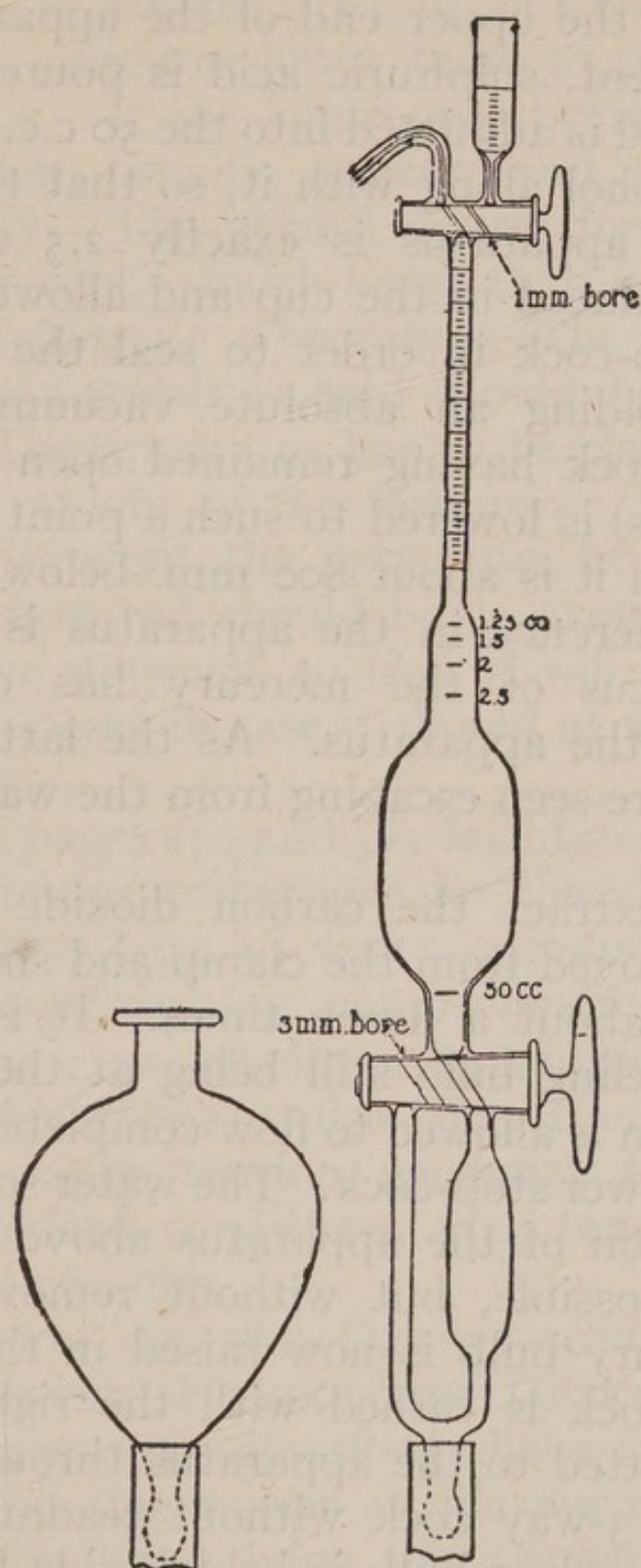
FIG. 3

llowed to flow down into the upper stem of the apparatus.
The cup is now washed with two portions of about 0.5 c.c.
each of water, care being taken that no air enters the appa-

ratus with the liquid. One small drop of octyl alcohol, to prevent foaming, is now admitted into the capillary connecting the cup with the upper end of the apparatus and about 1 c.c. of 5 per cent. sulphuric acid is poured into the cup. Enough of the acid is admitted into the 50 c.c. chamber, carrying the octyl alcohol along with it, so that the total volume of water in the apparatus is exactly 2.5 c.c. A drop of mercury is now placed in the cup and allowed to flow down to the upper stop-cock in order to seal the same and make it capable of holding an absolute vacuum. The leveling bulb (the lower cock having remained open from the beginning of operations) is lowered to such a point that the surface of the mercury in it is about 800 mm. below the lower stop-cock, and the mercury in the apparatus is allowed to fall until the meniscus of the mercury has dropped to the 50 c.c. mark on the apparatus. As the latter is evacuated, bubbles of CO_2 are seen escaping from the water mixture into the vacuum.

In order to extract the carbon dioxide completely the apparatus is removed from the clamp and shaken by turning it upside down about a dozen times. It is then replaced, the mercury leveling bulb still being at the low level, and the water solution is allowed to flow completely into the small bulb below the lower stop-cock. The water solution is drained out of the portion of the apparatus above the stop-cock as completely as possible, but without removing any of the gas. The mercury bulb is now raised in the left hand and the lower stop-cock is turned with the right hand so that mercury is admitted to the apparatus through the left-hand entrance of the 3-way cock without readmitting the water solution. The leveling bulb is held beside the apparatus so that the mercury level in it is even with that in the apparatus and the gas in the latter is under atmospheric pressure. A few hundredths of 1 c.c. of water will float on the mercury in the apparatus, but this may be disregarded in leveling

The volume of gas above the short column of water referred to is at once read off.

Plasma of normal adults yields 0.65 to 0.9 c.c. of gas, indicating 53 to 77 volume per cent. of CO_2 chemically bound by the plasma. Figures lower than 50 per cent. in adults indicate acidosis. If the figure goes below 30 symptoms of acid intoxication usually appear, and with further fall, rapidly intensify. The normal figures for infants appear to be 40 to 55 per cent.—much lower than for adults.

Caution in Setting up Apparatus.—The jaws of the clamp in which the apparatus is held should be lined with thick soft rubber. The apparatus has to be clamped very tightly because of the weight of the mercury.

In order to prevent the apparatus from slipping out of the clamp an iron rod should be so arranged as to project under the lower stopcock, so that it will support the apparatus from this point in case it should at any time slip down from the clamp.

The table on pages 270 and 271 facilitates calculation of the results. It contains corrections for the air (about 0.05 c.c.) dissolved by the 2.5 c.c. of water introduced into the apparatus, for an approximately equal volume of CO_2 physically dissolved by the 1 c.c. of plasma in addition to that chemically bound as bicarbonate, also corrections for temperature, pressure, and vapor tension necessary to reduce the gas volume to standard conditions, viz., temperature of $0°$ C. and pressure of 760 mm.

DETERMINATION OF THE OXYGEN-BINDING CAPACITY OF BLOOD (GASOMETRIC HEMOGLOBIN DETERMINATION).—VAN SLYKE.—The apparatus described above for determining the carbonic acid binding power of plasma may be used with equal facility for determining the oxygen binding power of blood.

Five to 10 c.c. of blood is introduced into a separatory funnel or bottle and distributed in a thin layer about the

TABLE FOR CALCULATION OF CARBON DIOXIDE COMBINING POWER OF PLASMA.[1]

Observed vol. gas $\times \dfrac{B^1}{760}$	C.c. of CO_2, reduced to 0°, 760 mm., bound as bicarbonate by 100 c.c. of plasma.				Observed vol. gas $\times \dfrac{B}{760}$	C.c. of CO_2, reduced to 0°, 760 mm., bound as bicarbonate by 100 c.c. 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] $\dfrac{B}{760} = \dfrac{\text{Barometric pressure}}{760}$

TABLE (*continued*).

Observed vol. gas $\times \frac{B}{760}$	C.c. of CO_2, reduced to 0°, 760 mm., bound as bicarbonate by 100 c.c. of plasma.				Observed vol. gas $\times \frac{B}{760}$	C.c. of CO_2, reduced to 0°, 760 mm., bound as bicarbonate by 100 c.c. of plasma.			
	15°	20°	25°	30°		15°	20°	25°	30°
0.51	39.1	39.5	40.0	40.3	0.91	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

inner wall, so that maximum contact with the air is assured. The vessel is rotated for three or four minutes so that the blood is kept in a thin layer, or it may be shaken 5 or more minutes on a mechanical shaker. The blood is then transferred to a cylinder or heavy-walled tube. The blood gas apparatus is now prepared by introducing into it 15 drops of redistilled caprylic alcohol and 6 c.c. of ammonia solution made by diluting 4 c.c. of concentrated ammonia to a liter. If saponin is available the diluted ammonia should be made to contain about 1 mg. per cubic centimeter. The apparatus is evacuated and the air extracted from the ammonia by shaking for about fifteen seconds. The extracted air is expelled and the process completed to make sure that no air is left in the solution. Just before the blood is introduced about 2 c.c. of the air-free ammonia is forced up into the cup of the apparatus. The aërated blood is now thoroughly stirred with a rod to assure even distribution of the corpuscles, and 2 c.c. is drawn into a pipette and run under the ammonia in the cup of the appa-

ratus. All but a few drops of the liquid in the cup is now run into the 50 c.c. chamber, the ammonia following the blood and washing it in. A few additional drops of the ammonia may be added from a dropper to make the washing complete.

The blood and ammonia in the apparatus are mixed and allowed to stand until the blood is *completely laked*. This requires about thirty seconds when saponin is present and five minutes when it is not. After laking is complete 0.4 c.c. of a saturated (40 grams to 100 c.c. of water) potassium ferricyanide solution is introduced to set free the oxygen combined with the hemoglobin. (The cyanide solution is made air-free by boiling or by shaking in an evacuated flask and is kept in a burette under a layer of paraffin oil 2 or 3 cm. thick to exclude air.) The apparatus is now evacuated until only a few drops of mercury remain above the lower stop-cock, and is shaken, preferably with a rotary motion, to whirl the blood in a thin layer about the wall of the chamber. If the blood was completely laked before the cyanide was added, extraction of the oxygen is completed in half a minute. The water solution is now drawn down into the bulb of the apparatus below the lower cock, and the extracted gases measured as in the determination of carbon dioxide. After the gas volume has been read the chamber is evacuated, and the blood readmitted and shaken again for thirty seconds in the vacuum. The reading is then repeated. If it shows an increase a third extraction should be performed.

In order to determine the oxygen bound by the hemoglobin it is necessary to subtract from the gas measured the volume of air physically dissolved by 2 c.c. of blood at atmospheric pressure and the prevailing room temperature. The volume of gas thus corrected may be reduced to standard conditions, $0°$, 760 mm., by multiplying by $(0.999.-$

$0.0046t) \times \dfrac{\text{barometer}}{760}$, t being the temperature in degrees

Centigrade. If this result is multiplied by 50 it gives the cubic centimeters of oxygen bound by the hemoglobin in 100 c.c. of blood. The amounts of air dissolved are given in the table below, which also gives the factors by which one may directly transpose the readings into terms of percentage of normal hemoglobin on the basis of Haldane's average, viz., 18.5 per cent. oxygen = 100 per cent. hemoglobin

FACTORS FOR CALCULATING HEMOGLOBIN FROM OXYGEN BOUND BY 2 C.C. OF BLOOD.

Temperature. C°.	Air dissolved by 2 c.c. of blood. Subtract from gas volume read in apparatus in order to obtain *corrected gas volume*, representing O_2, set free from hemoglobin. Cc.	Factor by which corrected gas volume is multiplied in order to give per cent. hemoglobin, calculated on the basis: 18.5 per cent. oxygen = 100 per cent. hemoglobin. Per cent.
15	0.037	$251 \times \frac{B}{760}$
16	0.036	250 "
17	0.036	249 "
18	0.035	247 "
19	0.035	246 "
20	0.034	245 "
21	0.033	244 "
22	0.033	242 "
23	0.032	241 "
24	0.032	240 "
25	0.031	239 "
26	0.030	237 "
27	0.030	236 "
28	0.029	235 "
29	0.029	234 "
30	0.028	233 "

It is advisable after one 2 c.c. portion of a blood sample has been analyzed to aërate the remainder of the sample a second time and repeat the determination in order to make certain that the first sample was completely saturated with oxygen.

Example.—
   Observed gas volume = 0.45 c.c. at 20°, 760 mm.
   Correction for dissolved air = 0.034.
   Corrected gas volume = 0.416 c.c.
   Hemoglobin = 0.416 × 245 = 102 per cent.

COLORIMETRIC DETERMINATION OF HEMOGLOBIN.—PALMER.
—*Apparatus and Reagents.—*
   0.1 c.c. pipette (calibrated to *contain* 0.1 c.c.).
   10 c.c. volumetric flask.
   Dilute NH_4OH solution (4 c.c. strong ammonia in 1 liter
      of water).
   Colorimeter.[1]
   Standard hemoglobin solution.[2]

*Technique.—*Fill a 10 c.c. volumetric flask about half-full
of the dilute ammonia. Draw blood to the mark in
0.1 c.c. pipette in usual manner. Transfer to the volu-
metric flask containing the ammonia water, drawing the
solution into the pipette two or three times to wash out all
blood. Next fill the volumetric flask to the mark with the
ammonia solution. Transfer contents to a large-sized test-
tube and bubble briskly illuminating gas or carbon monoxide
through the hemoglobin solution for at least thirty seconds
(this operation should, of course, be carried out in a hood
or otherwise so conducted that danger of carbon monoxide
poisoning is avoided). Compare in colorimeter against
standard 1 per cent. carbon monoxide hemoglobin solu-
tion.

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[1] The Duboscq colorimeter is desirable, as the color matching is very
sharp and accurate with this instrument. The Hellige may be used,
in which case only 20 mg. of blood is necessary, taken in the usual Sahli
pipette and diluted to 2 c.c. The Sahli pipette should be calibrated
before using, as frequently we find as much as 10 to 15 per cent. error in
those sold with the usual Sahli apparatus.

[2] See Suggestions on page 275.

PREPARATION OF CARBON MONOXIDE GAS FOR USE IN THE HEMOGLOBIN DETERMINATION.—Four grams of zinc powder, 6 grams calcium carbonate and a few fibers of asbestos are mixed and placed in a 30 to 50 c.c. *hard* glass test-tube. The tube is so arranged that the gas evolved may be collected over water and the mixture of zinc and calcium carbonate is given the full heat of a Bunsen burner. The above amounts yield over 1200 c.c. of carbon monoxide gas. This may be diluted ten times with air and used in place of illuminating gas for converting hemoglobin to carbon monoxide hemoglobin for the Palmer colorimetric hemoglobin determination.

Suggestions.—1. 0.1 c.c. pipettes are easily made of thick-walled millimeter tubing in which a bulb may be blown and calibrated with mercury or water.

2. A standard made up to contain a 20 per cent. solution of blood having an oxygen capacity of 18.5 per cent. is kept in the ice-chest, saturated with CO (illuminating gas). From this a 1 per cent. solution for routine use may be made. Seal in the cork with paraffin. This concentrated solution will keep for months.

3. The standard 1 per cent. solution for routine use is made up in 100 and 200 c.c. lots and kept sealed in a bottle which should be protected from light. With ordinary care, if kept in the ice-chest and protected from light, it will keep for weeks. At room temperature it keeps for about a week.

4. In making standards or dilutions always use the specified dilute ammonia solution.

5. The standard may best be prepared from blood, the oxygen capacity of which has been determined as described in the preceding section.

DETERMINATION OF SUGAR IN BLOOD.—LEWIS AND BENEDICT.—*Principle.*—The blood proteins are precipitated by picrate. The filtrate is made more alkaline and heated,

18

whereby the glucose reacts with the picrate, producing a reddish-brown color proportional in intensity to the glucose present.   The glucose is estimated by comparing the color of the solution in a colorimeter with that of a standard. The standard solution may be made from picramic acid, which is presumably the colored product formed by the action of glucose on picrates, or it may be made from a known solution of pure glucose, or even from dichromate.

Reagents.—

Picrate solution.

> 36 grams dry picric acid dissolved in 500 c.c. 1 per cent. NaOH plus 400 c.c. hot water; solution cooled and diluted to 1 liter.

Sodium carbonate solution.

> 20 grams Na_2CO_2 per 100 c.c. solution.

Standard picramic acid or dichromate, as described below.

Technic.—Two cubic centimeters of blood is drawn into an Ostwald pipette containing a little powdered potassium oxalate, and discharged into a 25 c.c. graduated flask, or into a large test-tube graduated at 12.5 c.c. and at 25 c.c.   The pipette is twice rinsed out with distilled water, these washings being added to the blood.   After a minute or two the blood is practically completely laked.   The solution of sodium picrate is added to the 25 c.c. mark (using a few drops of alcohol to dispel foam if necessary) and the mixture thoroughly shaken.   After a minute or two (or longer) the mixture is poured upon a dry filter, and the clear filtrate collected in a dry beaker.   Exactly 8 c.c. of the filtrate is measured into a large test-tube bearing graduations at the 12.5 c.c. and 25 c.c. mark, and 1 c.c. of 20 per cent. (anhydrous) sodium carbonate solution is added.   The tube is plugged with cotton and immersed in boiling water for ten minutes.   (Longer heating up to half an hour makes no change in the color.)   It is then removed, and the contents are cooled under running water and diluted to 12.5

c.c. or to 25 c.c., depending on the depth of color. At any time within half an hour the colored solution is compared in a colorimeter with a suitable standard solution, the standard being set at a height of 15 mm.

Occasionally the final filtrates in this or other picric acid methods develop a little turbidity during heating. Unless such turbidity is fairly marked it is of no account. When desired the final colored solution may be filtered through a small folded filter into the colorimeter cup.

The standard solution may be simultaneously prepared from pure glucose by treating 0.64 mg. of glucose in 4 c.c. of water with 4 c.c. of the picrate solution and 1 c.c. of the carbonate, and heating for ten minutes in boiling water, then diluting to 12.5 c.c.

A permanent standard solution may be prepared from picramic acid or from potassium dichromate. The latter standard does not match the unknown with absolute exactness, but can be employed with satisfactory results when pure picramic is not obtainable.

The picramic acid standard is best prepared from a stock solution containing 100 mg. of picramic acid and 200 mg. of sodium carbonate per liter; 126 c.c. of this solution is treated with 1 c.c. of the 20 per cent. carbonate solution, and 15 c.c. of the picrate solution, and diluted to 300 c.c. with distilled water. This solution matches exactly the color from 0.64 mg. of glucose, when treated as in the above method, and diluted to 12.5 c.c.

The standard prepared from potassium dichromate contains 800 mg. of pure potassium dichromate in a liter of water.

If desired, 1 c.c. of blood may be used instead of 2, making the initial dilution to 12.5 c.c. instead of to 25 c.c. In this case the test-tube graduated at 12.5 c.c. and 25 c.c. is convenient for the dilution. When 1 c.c. of blood is employed there is not sufficient filtrate for duplicates.

The calculation is as follows:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \div 10 = \text{percentage of sugar in the original blood.}$$

When the final dilution is made to 25 c.c. instead of 12.5 c.c. the final figure is of course multiplied by two.

GASTRIC CONTENTS.—*Test Meals.*—Ewald meals should be sent to the laboratory as soon as expressed. The amount of free acid should be determined immediately; the rest of the examination may be deferred. If a delay is necessary, the specimen should be put on ice. An analysis includes tests for:

1. Total acidity.
2. Free hydrochloric acid.
3. Lactic acid (if free HCl is absent or very low).
4. Blood.
5. Rennin.
6. Bile.

The contents of the cup are measured and note is made of the color, odor, condition of the bread (whether in fragments as chewed or well macerated), and amount of mucus present (whether well mixed with the meal). Anything unusual in the appearance of the specimen is noted, such as the presence of excess of water, or of undigested egg or other unexpected articles of diet. A drop of sediment is examined under the microscope for food elements, blood cells, pus, yeasts, sarcines, and Boas-Oppler bacilli (coarse Gram-positive bacilli, motile, and occurring in chains; very numerous when present). The meal is then carefully strained through two layers of gauze placed in a funnel.

1. *Total Acidity.* — Ten cubic centimeters is carefully measured with a pipette into a flat porcelain dish, and 2 or 3 drops of phenolphthalein is added. Decinormal sodium hydrate is then added from a burette until the red color when spread evenly does not become deeper on addition of another drop. The number of cubic centimeters

used is multiplied by 10 and the result used to express the acidity. The usual normal reading is between 40 and 60, but 80 is not abnormal.

2. *Free Hydrochloric Acid.*—Amido-benzol paper is touched with the fluid. If it turns red, free mineral acid is present.

Ten cubic centimeters of the strained juice is measured into another porcelain dish with a pipette and a drop of Töpfer's reagent (dimethyl-amido-azo-benzol (see Indicators)) is added. Decinormal NaOH is then added until the orange-red color changes to a bright yellow—the end-reaction. The result is again expressed in the number of cubic centimeters which would be required to neutralize 100 c.c. of gastric contents. The normal amount is given as from 20 to 60, with an average of 40.

If the fluid is small in amount, the free HCl should be determined first, and then a few drops of phenolphthalein added to the same sample and titration continued to determine the total acidity. The amount of alkali used in neutralizing to Töpfer's reagent must of course be included in this reading. If necessary 5 c.c. or even less may be used for titration, but this increases the error.

3. *Lactic Acid.*—Specimens with a low free acidity should be tested for lactic acid.

Kelling's Test.—Add a few drops of FeCl$_3$ solution to a test-tube of distilled water sufficient to produce a very faint yellow. Divide this into two portions and to one add a few drops of the juice. Lactic acid will produce a distinct canary yellow in the tube to which it is added.

4. *Blood.*—If brown, questionable particles are seen in the sediment; these should be selected for the test. If not, the fluid should be tested as follows: Take 3 c.c. of fluid, 3 c.c. of glacial acetic acid, and 2 c.c. of ether; shake and allow to separate. If large amounts of fat are present extract with neutral ether first. Add 5 to 10 drops of fresh guaiac tincture and 2 c.c. of H$_2$O$_2$. A blue color in the ether indicates blood.

5. *Rennin.*—Take a few drops of the test meal and add 10 c.c. of milk. If HCl is absent or low in amount mix 1 drop of 1 per cent. calcium chloride solution with the gastric juice before adding it to the milk.

6. *Bile.*—A yellow juice may be tested for bile by layering under alcoholic iodine as for urine; a green juice by layering over yellow nitric acid, when a red color may appear from the further oxidation of the biliverdin; but, as a rule, inspection is sufficient.

Vomitus.—Examined only as ordered. The complete routine examination is not necessary.

FAT AND FATTY ACIDS IN FECES.—FOLIN AND WENTWORTH. —*Principle.*—The soaps present in the stool, which are insoluble in ether alone, are changed by adding HCl to the ether into ether-soluble free fatty acids. The dried material, therefore, is extracted in a Soxhlet apparatus with an ether hydrochloric acid mixture. The extract contains neutral fats and fatty acids. The fatty acids are determined by titration with sodium alcoholate.

Reagents.—

$\frac{N}{10}$ sodium alcoholate solution.[1]

Petroleum ether, boiling point 30° to 60° C.

Benzol.

Anhydrous ether—hydrochloric acid solution.[2]

Soxhlet extraction apparatus.

[1] The sodium alcoholate solution is prepared by dissolving about 2.3 grams of metallic sodium in about 1 liter of absolute alcohol. It is standardized in the usual manner against decinormal hydrochloric acid.

[2] The ethereal hydrochloric acid solution used for extraction should be decinormal or a little stronger. It is prepared by dropping concentrated sulphuric acid on about 10 grams of powdered sodium chloride and leading the gas into about 1 liter of ether. The ether must be of the absolute sodium dried variety, as both water and alcohol must be carefully excluded. The hydrochloric acid content of the ether is determined by titration (in the presence of water) and is then diluted to the desired concentration by the addition of more anhydrous ether.

Technic.—The thoroughly dried stool is pulverized and sifted through a 40-mesh sieve. All should go through the sieve. The whole is then sifted through once more in order to ensure thorough mixing of the sample. The thorough powdering of the stools is an important detail, for without it, it is well-nigh impossible to obtain complete extraction.

One gram of powder is then weighed out, wrapped up in a piece of fat-free filter paper and the whole transferred to a fat-free filter paper "thimble." This is inserted in the extraction apparatus, which is then attached to a 250 c.c. Erlenmeyer flask containing about 150 c.c. of the ethereal hydrochloric acid solution. The boiling of the ether should then be kept up for about twenty hours. After disconnecting the flask the ether is distilled off. With it goes the hydrochloric acid, provided that no alcohol or water is present. (Any traces of HCl which may remain are removed during the ligroin treatment.) When practically all the ether has been thus removed about 50 c.c. of low boiling petroleum ether is added and the flask is set aside overnight. The petroleum ether should have a boiling-point of 30° to 60° C.; *i. e.*, when distilled all should go over below 60°.

The following day the petroleum-ether solution is filtered through a small plug of absorbent cotton inserted in the stem of a suitable funnel or "adapter." The filtrate and washings are collected in a weighed, tall, 100 c.c. beaker. The solvent is boiled off, the residue is dried at about 95° C. for five hours, cooled, and weighed. This gives the total weight of the neutral fats and the fatty acids.

The fatty residue is then dissolved in 50 c.c. of benzol, 1 or 2 drops of a 1 per cent. alcoholic solution of phenolphthalein is added, and the mixture is heated until the boiling-point is nearly reached. It is then immediately titrated with the standardized sodium alcoholate solution. The titration should be continued until the maximum color of the indicator is obtained. The subsequent more or less rapid

fading away of the color does not indicate that the true end-point was not reached. The fading seems to be due mainly to the fact that on cooling the soap which is formed is transformed into basic soap, thereby setting free a little of the acid.

Each cubic centimeter of decinormal alkali solution used corresponds to 28.4 mg. of stearic acid, and all results thus far obtained indicate that the fatty acid in stools consists mainly of stearic acid.

ARSENIC DETERMINATION IN URINE, BLOOD, TISSUES, ETC.—Destroy the organic matter present by heating 5 grams of blood or tissue, or 30 c.c. of urine, with 15 c.c. of concentrated, arsenic-free sulphuric acid in a Kjeldahl flask for a number of hours, until the solution becomes clear or is at most slightly tinged with brown. Cool, dilute with 10 c.c. of water, and rinse into a 50 c.c. flask, filling the latter to the mark with the washings. Use aliquot parts of 15 c.c. for the Marsh test or, more conveniently, the Sanger-Black modification of the Gutzeit determination (described in Treadwell-Hall's *Analytical Chemistry*).

SANITARY EXAMINATION OF MILK.

SANITARY BACTERIOLOGICAL EXAMINATION OF MILK.
A. TOTAL COUNT.

I. PLATE METHOD.—For preparation of apparatus and media see p. 319, under Water.

The collection of samples, plating, counting and reporting of results are carried out in the same manner as in water examination. (See p. 319.) Since milk counts run high, dilutions of 1 to 100, 1 to 1000 and 1 to 10,000 are made in routine work. Agar plates incubated for twenty-four hours at 37° C. are used for milk work.

Routine Procedure.—

1st Day: 1. Prepare dilutions as required.

2. Make two agar plates from each dilution and incubate at 37° C.

2d Day: Count agar plates made on first day.

II. DIRECT MICROSCOPIC METHOD.—*Apparatus.*—

1. Capillary pipette accurately calibrated to discharge 0.01 c.c.

2. Slide marked in square centimeters. Engine ruled slides are preferred. Hand ruling with diamond point or grease pencil may be used in emergency.

3. Microscope with 2 or 1.9 mm. oil-immersion objective and 6.4 × eyepiece and mechanical stage.

4. Special ocular micrometer marked with cross-hairs and a circle 8 mm. in diameter.

5. Stiff platinum wire, mounted.

6. Stage micrometer.

Calibration of Ocular Micrometer.—With the stage and ocular micrometers in position, adjust the draw tube of the microscope so that the diameter of the circle in the ocular measures 0.146 mm. on the stage. The number of bacteria seen in one field then, multiplied by 600,000, equals the number of bacteria per cubic centimeter, and the total number seen in thirty fields multipled by 20,000 equals the number in 1 c.c.

Procedure.—One hundredth of a cubic centimeter of milk or cream is measured by means of a clean capillary pipette accurately calibrated to discharge this quantity of milk. The milk or cream is deposited on a clean glass slide. By means of a stiff needle the drop of liquid is spread evenly over an area of 1 sq. cm. and dried quickly in a warm place protected from dust, flies, and cockroaches. The surface on which the slides rest must be level so that the films may dry evenly.

The dry smears are then prepared for microscopic examination by immersing the slide in xylol or other fat solvent for one minute or longer if desired. After this the slide is drained and dried, immersed in 70 to 90 per cent. grain or denatured alcohol for one or more minutes, then transferred to a fresh, saturated, aqueous solution of methylene blue. Old or unfiltered solutions are to be avoided, as they may contain troublesome precipitates. The slides remain in this solution for five seconds to one minute or longer, depending on the effect desired. They are then rinsed in water to remove the surplus stain and decolorized in alcohol. This takes several seconds to minutes, during which time the slide should be under observation in order that it may be removed from the alcohol before decolorization has proceeded too far. When the decolorization is completed the general background of the film should have a pale blue tint. When staining has been prolonged a deep blue margin or deep blue central patches may persist. These deeply stained areas do not contain more bacteria than other

parts of the film and may be removed if troublesome, by decolorizing and restaining lightly. After drying, the slides are ready for microscopic examination.

In making exact counts the following more or less arbitrary rules have been drawn up:

1. Incompletely divided forms are to be counted as two individuals.

2. As careful estimates as possible are to be made of the compact masses of micrococci or other forms, but all counts made from smears containing such clumps shall be regarded as of doubtful accuracy.

3. Bacteria within cells are to be counted.

4. Single individual objects in high-grade milk having the appearance of bacteria are to be regarded as doubtful bacteria, especially if they have the appearance of cocci or if they differ in morphology from the other undoubted bacteria present. These bodies may be chromatin masses from the disintegrating nuclei of tissue cells, or possibly dead udder cocci. They are rarely, if ever, present in large numbers and are never troublesome except occasionally in high-grade milk, and then only when exact counts are desired.

In making counts of groups of bacteria under the microscope, it is our custom to count as separate groups all masses of bacteria which look as if they would break apart in making dilutions so as to form separate colonies on the plates, whether containing one bacterium or thousands. This rule leaves much to be desired, as its interpretation depends entirely upon the judgment of the individual who makes the count; but no better statement of the case has suggested itself.

The number of fields which should be counted is dependent upon the accuracy desired. Experience has shown that it is scarcely over worth while for one person to count over one hundred fields on a smear. Additional accuracy can be better secured by duplicate sampling or by having two or more persons make counts from the same preparation.

A view of thirty fields gives a sufficiently accurate count in practically all cases and remarkably constant group counts are obtained in high-count milks when only five fields are counted. Counts of individuals are much more apt to be variable than are counts of groups because of the widely variable number of individuals in a group.

Certain chances of error are present in the microscopic technic, the most important of which seem to be:

1. Faulty measurement of the original sample of milk.

2. Growth of bacteria after the sample is taken, especially in the drop of milk while it is drying.

3. Inaccurate counting due to carelessness, poor preparations, differences in judgment as to what constitutes an individual or group, or to mistaking objects for bacteria which are not bacteria.

4. Irregular distribution of bacteria in clumps of irregular sizes. When a milk contains clumps of bacteria of large size, it is impossible to make a satisfactory count of the number of individual bacteria.

Sources of error commonly urged which do not seem to play any important part in causing errors are:

1. Error due to small amount of milk examined. This error averages no larger for the microscopic technic than for the plating technic. The amount of milk ordinarily examined by the microscope is less than that examined by the plate count in low-count milks but is larger in the case of high-count milks.

2. Errors due to washing bacteria out of smears, or to adding them by the use of unsterilized pipettes, washing from smear to smear, and the like.

3. Errors due to the counting of dead bacteria. Very few dead bacteria occur in fresh, unpasteurized milks. Moreover, if they do occur, they are as significant in interpreting the past history of the milk as are living bacteria.

B. Determination of the Number of Streptococci.—To estimate the number of streptococci in milk the method recommended

as the simplest and most reliable is to add diluted fractions of the milk, 1, 0.1, 0.01, 0.001 c.c., etc., to tubes of glucose neutral red broth. Ordinary broth will do, but the neutral red broth is preferable and gives better results. The tubes are incubated for two days at 37° C. and then examined, in hanging-drop preparation, for streptococcus chains. The deposit should be selected for examination, and several hanging-drop preparations made. A positive result should only be recorded when quite definite chains of cocci are detected, or, in doubtful cases, when stained preparations show such definite chains.

To isolate the streptococci, brush diluted loopfuls of the positive tubes over plates containing nutrient agar. Incubate for twenty-four hours, and if necessary for two days, at 37° C. Subcultivate the colonies with the characters of streptococcus colonies into broth or upon sloped agar in tubes containing condensation water. In cases in which streptococci are likely to be scanty, part of the centrifugalized deposit may be used to inoculate the agar plates.

The tests recommended to differentiate the streptococcus strains isolated are the following: Morphology, growth upon sloped nutrient agar, growth in nutrient broth, growth upon gelatin slope, action upon litmus milk, the production of acid in lactose, saccharose, salicin, mannite, raffinose, and inulin.

The sugar-alcohol media for the differentiation of streptococci were introduced by Gordon.

The presence or absence of streptococci in milk may also be studied by a careful examination of the centrifugalized deposit stained by methylene blue. Failure to find streptococcus chains does not mean they are absent, but only suggests they are not present in considerable numbers. The stained deposits from samples of vended milk, usually show numerous streptococci, but in those made from fresh normal milk samples they are, as a rule, not to be demonstrated.

Determination of Leukocytes.—1. If the direct microscopic method is used for determination of the total count, the leukocytes are best determined in the same smears and by the same method of enumeration.

2. *Savage Method. Apparatus.*—Centrifuge, centrifuge tubes, microscope, Thoma-Zeiss blood-counting cell.

Procedure.—The ordinary Thoma-Zeiss blood-counting chamber is employed. Direct counting of the cells is impossible owing to the opacity caused by the large amount of fat; 1 c.c. of the milk is accurately transferred to a centrifuge tube (about 15 c.c. capacity) of the long-necked flask type, and freshly filtered Toisson's solution is poured in to almost fill the tube. The two fluids are well mixed, and then centrifugalized for ten minutes. The cream is well broken up by a clean glass rod, to disentangle leukocytes carried to the surface, and the mixture centrifugalized for an additional five minutes. All the fluid is then removed down to the 1 c.c. mark, great care being taken not to disturb the deposit. This can be conveniently and readily done by means of a fine glass tube connected to an exhaust pump. Theoretically, all the cellular elements present in the original 1 c.c. of milk are now present in the 1 c.c. of fluid. The deposit is thoroughly well mixed (with a wire), and distributed through the 1 c.c. A sufficient quantity is placed on the ruled squares of the Thoma-Zeiss apparatus, and the cover-glass put on. The number of cells is counted in a number of different fields of vision, moving regularly from one field vision to another. The diameter of the field of vision is ascertained before counting by drawing out the microscope tube until an exact number of sides of the squares spans a diameter of the field of vision.

The number of cellular elements per cubic millimeter of milk equals $\dfrac{56,000y}{11d}$, where y equals the average number per field of vision, d equals the number of squares which just spans the diameter. d is determined once for all by marking

the microscope draw tube so that only twenty fields have to be counted and the figures substituted in the formula.

Sediment. Examination of the Stained Centrifugalized Deposit.—To obtain comparable results the sediment from a definite amount of milk should be examined after centrifugalization for a definite period; 10 c.c. of milk centrifugalized for ten minutes is convenient. Part of the deposit is spread thinly but uniformly over a cover-slip, dried in air, fixed in the flame, or preferably by soaking in a mixture of equal parts alcohol and ether for one minute, stained by methylene blue and mounted in balsam.

The preparation may be utilized to gain an idea of the general bacterial content, whether streptococci are present, and if so in what numbers and whether intracellular, while, if considered necessary, a differential count may be made of the cellular elements present. For this purpose not less than 200 should be enumerated. With care a rough but valuable estimate can be obtained from this examination as to the probable number of bacteria in the sample.

CHEMICAL EXAMINATION OF MILK.

Fat Determination by Babcock Centrifugal Method. —1. *Apparatus.*—(*a*) Babcock milk-test bottles, graduated to 10 per cent.

(*b*) A centrifuge with sockets for from 2 to 32 bottles, according to the number of tests to be made, and capable of being run at 600 to 1200 revolutions per minute, according to the diameter of the machine.

(*c*) Pipettes, 17.6 c.c.

(*d*) Graduates, 17.5 c.c.

2. *Determination.*—Pipette off 17.6 c.c. of the carefully mixed sample into a Babcock test bottle and cautiously add 17.5 c.c. of sulphuric acid (sp. gr., 1.82 to 1.83). Both acid and milk should be at a temperature of 15° to 20° C. (60° to 70° F.). Mix the contents of the Babcock bottle

with a gentle rotary motion and continue the agitation until all the curd has been dissolved. Whirl in a centrifuge for five minutes at the required speed for the machine used. Add boiling-hot water, filling to the neck of the bottles, and whirl again for two minutes. Again add boiling water so as to bring the fat within the scale on the neck of the bottles, and after whirling for one minute more read the length of the fat column from the bottom of the lower meniscus to the top of the upper meniscus, care being taken to make the readings at a temperature between 55° and 65° C. (130° to 150° F.), at which point the fat is wholly liquid. The readings give the percentage of 0.75 fat in the milk direct.

The speed at which the centrifuge should be run is shown in the following table:

Diameter of wheel, inches.	Speed of centrifuge, R. P. M.
10	1074
12	980
14	909
16	848
18	800
20	759
22	724
24	693

Certain difficulties are sometimes experienced in obtaining a representative sample of partially churned or of sour milk. Partially churned milk should be heated to about 45° C. (110° F.) in a water-bath, vigorously agitated, and a charge for test immediately measured out. Soured milk should be treated with 5 or 10 per cent. by volume of strong caustic soda or caustic potash solution and the mixture thoroughly agitated until completely liquid. The charge for test is then immediately measured and a correction made in the final percentage for the volume occupied by the alkali solution. When alkali has been added, the addition of

acid should be made more cautiously, and a correspondingly larger amount of acid should be employed.

FAT DETERMINATION BY ROESE-GOTTLIEB EXTRACTION METHOD.—This method can be used with preparations such as ice-cream and sweetened condensed milk, to which the Babcock method is not applicable. It can, of course, also be used for milk, and can be applied to blood serum or plasma for clinical purposes.

When ice-cream or sweetened condensed milk is analyzed a 40-gram sample is diluted to 100 c.c.

Of the sample thus diluted, or of milk or blood plasma, 10 c.c. is pipetted into a Röhrig tube, or a glass cylinder 2 cm. in diameter and 40 cm. high, to which a narrow siphon can be fitted; dilute with 0.5 c.c. of water, add 1.25 c.c. of concentrated ammonium hydroxide (2 c.c. if the sample is sour) and mix thoroughly. Add 10 c.c. of 95 per cent. alcohol and shake well. Then add 25 c.c. of washed ethyl ether, shake vigorously for half a minute, add 25 c.c. of petroleum ether, preferably redistilled below 60° C., and shake again for half a minute. Let stand twenty minutes or until the upper liquid is clear and its lower level constant.

Draw off as much as possible of the ethereal liquid, usually 0.5 to 0.8 c.c. is left, through a small filter paper into a weighed flask. Extract the liquid remaining in the tube in the same manner as before, except that only 15 c.c. each of the ethers is used, draw off through the same filter into the flask and wash with a few cubic centimeters of the mixed ethers (1 to 1). Evaporate the drawn off and filtered liquid slowly and dry at 100° to constant weight. The ether used must be tested for residue upon evaporation and a correction introduced if necessary.

The dried and weighed fats are dissolved in a little petroleum ether; if a residue be found (due to a trace of the aqueous liquid which may have passed the filter) it must be washed in the flask, dried and its weight deducted from that of the crude fat.

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This method is applicable to condensed milk, cream, milk, skim-milk, buttermilk and whey. With substances of low fat content the second extraction may be omitted, the weight of the fat being increased to correspond to the entire volume of ethereal liquid measured in the tube.

CALCULATION OF TOTAL SOLIDS IN MILK.—The milk should be at least three or four hours old before it is used for specific gravity determination. The specific gravity of the milk is then taken by means of a lactometer of high sensitiveness. The percentage of "solids not fat" in the milk may then be calculated by means of Babcock's formula, which is as follows:

$$\text{Plasma solids} = \left(\frac{100S - Sf}{100 - 1.0753Sf} - 1 \right) \times (100 - f)\, 2.5$$

where S equals the specific gravity of the milk and f equals the percentage of fat. This when added to the percentage of fat will give the percentage of total solids. The percentage of total solids may also be taken directly from the following table, interpolations being made where necessary.

TABLE FOR DETERMINING TOTAL SOLIDS IN MILK FROM ANY GIVEN SPECIFIC GRAVITY AND PERCENTAGE OF FAT.

PROPORTIONAL PARTS.

Lactometer fraction.	Fraction to be added to total solids.
0.1	0.03
0.2	0.05
0.3	0.08
0.4	0.10
0.5	0.13
0.6	0.15
0.7	0.18
0.8	0.20
0.9	0.23

Directions for Using the Table.—If the specific gravity as expressed in Quevenne degrees is a whole number, the percentage of total solids is found at the intersection of the

TABLE FOR DETERMINING TOTAL SOLIDS IN MILK FROM ANY GIVEN SPECIFIC GRAVITY AND PERCENTAGE OF FAT (PER CENT. TOTAL SOLIDS).

Per cent. of fat	Lactometer reading at 60° F. (Quevenne degrees).										
	26	27	28	29	30	31	32	33	34	35	36
2.00	8.90	9.15	9.40	9.65	9.90	10.15	10.40	10.66	10.91	11.16	11.41
2.05	8.96	9.21	9.46	9.71	9.96	10.21	10.46	10.72	10.97	11.22	11.47
2.10	9.02	9.27	9.52	9.77	10.02	·10.27	10.52	10.78	11.03	11.28	11.53
2.15	9.08	9.33	9.58	9.83	10.08	10.33	10.58	10.84	11.09	11.34	11.59
2.20	9.14	9.39	9.64	9.89	10.14	10.39	10.64	10.90	11.15	11.40	11.65
2.25	9.20	9.45	9.70	9.95	10.20	10.45	10.70	10.96	11.21	11.46	11.71
2.30	9.26	9.51	9.76	10.01	10.26	10.51	10.76	11.02	11.27	11.52	11.77
2.35	9.32	9.57	9.82	10.07	10.32	10.57	10.82	11.08	11.33	11.58	11.83
2.40	9.38	9.63	9.88	10.13	10.38	10.63	10.88	11.14	11.39	11.64	11.85
2.45	9.44	9.69	9.94	10.19	10.44	10.69	10.94	11.20	11.45	11.70	11.95
2.50	9.50	9.75	10.00	10.25	10.50	10.75	11.00	11.26	11.51	11.76	12.01
2.55	9.56	9.81	10.06	10.31	10.56	10.81	11.06	11.32	11.57	11.82	12.07
2.60	9.62	9.87	10.12	10.37	10.62	10.87	11.12	11.38	11.63	11.88	12.13
2.65	9.68	9.93	10.18	10.43	10.68	10.93	11.18	11.44	11.69	11.94	12.19
2.70	9.74	9.99	10.24	10.49	10.74	10.99	11.24	11.50	11.75	12.00	12.25
2.75	9.80	10.05	10.30	10.55	10.80	11.05	11.31	11.56	11.81	12.06	12.31
2.80	9.86	10.11	10.36	10.61	10.86	11.11	11.37	11.62	11.87	12.12	12.37
2.85	9.92	10.17	10.42	10.67	10.92	11.17	11.43	11.68	11.93	12.18	12.43
2.90	9.98	10.23	10.48	10.73	10.98	11.23	11.49	11.74	11.99	12.24	12.49
2.95	10.04	10.29	10.54	10.79	11.04	11.30	11.55	11.80	12.05	12.30	12.55
3.00	10.10	10.35	10.60	10.85	11.10	11.36	11.61	11.86	12.11	12.36	12.61
3.05	10.16	10.41	10.66	10.91	11.17	11.42	11.67	11.92	12.17	12.42	12.68
3.10	10.22	10.47	10.72	10.97	11.23	11.48	11.73	11.98	12.23	12.48	12.74
3.15	10.28	10.53	10.78	11.03	11.29	11.54	11.79	12.04	12.29	12.55	12.80
3.20	10.34	10.59	10.84	11.09	11.35	11.60	11.85	12.10	12.35	12.61	12.86
3.25	10.40	10.65	10.90	11.16	11.41	11.66	11.91	12.16	12.42	12.67	12.92
3.30	10.46	10.71	10.96	11.22	11.47	11.72	11.97	12.22	12.48	12.73	12.98
3.35	10.52	10.77	11.03	11.28	11.53	11.78	12.03	12.28	12.54	12.79	13.04
3.40	·10.58	10.83	11.09	11.34	11.59	11.84	12.09	12.34	12.60	12.85	13.10
3.45	10.64	10.89	11.15	11.40	11.65	11.90	12.15	12.40	12.66	12.91	13.16
3.50	10.70	10.95	11.21	11.46	11.71	11.96	12.21	12.46	12.72	12.97	13.22
3.55	10.76	11.02	11.27	11.52	11.77	12.02	12.27	12.52	12.78	13.03	13.28
3.60	10.82	11.08	11.33	11.58	11.83	12.08	12.33	12.58	12.84	13.09	13.34
3.65	10.88	11.14	11.39	11.64	11.89	12.14	12.39	12.64	12.90	13.15	13.40
3.70	10.94	11.20	11.45	11.70	11.95	12.20	12.45	12.70	12.96	13.21	13.46
3.75	11.00	11.26	11.51	11.76	12.01	12.26	12.51	12.76	13.02	13.27	13.52
3.80	11.06	11.32	11.57	11.82	12.07	12.32	12.57	12.82	13.08	13.33	13.58
3.85	11.12	11.38	11.63	11.88	12.13	12.38	12.63	12.88	13.14	13.39	13.64
3.90	11.18	11.44	11.69	11.94	12.19	12.44	12.69	12.94	13.20	13.45	13.70
3.95	11.24	11.50	11.75	12.00	12.25	12.50	12.75	13.00	13.26	13.51	13.77
4.00	11.30	11.56	11.81	12.06	12.31	12.56	12.81	13.06	13.32	13.57	13.83
4.05	11.36	11.62	11.87	12.12	12.37	12.62	12.87	13.12	13.38	13.63	13.89
4.10	11.42	11.68	11.93	12.18	12.43	12.68	12.93	13.18	13.44	13.69	13.95
4.15	11.48	11.74	11.99	12.24	12.49	12.74	12.99	13.25	13.50	13.76	14.01
4.20	11.54	11.80	12.05	12.30	12.55	12.80	13.05	13.31	13.56	13.82	14.07
4.25	11.60	11.86	12.11	12.36	12.61	12.86	13.12	13.37	13.62	13.88	14.13
4.30	11.66	11.92	12.17	12.42	12.67	12.92	13.18	13.43	13.68	13.94	14.19
4.35	11.72	11.98	12.23	12.48	12.73	12.98	13.24	13.49	13.74	14.00	14.25
4.40	11.78	12.04	12.29	12.54	12.79	13.04	13.30	13.55	13.80	14.06	14.31
4.45	11.84	12.10	12.35	12.60	12.85	13.10	13.36	13.61	13.86	14.12	14.37

vertical column headed by this number with the horizontal column corresponding to the percentage of fat.

If the specific gravity as expressed in Quevenne degrees is a whole number and a decimal, the percentage of total solids corresponding to the whole number is first found, and to this is added the fraction found opposite the tenth under "Proportional Parts." Two examples may suffice for illustration: (1) Fat, 3.8 per cent.; specific gravity, 32. Under column headed 32, 12.57 per cent. is found, corresponding to 3.8 per cent. fat. (2) Fat, 3.8 per cent.; specific gravity, 32.5. The percentage of total solids corresponding to this percentage of fat and a specific gravity of 32 is 12.57. Under "Proportional Parts" the fraction 0.13 appears opposite 0.5. This added to 12.57 makes 12.70, which is the desired percentage.

An inspection of the table shows that the percentage of total solids increases practically at the rate of 0.25 for each lactometer degree and 1.2 for each per cent. of fat. This gives rise to Babcock's simple formula: Total solids = $\frac{1}{4}$ L + 1.2 F. (L = lactometer reading in Quevenne degrees and f = percentage of fat.)

To illustrate the use of the formula the following example is given: Fat, 4 per cent.; specific gravity, 32. In this case one-quarter of 32 is 8; 1.2 multiplied by 4 is 4.8; 8 plus 4.8 equals 12.8, which represents the percentage of total solids.

TEST FOR FORMALDEHYDE.—Shake the milk with an equal volume of strong alcohol and use the filtrate.

Mix 5 c.c. of this filtrate with 0.03 gm. of phenylhydrazine and add 4 or 5 drops of a 1 per cent. ferric chloride solution. Add slowly, and with agitation, in a bath of cold water to prevent heating the liquid, 1 to 2 c.c. of concentrated sulphuric acid. Dissolve the precipitate by the addition either of concentrated sulphuric acid (keeping the mixture cool) or alcohol. In the presence of formaldehyde a red color develops. The method gives reliable reactions for formaldehyde in dilutions of 1 to 50,000 to 1 to 150,000. Acetaldehyde and benzaldehyde give no reaction.

SANITARY EXAMINATION OF WATER AND SEWAGE.

SANITARY ANALYSIS OF WATER.

A complete sanitary analysis of a water covers:

1. A physical examination to determine color, turbidity, odor, and taste.

2. A microscopic examination to determine the numbers and character of particles in suspension, especially algæ.

3. A chemical analysis to determine the nature and amount of chemical substances.

4. A bacteriological examination to estimate number and kind of bacteria.

5. A sanitary survey of watershed, including methods of collecting, handling, storing, and distributing the water.

6. Clinical experience, which is a very important test.

QUANTITY OF WATER REQUIRED.—Minimum quantity for ordinary physical, chemical and microscopic analysis is two liters; for bacteriological analysis, 100 c.c.

COLLECTION OF SAMPLES AND DIRECTIONS FOR SHIPPING.— See page 20. Sample bottles packed in mailing cases can always be secured from the Departmental Laboratories upon application.

PHYSICAL EXAMINATION.—The usual determinations are odor, color and turbidity.

Procedures are copied from Standard Method of Water Analysis, American Public Health Association.

Odor.—This observation is made both before and after heating the sample to boiling point, and after thorough shaking in each case. Odors are defined by such descriptive terms as "mouldy," "marshy," etc., and the intensity is expressed by some such notation as "faint," "distinct," "decided," etc.

Odors in water are very objectionable. As a rule the most objectionable odors develop in surface waters and are caused by the growth of algæ, diatomes, protozoa, and other microscopic organisms. In case of deep wells, hydrogen sulphide and other inorganic compounds may give odors to the water. Odors and tastes develop in impounding reservoirs from stagnation and putrefaction of organic matter.

Certain organisms can be distinguished by their odor, as the "fishy" odor of uroglena, "aromatic" odor of asterionella and "pig-pen" odor of anabena, which is one of the blue-green algæ. Odors caused by undecomposed organisms are due to compounds of the nature of essential oils. Algæ are responsible for many bad tastes and odors in water.

Color.—Color in water is usually from vegetable origin, such as dead leaves, bark and roots. Color means the "true color" or that due only to substances in solution. Water containing matter in suspension should be filtered before the observation is made until no visible turbidity remains. Either filter paper or the Berkefeld filter should be used.

The platinum-cobalt method of measuring color is considered the standard. The unit of color is that produced by one part per million of platinum.

A standard solution having a color of 500 is made as follows: 1.246 grams of potassium platinic chloride ($PtCl_42KCl$), or an amount equivalent to 0.5 gram of platinum; 1.00 gram crystallized cobalt chloride ($CoCl_26H_6O$), or an amount equivalent to 0.25 grams of cobalt.

Dissolve in 100 c.c. of concentrated hydrochloric acid.

Dilute to 1 liter with distilled water.

Dilute measured quantities of this solution with water in 50 c.c. Nessler tubes to prepare standards having colors of 0, 5, 10, 15, etc. The color of a sample of water is observed by filling a Nessler tube and by comparing with standards.

As platinum or its compounds is almost impossible to obtain at present, the color of the water to be tested may be compared with that of glass disks, which are calibrated to correspond with colors on the platinum scale. This method is known as the U. S. Geological Survey Standard. (This color comparison apparatus is listed as item A 1836, proposed revision, paragraph 846, Manual for Medical Department, 1916.)

Turbidity.—Turbidity is synonymous with muddiness, usually due to clay or silt.

The standard is the method introduced by the U. S. Geological Survey using Pear's Precipitated Fuller's Earth. The earth is ignited, ground, passed through a 200 mesh sieve and weighed. One gram suspended in one liter of water gives a turbidity of 1000. Standards for comparison are prepared from this stock suspension by dilution with distilled water. The usual standards are 0, 5, 10, 15, etc. Both sample and standard should be thoroughly shaken before making the comparison. For field determinations the U. S. Geological Survey Turbidity Rod is used.

CHEMICAL EXAMINATION.—A sanitary chemical analysis is not really an analysis at all properly so-called, but is a series of determinations made with a view of assisting the judgment.

The object of a sanitary chemical analysis is not to determine the amount of certain compounds of carbon, hydrogen and nitrogen, etc., in the water, because these compounds are in themselves dangerous, but to determine the presence or absence of, and if present, the amount of such compounds as will aid us in tracing the past history or the present condition of the particular water that is being studied.

Expression of Results.—Results are expressed in parts per million, abbreviated p.p.m.

1 milligram in 1000 c.c. equals 1 p.p.m.

1 p.p.m. equals 0.058 grain per U. S. gallon.

1 grain per gallon equals 17.1 p.p.m.

In a sanitary chemical analysis, the following determinations are usually made:

Nitrogen as albuminoid ammonia.
Nitrogen as free ammonia.
Nitrogen as nitrites.
Nitrogen as nitrates.
Oxygen consumed.
Oxygen dissolved.
Solids:
  Total.
  Volatile.
  Fixed.
Chlorin.
Hardness.
Reaction:
  Acidity.
  Alkalinity.

Determinations of substances that have toxic effect on the human system, such as heavy metals, alkaloids, etc., are sometimes necessary.

NITROGEN DETERMINATIONS.—These are readily made and offer a convenient means of measuring the successive steps of organic decomposition and inorganic oxidation. This circulation of nitrogen from the organic to the mineral state is known as nitrification and is brought about by the so-called nitrifying bacteria. The first step in the decomposition of organic matter produces nitrogen in the form of ammonia. The next step is oxidation to nitrite and the final step oxidation to nitrate.

Nitrogen as Free Ammonia.—Ammonia determinations are made by a method known as Nesslerization. This consists of comparing the color produced by Nessler's reagent in the unknown sample with colors of known standards. These standards are prepared by adding measured quantities of a standard ammonium chloride solution and making up to a definite volume with ammonia-free water.

Procedure.—*Distillation Method.*—Use a metal or a glass flask connected with a condenser so that the distillate may drop from the condenser tube directly into a Nessler tube or a flask. Free the apparatus from ammonia by boiling distilled water until the distillate shows no trace of ammonia. After this has been done, empty the distilling flask and measure into it 500 c.c. or a smaller portion diluted to 500 c.c. with ammonia-free water. If the sample is acid or if the presence of urea is suspected add about 0.5 gram of sodium carbonate before distillation. Omit this if possible as it tends to increase "bumping." Apply heat so that the distillation may proceed at the rate of not more than 10 c.c. nor less than 6 c.c. per minute. Collect the distillate in four Nessler tubes, 50 c.c. to each tube, or if the nitrogen is high, in a 200 c.c. graduated flask. These receptacles contain the ammonia nitrogen to be measured as hereafter described.

NESSLERIZATION.—*Reagents.*—1. Ammonia-free water.

2. Standard ammonium chloride solution. Dissolve 3.82 grams of ammonium chloride in ammonia-free water and dilute to 1 liter; dilute 10 c.c. of this to 1 liter with ammonia-free water. One c.c. equals 0.00001 gram of nitrogen.

3. *Nessler Reagent.*—Dissolve 50 grams of potassium iodide in a minimum quantity of cold water. Add a saturated solution of mercuric chloride until a slight precipitate persists permanently. Add 400 c.c. of 50 per cent. solution of potassium hydroxide, made by dissolving the potassium hydroxide and allowing it to clarify by sedimentation before using. Dilute to 1 liter, allow to settle and decant. This solution

should give the required color with ammonia within five minutes after addition and should not produce a precipitate with small amounts of ammonia within two hours.

Procedure.—Prepare a series of 16 Nessler tubes containing the following amounts of the standard ammonium chloride solution, diluted to 50 c.c. with ammonia-free water, namely: 0.0, 0.1, 0.3, 0.5, 0.7, 1.0, 1.4, 1.7, 2, 2.5, 3, 3.5, 4, 4.5, 5, and 6 c.c. These solutions will contain 0.00001 gram of nitrogen for each cubic centimeter of the standard solution.

Nesslerize the standards and the distillates by adding approximately 1 c.c. of Nessler reagent to each tube. Do not stir the contents of the tubes. The temperature of the tubes should be practically the same as that of the standards, otherwise the colors will not be directly comparable. Allow the tubes to stand at least ten minutes after Nesslerizing. Compare the color produced in the tubes with that in the standards by looking vertically downward through them at a white or mirrored surface placed at an angle in front of a window so that to reflect the light upward.

After the readings have been recorded add the results obtained by Nesslerizing each portion of the entire distillate. If 500 c.c. of the sample is distilled this sum, expressed in cubic centimeters and multiplied by 0.02, will give the number of parts per million of ammonia nitrogen in the sample. If x c.c. of sample is used multiply the sum of the readings by $\frac{10}{x}$.

Example: the results of Nesslerization were as follows:

1st	50 c.c. =	1.4 c.c. of standard solution.	
2d	50 c.c. =	1.0 c.c.	" "
3d	50 c.c. =	1.6 c.c.	" "
4th	50 c.c. =	0.0 c.c.	" "
5th	50 c.c. =	0.0 c.c.	" "
Total	500 c.c. =	3.0 c.c.	" "

Then the ammonia nitrogen in parts per million is equal to $(3.0 \times 0.02) = 0.06$.

Suppose only 400 c.c. of sample were used and with results as follows:

1st	50 c.c.	—	1.8 c.c.	of standard solution.
2d	50 c.c.	—	1.2 c.c.	"	"
3d	50 c.c.	—	0.8 c.c.	"	"
4th	50 c.c.	—	0.2 c.c.	"	"
5th	50 c.c.	—	0.0 c.c.	"	"
6th	50 c.c.	—	0.0 c.c.	"	"

Total 400 c.c.   4.0 c.c.   "   "

Ammonia nitrogen in parts per million is equal to $(4.0 \times \frac{10}{400})$ = 0.1.

If the ammonia is known to be high the distillate may be collected in 200 c.c. flasks and an aliquot part Nesslerized.

Nitrogen as Albuminoid Ammonia.—Nitrogen as albuminoid ammonia is an approximate measure of nitrogenous organic matter from two sources, vegetable and animal. It does not exist as such but is the nitrogen driven off in the form of ammonia when water is distilled with an alkaline permanganate solution. The amount is an index of the matter existing in an unstable condition.

Reagents.—Alkaline potassium permanganate. Pour 1200 c.c. of distilled water into a porcelain dish of 2500 c.c. capacity, boil ten minutes and turn off the gas. Add 16 grams of C. P. potassium permanganate and stir until solution is complete. Then add 800 c.c. of 50 per cent. clarified solution of potassium hydroxide or an equivalent amount of sodium hydroxide and enough distilled water to fill the dish. Boil down to 2000 c.c. Test this solution for ammonia by making a blank determination. Correct determinations by the amount of this blank.

Procedure.—After the collection of the distillate for ammonia nitrogen add 50 c.c. (or more if necessary to ensure the complete oxidation of the organic matter) of alkaline potassium permanganate and continue the distillation until

at least four portions, and preferably five portions, of 50 c.c. each, of distillate have been collected in separate tubes. Determine the albuminoid nitrogen in the distillate by Nesslerization. If the albuminoid nitrogen is known to be high it is convenient to collect the distillate in a 200 c.c. flask and to Nesslerize an aliquot part of it.

Dissolved albuminoid nitrogen may be determined in a sample from which suspended matter has been removed by filtration either through filter paper or through a Berkefeld filter. Suspended albuminoid nitrogen is the difference between the total and the dissolved albuminoid nitrogen.

Nitrogen as Nitrites.—*Reagents.*—1. Sulfanilic acid solution: Dissolve 8.00 grams of the purest sulfanilic acid in 1000 c.c. of $\frac{5}{N}$ acetic acid (sp. gr. 1.041) or in 1000 c.c. of water containing 50 c.c. of concentrated hydrochloric acid. This is practically a saturated solution.

2. A-naphthylamine acetate or chloride solution: Dissolve 5.00 grams solid a-naphthylamine in 1000 c.c. of $\frac{5}{N}$ acetic acid or in 1000 c.c. of water containing 8 c.c. of concentrated hydrochloric acid. Filter the solution through washed absorbent cotton or an alundum filter.

3. Sodium nitrite stock solution: Dissolve 1.1 gram silver nitrite in nitrite-free water; precipitate the silver with sodium chloride solution and dilute the whole to 1 liter.

4. Standard sodium nitrite solution: Dilute 100 c.c. of solution No. 3 to 1 liter, then dilute 50 c.c. of this solution to 1 liter with sterilized nitrite-free water, add 1 c.c. of chloroform, and preserve in a sterilized bottle. One c.c. = 0.0005 mg. nitrogen.

Procedure.—Place in a standard Nessler tube 50 c.c. of the sample, decolorized if necessary, with nitrite-free aluminum hydroxide or a smaller amount diluted to 50 c.c. At the same time prepare in Nessler tubes a set of standards, by diluting to 50 c.c. with nitrite-free water, various amounts of the standard nitrite solution. The following amounts of

standard solution are suggested: 0.0, 0.1, 0.2, 0.4, 0.7, 1, 1.4, 1.7, 2, and 2.5 c.c. Add 1 c.c. of the sulfanilic acid solution and 1 c.c. of the a-naphthylamine acetate or hydrochloride solution to the sample and to each standard. Mix thoroughly and allow to stand ten minutes; then compare the sample with the standards. Do not allow the sample to stand more than one-half hour before making the comparison. If the color of the sample is deeper than that of the highest standard repeat the test on a diluted sample. If 50 c.c. of the sample is used 0.01 times the number of c.c. of the standard matched equal parts per million of nitrite nitrogen. Satisfactory results can be obtained by using either hydrochloric or acetic acid in preparing the test solutions, but the speed of the reaction is more rapid if acetic acid is used.

Nitrogen as Nitrates. — *Reagents.* — 1. Phenoldisulphonic acid: Dissolve 25 grams of pure white phenol in 150 c.c. of pure concentrated sulphuric acid. Add 75 c.c. of fuming sulphuric acid (15 per cent. SO_3), stir well, and heat for two hours at about 100° C.

2. Potassium hydroxide solution: Prepare an approximately $\frac{12}{N}$ solution, 10 c.c. of which will neutralize about 4 c.c. of the phenoldisulphonic acid.

3. Standard nitrate solution: Dissolve 0.72 gram of pure recrystallized potassium nitrate in 1 liter of distilled water. Evaporate cautiously to dryness 10 c.c. of the solution on the water-bath. Moisten residue quickly and thoroughly with 2 c.c. of phenoldisulphonic acid and dilute to 1 liter. This is the standard solution, 1 c.c. of which equals 0.001 mg. of nitrate nitrogen.

4. Standard silver sulphate solution: Dissolve 4.4 grams of silver sulphate free from nitrate in 1 liter of water. One c.c. of this solution is equal to 1 mg. of chloride.

Procedure.—The alkalinity, chloride, and nitrite content, and color of the sample must first be determined. Measure into an evaporating dish 100 c.c. of the sample or if the nitrate

is very high such volume of the sample as has been found by previous determination to contain about 0.01 mg. of nitrate nitrogen. Add sufficient $\frac{N}{50}$ H_2SO_4 (1 c.c. of this solution is equivalent to 1 mg. of $CaCO_3$) to neutralize the alkalinity. If the sample contains more than 30 p.p.m. of chloride, the amount should be reduced to about this figure by adding standard silver sulphate solution, 1 c.c. of which is equivalent to 1 mg. of chloride. Heat the mixture to boiling, add a little aluminum hydroxide, stir, filter, and wash with small amounts of hot water. Evaporate the filtrate to dryness, and add 2 c.c. of the phenoldisulphonic acid, rubbing with a glass rod to ensure intimate contact. If the residue becomes packed or appears vitreous because of the presence of much iron, heat the dish on the water-bath for a few minutes. Dilute the mixture with distilled water, and add slowly a strong solution of potassium hydroxide or ammonium hydroxide until the maximum color is developed. Transfer the solution to a Nessler tube, filtering if necessary. If nitrate is present a yellow color will be formed. Compare the color with that of standards made by adding 2 c.c. of strong potassium hydroxide or ammonium hydroxide to various amounts of standard nitrate solution and diluting them to 50 c.c. in Nessler tubes. The following amounts of standard nitrate solution are suggested: 0, 0.5., 1, 1.5, 2, 4, 6, 8, 10, 15, 20, and 40 c.c. These standards may be kept several weeks without deterioration. If 100 c.c. of water is used the number of cubic centimeters of the standard multiplied by 0.01 is equal to parts per million of nitrate nitrogen.

Standards that will remain permanent for several years if stored in the dark may be prepared from tripotassium nitrophenoldisulphonate.

If nitrite nitrogen is present in excess of 1 part per million it should be oxidized by heating the samples a few minutes with a few drops of hydrogen peroxide free from nitrate repeatedly added or by adding dilute potassium perman-

ganate in the cold until a faint pink coloration appears; the nitrogen equivalent of the nitrite thus oxidized to nitrate is then subtracted from the final nitrate nitrogen reading.

OXYGEN CONSUMED.—This is a measure of the carbonaceous organic matter which is partly oxidized by potassium permanganate. The expression is synonymous with oxygen required, oxygen absorbed and oxygen consuming capacity.

Reagents.—1. Dilute sulphuric acid: Dilute 1 part of concentrated sulphuric acid with 3 parts of distilled water and free the solution from oxidizable matter by adding potassium permanganate until a faint pink color persists after the solution has stood several hours.

2. Standard ammonium oxalate: Dissolve o.888 gram of the pure salt in 1 liter of distilled water. One c.c. is equivalent to o.1 mg. of oxygen. An equivalent quantity of oxalic acid or sodium oxalate may be used.

3. Standard potassium permanganate: Dissolve o.4 gram of the crystallized salt in 1 liter of distilled water. Add 10 c.c. of the dilute sulphuric acid (Solution No. 1) and 10 c.c. of this solution of potassium permanganate to 100 c.c. of distilled water, and digest thirty minutes. Add 10 c.c. of the ammonium oxalate solution (Solution No. 2) and then add potassium permanganate until a pink coloration appears. This destroys the oxygen-consuming capacity of the water used. Now add another 10 c.c. of ammonium oxalate solution and titrate with potassium permanganate. Adjust the potassium permanganate solution so that 1 c.c. is equivalent to 1 c.c. of ammonium oxalate solution or o.1 mg. of available oxygen.

Acid Digestion.—Place in a flask 100 c.c. of the water, or, if the water is of high organic content, a smaller portion diluted to 100 c.c. Add 10 c.c. of sulphuric acid solution and 10 c.c. of standard potassium permanganate and digest the liquid exactly thirty minutes in a bath of boiling water the level of which is kept above the level of the contents of

the flask. If the quantity of permanganate is insufficient for complete oxidation repeat the digestion with a larger quantity; at least 5 c.c. excess of the standard permanganate should be present when the ammonium oxalate solution is added. Remove the flask, add 10 c.c. of the ammonium oxalate solution, and titrate with the standard permanganate until a faint but distinct color is obtained. If 100 c.c. of water is used the number of cubic centimeters of potassium permanganate solution in excess of the number of cubic centimeters of ammonium oxalate solution is equal to parts per million of oxygen consumed.

OXYGEN DISSOLVED.—The solubily of oxygen in water is a function of the temperature and pressure.

The following table indicates the parts per million of oxygen required to saturate water under an atmospheric pressure of 760 mm. and for various temperatures. Results are expressed in parts per million and percentage saturation:

Temperature ° C	p.p.m.	Temperature ° C.	p.p.m.
0	14.62	16	9.95
1	14.23	17	9.74
2	13.84	18	9.54
3	13.48	19	9.35
4	13.13	20	9.17
5	12.80	21	8.99
6	12.48	22	8.83
7	12.17	23	8.68
8	11.87	24	8.53
9	11.59	25	8.38
10	11.33	26	8.22
11	11.08	27	8.07
12	10.83	28	7.92
13	10.60	29	7.77
14	10.37	30	7.63
15	10.15		

Reagents.—1. Sulphuric acid, concentrated: Sp. gr. 1.83–1.84.

2. Potassium permanganate: Dissolve 6.32 grams of the salt in water and dilute the solution to 1 liter.

3. Potassium oxalate: A 2 per cent. solution.

4. Manganous sulphate: Dissolve 480 grams of the salt in water and dilute the solution to 1 liter.

5. Alkaline potassium iodide: Dissolve 700 grams of potassium hydroxide and 150 grams of potassium iodide in water and dilute the solution to 1 liter.

6. Hydrochloric acid: Concentrated (sp. gr. 1.18 to 1.19).

7. Sodium thiosulphate: A $\frac{N}{40}$ solution. Dissolve 6.2 grams of chemically pure recrystallized sodium thiosulphate in water and dilute the solution to 1 liter with freshly boiled distilled water. Each cubic centimeter is equivalent to 0.2 mg. of oxygen or to 0.1395 c.c. of oxygen at 0° C. and 760 mm. pressure. Inasmuch as this solution is not permanent, it should be standardized occasionally against a $\frac{N}{40}$ solution of potassium bichromate. The keeping qualities of the thiosulphate solution are improved by adding to each liter 5 c.c. of chloroform and 1.5 grams of ammonium carbonate.

8. Starch solution: Mix a small amount of clean starch with cold water until it becomes a thin paste and stir this mass into 150 to 200 times its weight of boiling water. Boil for a few minutes then sterilize. It may be preserved by adding a few drops of chloroform.

Collection of Sample.—Collect the sample in a narrow-necked glass-stoppered bottle of 250 to 270 c.c. capacity. The following procedure should be followed in order to avoid entrainment or absorption of atmospheric oxygen. In collecting from a tap fill the bottle through a glass or rubber tube extending well into the tap and to the bottom of the bottle. To avoid air bubbles allow the bottle to overflow for several minutes, and then carefully replace the glass stopper so that no air bubble is entrained. In collecting from the

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surface of a pond or tank connect the sample bottle to a bottle of 1 liter capacity. Provide each bottle with a two-hole rubber stopper having one glass tube extending to the bottom and another glass tube entering but not projecting into the bottle. Connect the short tube of the sample bottle with the long tube of the liter bottle. Immerse the sample bottle in the water and apply suction to the outlet of the liter bottle. To collect a sample at any depth arrange the elevation then the inlet tube of the sample bottle. Lower the two bottles, in any convenient form of cage properly weighted, to the desired depth. Water entering during the descent will be flushed through into the liter bottle. When air bubbles cease rising to the surface raise the bottles. Finally replace the perforated stopper of the sample bottle with a glass stopper in such manner as to avoid entraining bubbles of air.

Procedure.—Remove the stopper from the bottle and add, first, 0.7 c.c. of the concentrated sulphuric acid and then 1 c.c. of the potassium permanganate solution. These and all other reagents should be introduced by pipette under the surface of the liquid. Insert the stopper and mix by inverting the bottle several times. After twenty minutes have elapsed destroy the excess of permanganate by adding 1 c.c. of the potassium oxalate solution, the bottle being at once restoppered and its contents mixed. If a noticeable excess of potassium permanganate is not present at the end of twenty minutes, again add 1 c.c. of the potassium permanganate solution. If this is still insufficient use a stronger potassium permanganate solution. After the liquid has been decolorized by the addition of potassium oxalate add 1 c.c. of the manganous sulphate solution and 3 c.c. of the alkaline potassium iodide solution. Allow the precipitate to settle. Add 2 c.c. of the hydrochloric acid and mix by shaking.

The procedure to this point must be carried out in the

field, but after the acid has been added and the stopper replaced there is no further change, and the rest of the test may be performed within a few hours, whenever convenient. Transfer 200 c.c. of the contents of the bottle to a flask and titrate with $\frac{N}{40}$ sodium thiosulphate, using a few cubic centimeters of the starch solution as indicator toward the end of the titration. Do not add the starch solution until the color has become faint yellow, and titrate until the blue color disappears.

The use of potassium permanganate is made necessary by high nitrite or organic matter. The procedure outlined must be followed in all work on sewage and partly purified effluents or seriously polluted streams or samples whose nitrite nitrogen exceeds 0.1 part per million. In testing other samples the procedure may be shortened by beginning with the addition of the manganous sulphate solution and proceeding from that point as outlined, except that only 1 c.c. of alkaline potassium iodide need be added.

Calculation of Results.—Oxygen shall be reported in parts per million by weight. It is sometimes convenient to know the number of cubic centimeters per liter of the gas at 0° C. temperature and 760 mm. of pressure and also to know the percentage which the amount of gas present is of the maximum amount capable of being dissolved by distilled water at the same temperature and pressure. If 200 c.c. of the sample is taken the number of cubic centimeters of $\frac{N}{40}$ thiosulphate used is equal to parts per million of oxygen. Corrections for volume of reagents added amount to less than 3 per cent. and are not justified except in work of unusual precision. To obtain the result in cubic centimeters per liter multiply the number of cubic centimeters of thiosulphate used by 0.698. To obtain the result in percentage of saturation divide the number of cubic centimeters of thiosulphate by the figure in the Table opposite the temperature of the water. The term "saturation" refers to a condition of equilibrium

between the solution and an oxygen pressure in the atmosphere corresponding to 158.8 mm., or approximately one-fifth atmosphere. The true saturation or equilibrium between the solution and pure oxygen is nearly five times this value, and consequently values in excess of 100 per cent. saturation frequently occur in the presence of oxygen-forming plants.

Solids.—The total solids furnishes an index of the total quantity of foreign impurities and further furnishes a rough index of the relative quantity of inorganic and organic substances which make up these impurities. The loss on ignition represents the amount of organic matter in the water. The weight of the substance remaining represents the inorganic matter.

Total Solids.—Ignite and weigh a clean platinum dish and measure into it 100 c.c. of the thoroughly shaken sample. Evaporate to dryness on a water-bath. Then heat the dish in an oven at 103° C. or 180° C. for one hour. Cool in a desiccator and weigh. The temperature of drying should be mentioned in the report. The increase in weight gives the total solids or residue on evaporation. If 100 c.c. of the sample was taken this weight expressed in milligrams and multiplied by 10 is equal to parts per million of residue on evaporation.

Fixed and Volatile Solids.—Procedure.—Ignite the residue in the platinum dish at a low red heat. If great accuracy is desired this should be done in an electric muffle furnace or in a radiator, which consists of a platinum or a nickel dish large enough to allow an air space of about half an inch between it and the dish within it, the inner dish being supported by a triangle of platinum wire laid on the bottom of the outer dish. A disk of platinum or nickel-foil large enough to cover the outer dish is suspended over the inner dish to radiate the heat into it. The larger dish is heated to bright redness until the residue is white or nearly so. Allow the

dish to cool and moisten the residue with a few drops of distilled water. Dry the residue in the oven, cool in a desiccator and weigh. The fixed residue on evaporation is the difference between this weight and the weight of the dish.

The loss on ignition is the difference between the total residue on evaporation and the fixed residue on evaporation.

If the odor and color on ignition of some residue give helpful clues to the character of the organic matter, record them.

REACTION.—The reaction of natural water is commonly slightly alkaline, although waters holding much free acid in solution, usually sulphuric acid, are by no means rare.

ALKALINITY.—The alkalinity of a natural water represents its content of carbonate, bicarbonate, borate, silicate, phosphate and hydroxide, etc. Alkalinity is determined by neutralization with standard sulphuric acid or potassium bisulphate in the presence of phenolphthalein and either methyl orange, erythrosine, or lacmoid as indicators. Methyl orange may be used except in waters containing aluminum sulphate or iron sulphate. The relations between estimates in the presence of these indicators and the carbonate, bicarbonate and hydroxide radicles are indicated in Table on page 312. The alkalinity of carbonates in the presence of phenolphthalein is different from that in the presence of methyl orange, partly because of loss of carbon dioxide and partly because of defects in phenolphthalein as an indicator in such conditions.

Reagents.—1. Sulphuric Acid or Potassium Bisulphate: A $\frac{N}{50}$ solution.

2. Phenolphthalein Indicator: Dissolve 5 grams of a good quality of phenolphthalein in 1 liter of 50 per cent. alcohol. Neutralize with $\frac{N}{10}$ potassium hydroxide. The alcohol should be diluted with boiled distilled water.

3. Methyl Orange Indicator: Dissolve 0.5 gram of a good grade of methyl orange in 1 liter of distilled water. Keep the solution in the dark.

4. Lacmoid Indicator: Dissolve 2 grams of lacmoid in 1 liter of 50 per cent. alcohol. Dilute the alcohol with freshly boiled distilled water.

5. Erythrosine Indicator: Dissolve 0.5 gram of erythrosine (the sodium salt) in 1 liter of freshly distilled water.

RELATIONS BETWEEN ALKALINITY TO PHENOLPHTHALEIN AND THAT TO METHYL ORANGE, ERYTHROSINE, OR LACMOID, IN PRESENCE OF BICARBONATE, CARBONATE, AND HYDROXIDE.

Result of titration.	Value of radicle expressed in terms of calcium carbonate.		
	Bicarbonate.	Carbonate.	Hydroxide.
$P = 0$	T	0	0
$P < \frac{1}{2}T$	$T-2P$	$2\,P$	0
$P = \frac{1}{2}T$	0	$2\,P$	0
$P > \frac{1}{2}T$	0	$2(T-P)$	$2P-T$
$P = T$	0	0	T

T = total alkalinity in presence of methyl orange, erythrosine, or lacmoid.

P = alkalinity in presence of phenolphthalein.

Procedure with Phenolphthalein.—Add 4 drops of phenolphthalein indicator to 50 to 100 c.c. of the sample in a white porcelain casserole or an Erlenmeyer flask over a white surface. If the solution becomes colored, hydroxide or normal carbonate is present. Add $\frac{N}{50}$ sulphuric acid from a burette until the coloration disappears.

The phenolphthalein alkalinity in parts per million of calcium carbonate is equal to the number of cubic centimeters of $\frac{N}{50}$ sulphuric acid used multiplied by 20 if 50 c.c. of the sample was used, or by 10 if 100 c.c. was used.

Procedure with Methyl Orange.—Add 2 drops of methyl orange indicator to 50 to 100 c.c. of the sample or to the solution to which phenolphthalein has been added, in a white porcelain casserole or an Erlenmeyer flask, over a white surface. If the solution becomes yellow, hydroxide, normal carbonate or bicarbonate is present. Add $\frac{N}{50}$ sul-

phuric acid from a burette until the faintest pink coloration appears. The methyl orange alkalinity in parts per million of calcium carbonate is equal to the total number of cubic centimeters of $\frac{N}{50}$ sulphuric acid used multiplied by 20 if 50 c.c. of the sample was used or by 10 if 100 c.c. was used.

Procedure with Erythrosine.—Add 5 c.c. of neutral chloroform and 1 c.c. of erythrosine indicator to 50 or 100 c.c. of the sample in a 250 c.c. clear glass-stoppered bottle. If the chloroform becomes rose colored on shaking, hydroxide, bicarbonate or normal carbonate is present. Add $\frac{N}{50}$ sulphuric acid from a burette until the chloroform becomes colorless. A white surface behind the bottle facilitates detection of a trace of color as the end-point is approached. The calculation is the same as with phenolphthalein alkalinity.

ACIDITY.—Waters may have an acid reaction because of the presence of free carbon dioxide, mineral acids or some of their salts, especially that of iron and aluminum.

Reagents.—1. $\frac{N}{50}$ sodium carbonate: Dissolve 1.06 grams of anhydrous sodium carbonate in 1 liter of boiled distilled water that has been cooled in an atmosphere free from carbon dioxide. Preserve this solution in bottles of resistant glass protected from the air by tubes filled with soda-lime. One c.c. is equivalent to 1 mg. of $CaCO_3$.

2. $\frac{N}{22}$ sodium carbonate: Dissolve 2.41 grams of anhydrous sodium carbonate in 1 liter of boiled distilled water that has been cooled in an atmosphere free from carbon dioxide. Preserve this solution in bottles of resistant glass protected from the air by tubes filled with soda-lime. One c.c. is equivalent to 1 mg. of CO_2.

3. Phenolphthalein indicator (see page 311).

4. Methyl orange indicator (see page 311).

TOTAL ACIDITY. — *Procedure.* — Add 4 drops of phenolphthalein indicator or 50 to 100 c.c. of the sample in a white porcelain casserole to an Erlenmeyer flask over a white surface. Add $\frac{N}{50}$ sodium carbonate until the solution turns

pink. The total acidity in parts per million of calcium carbonate is equal to the number of cubic centimeters of $\frac{N}{50}$ sodium carbonate used multiplied by 20 if 50 c.c. of the sample was used or by 10 if 100 c.c. was used.

FREE CARBON DIOXIDE.—Carbon dioxide may exist in water in three forms: free carbon dioxide, bicarbonate and carbonate. One-half the carbon dioxide as bicarbonate is known as the half-bound carbon dioxide. The carbon dioxide as carbonate plus one-half that as bicarbonate is known as bound carbon dioxide.

Procedure.—Pour 100 c.c. of the sample into a tall narrow vessel, preferably a 100 c.c. Nessler tube. Add 10 drops of phenolphthalein indicator and titrate rapidly with $\frac{N}{22}$ sodium carbonate, stirring gently, until a faint but permanent pink color is produced. The free carbon dioxide (CO_2) in parts per million is equal to ten times the number of cubic centimeters of $\frac{N}{22}$ sodium carbonate used.

Because of the ease with which free carbon dioxide escapes from water, particularly when the gas is present in large amount, a special sample should be collected for this determination, which should preferably be made at the time of collection. If the analysis cannot be made at the time of collection approximate results with water not too high in free carbon dioxide may be obtained on samples collected in bottles completely filled so as to leave no air space under the stopper. Bottled samples should be kept, until tested, at a temperature lower than that of the water when collected. If mineral acids or certain salts are present correction must be made. At best the results of the titration are uncertain because the proper end-point for correct results differs in color with different types of water.

CHLORIDE.—Comparison of the chloride content of a water with that of other waters in the vicinity known to be unpolluted frequently affords useful information as to its sanitary quality. If, however, the chloride normally exceeds

20 parts per million because of chloride-bearing mineral deposits the chloride content of a water has little sanitary significance.

Reagents.—1. Standard Sodium Chloride Solution: Dissolve 16.48 grams of pure fused sodium chloride in 1 liter of distilled water. Dilute 100 c.c. of this stock solution to 1 liter in order to obtain a standard solution, each cubic centimeter of which contains 0.001 gram of chloride.

2. Standard Silver Nitrate Solution: Dissolve about 2.4 grams of silver nitrate crystals in 1 liter of distilled water. Standardize this with the standard salt solution and adjust, correcting for volume so that 1 c.c. will be exactly equivalent to 0.0005 gram of chloride.

3. Potassium Chromate Indicator: Dissolve 50 grams of neutral potassium chromate in a little distilled water. Add enough silver nitrate to produce a slight red precipitate. Filter and dilute the filtrate to 1 liter with distilled water.

Procedure.—Add 1 c.c. of potassium chromate indicator to 50 c.c. of the sample in a 6-inch white porcelain evaporating dish or a 150 c.c. Erlenmeyer flask over a white surface. Titrate with the silver nitrate solution under similar conditions of volume, light and temperature as were used in standardizing the silver nitrate until a faint reddish coloration is perceptible. The detection of the end-point is facilitated by comparison of the contents of the porcelain dish with those of another dish containing the same quantity of potassium chromate indicator in 50 c.c. of distilled water. Some analysts prefer to make the titration in a dark-room provided with a yellow light. The end-point is very sharp by electric light and also by daylight with photographic yellow glass. The titration may be made in Nessler tubes if the solutions are standardized under similar conditions.

If the amount of chloride is very high use 25 c.c., or even a smaller quantity, diluting the volume taken to 50 c.c. with distilled water. If the amount of chloride is very low concen-

trate 250 c.c. of the sample to 50 c.c. by evaporation. Rotate the liquid to make sure that no residue remains undissolved on the walls of the dish, and, if necessary, use a rubber-tipped glass rod to assist in this operation.

HARDNESS.—A water containing certain mineral constituents in solution chiefly calcium and magnesium which form insoluble compounds with soap is said to be hard. Carbon dioxide in water increases the solubility of calcium and magnesium carbonates forming bicarbonate. If carbon dioxide is removed from the water by boiling the bicarbonate is decomposed and calcium and magnesium are partly precipitated, and the water is softened to the extent that these substances are precipitated. The hardness thus removed is called the temporary hardness. The hardness remaining after boiling is due mainly to calcium and magnesium in equilibrium with sulphate chloride and nitrate and residual carbonate. Such is called permanent hardness.

Total Hardness by Soap Method.—The determination of hardness by the soap method roughly approximates the amount of calcium and magnesium in a water, though it actually measures the soap-consuming power of the water.

Reagents.—1. Standard Calcium Chloride Solution: Dissolve 0.2 gram of pure calcite (calcium carbonate) in a little dilute hydrochloric acid, being careful to avoid loss of solution by spattering. Evaporate the solution to dryness several times with distilled water to expel excess of acid. Dissolve the residue in distilled water and dilute the solution to 1 liter. One c.c. of this dilution is equivalent to 0.2 mg. of calcium carbonate.

2. Standard Soap Solution: Dissolve 100 grams of dry white Castile soap in 1 liter of 80 per cent. alcohol and allow this solution to stand several days before standardizing.

Method of Standardization.—Dilute 20 c.c. of the calcium chloride solution in a 250 c.c. glass-stoppered bottle to 50 c.c. with distilled water which has been recently boiled and

cooled. Add soap solution from a burette, 0.2 or 0.3 c.c. at a time, shaking the bottle vigorously after each addition until a lather remains unbroken for five minutes over the entire surface of the water while the bottle lies on its side. Then adjust the strength of the stock solution with 70 per cent. alcohol so that the resulting diluted soap solution will give a permanent lather when 6.40 c.c. of it is properly added to 20 c.c. of standard calcium chloride solution diluted to 50 c.c. Usually 75 to 100 c.c. of the stock soap solution is required to make 1 liter of the standard soap solution. The quantity of calcium carbonate equivalent to each cubic centimeter of the standard soap solution consumed in the titration is indicated in Table.

TOTAL HARDNESS IN PARTS PER MILLION OF $CaCO_3$ FOR EACH TENTH OF A CUBIC CENTIMETER OF SOAP SOLUTION WHEN 50 C.C. OF THE SAMPLE IS TITRATED.

Cubic centimeters of soap solution.	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
0.0	..	..	..	..	..	..	..	0.0	1.6	3.2
1.0	4.8	6.3	7.9	9.5	11.1	12.7	14.3	15.6	16.9	18.2
2.0	19.5	20.8	22.1	23.4	24.7	26.0	27.3	28.6	29.9	31.2
3.0	32.5	33.8	35.1	36.4	37.7	38.0	40.3	41.6	42.9	44.3
4.0	45.7	47.1	48.6	50.0	51.4	52.9	54.3	55.7	57.1	58.6
5.0	60.0	61.4	62.9	64.3	65.7	67.1	68.6	70.0	71.4	72.9
6.0	74.3	75.7	77.1	78.6	80.0	81.4	82.9	84.3	85.7	97.1
7.0	88.6	90.0	91.4	92.9	94.3	95.7	97.1	98.6	100.0	101.5

Procedure.—Measure 50 c.c. of the water into a 250 c.c. bottle and add to it soap solution in small quantities in precisely the same manner as described under the standardization of the soap solution. From the number of cubic centimeters of soap solution used obtain from the above Table or

from a plotted curve the total hardness of the water in parts per million of calcium carbonate.

To avoid mistaking the false or magnesium end-point for the true one when adding the soap solution to waters containing magnesium salts, read the burette after the titration is apparently finished and add about 0.5 c.c. more of soap solution. If the end-point was due to magnesium the lather will disappear. Soap solution must then be added until the true end-point is reached. Usually the false lather persists for less than five minutes.

If more than 7 c.c. of soap solution is required for 50 c.c. of the water take less of the sample and dilute it to 50 c.c. with distilled water which has been recently boiled and cooled. This step reduces somewhat the disturbing influence of magnesium, which consumes more soap than an equivalent weight of calcium.

INTERPRETATION OF RESULTS OF CHEMICAL DETERMINATIONS.—It should be borne in mind that each class of waters has its own characteristics.

For example, flowing surface waters usually contain small amounts of dissolved matter, more or less suspended matter, depending upon topographical and geological conditions and variable amounts of organic matter, either vegetable or animal, likewise depending upon conditions of population and vegetation. Impounded surface supplies differ from flowing surface supplies, inasmuch as they are always mixed with more or less ground water, and furthermore the element of storage is responsible for uniformity of composition and the bringing into play of physical and biological agencies, which aim for improvement.

Ground waters are usually free from suspended matter. On the other hand the dissolved matter is likely to be high on account of solubility of the various salts with which the water comes in contact. Organic matter, both animal and vegetable, is usually absent or very slight.

The significance of these facts must be judged separately in each particular case.

BACTERIOLOGICAL EXAMINATION OF WATER.

Preparation of Apparatus.—All glassware (sample bottles, pipettes, dilution bottles, Petri dishes, and fermentation tubes), whether new or previously used, must be carefully washed and rinsed, then sterilized by heating for one hour at 160° to 170° C.

For fermentation tests the Dunham tube is recommended. For testing 10 c.c. portions of water the 210 mm. × 27 mm. or equivalent size should be used for the outer tube and the 75 mm. × 10 mm. for the inner tube. These tubes should contain not less than 30 c.c. of media. For testing 1 c.c. portions of water smaller tubes and smaller quantities of media may be used.

Media.—The media used in routine work are lactose broth, gelatin, and agar. They are composed of the following ingredients:

LACTOSE BROTH.

Beef extract	3 grams
Peptone	5 grams
Lactose	10 grams
Water	1 liter

AGAR.

Beef extract	3 grams
Peptone	5 grams
Agar dried at 105° C.	15 grams
Water	1 liter

GELATIN.

Beef extract	3 grams
Peptone	5 grams
Gelatin dried at 105° C.	100 grams
Water	1 liter

The media must be filtered until clear. Do not clear with egg. (For further details see Standard Methods.)

The titer of these media should be 1+ acidity except lactose broth. All sugar media should be neutral to phenolphthalein.

Endo's Medium.—The Endo media used in water analysis is slightly different from the Endo medium used for the isolation of the typhoid organism.

Procedure to Make One Liter.—1. Add 5 grams of beef extract, 10 grams of peptone and 30 grams of agar dried for one-half hour at 105° C. before weighing, to 1000 c.c. of distilled water. Boil on a water-bath until all the agar is dissolved and then make up the loss by evaporation.

2. Cool the mixture to 45° C. in a cold water-bath, then warm to 65° C. in the same bath without stirring.

3. Make up lost weight and titrate, and if the reaction is not already between neutral and +1 adjust to neutral.

4. Filter through cloth and cotton until clear.

5. Distribute 100 c.c. or larger known quantities in flasks large enough to hold the other ingredients which are to be added later.

6. Sterilize in the autoclave at 15 pounds (120° C.) for fifteen minutes after the pressure reaches 15 pounds.

7. Prepare a 10 per cent. solution of basic fuchsin in 95 per cent. alcohol, allow to stand twenty hours, decant and filter the supernatant fluid. This is a stock solution.

8. When ready to make plates, prepare a 10 per cent. solution of anhydrous sodium sulphite. To 10 c.c. of the sodium sulphite solution add 2 c.c. of the fuchsin stock solution and steam five minutes in the Arnold or on a water-bath. To each 100 c.c. of the agar mixture add 1 gram of lactose, dissolve in streaming steam or on a water-bath and add 0.5 c.c. of the fuchsin sulphite solution. The lactose used must be chemically pure and the sulphite solution must be made up fresh every day.

9. Pour plates and allow to harden thoroughly in the incubator before use.

Collection of Sample.—Collect representative samples in sterilized sample bottles, recording time and place of sampling. Transport sample as quickly as possible to laboratory. When several hours are required for transportation the sample must be kept below 10° C.

Determination of Total Count.—In preparing plates, such amounts of the water under examination should be plated as will give from 25 to 250 colonies on a plate, and the aim should be always to have at least two plates giving colonies between these limits. Since it is impracticable to measure less than 0.1 c.c. of water directly, samples with a count higher than 2500 per c.c. must be so diluted with sterile tap or distilled water that 1 c.c. will contain about 2500 bacteria. It will be necessary to make a series of dilutions of samples from variable or unknown sources. When it is possible to obtain plates showing colonies within these limits, only such plates should be considered in recording results, except when the same amount of water has been planted in two or more plates, of which one gives colonies within these limits, while the others give less than 25 or more than 250. In such case the result recorded should be the average of all the plates planted with this amount of water. Ordinarily it is not desirable to plant more than 1 c.c. of water in a plate; therefore when the total number of colonies developing from 1 c.c. is less than 25, it is obviously necessary to record the results as observed, disregarding the general rule given above.

All sample and dilution bottles should be shaken vigorously twenty-five times before samples are removed for dilution or plating. Plating should be done immediately after the dilutions are made; 1 c.c. of the sample or dilution should be used for plating and should be placed in the Petri dish first; 10 c.c. of liquefied medium at a temperature of 40° C. should be added to the 1 c.c. of water in the Petri

dish. The cover of the Petri dish should be lifted just enough for the introduction of the pipette or culture medium, and the lips of all test-tubes or flasks used for pouring the medium should be flamed.

All gelatin plates should be incubated for forty-eight hours, at 20° C., in a dark, well-ventilated incubator in an atmosphere practically saturated with moisture.

All agar plates should be incubated for twenty-four hours, at 37° C., in a dark, well-ventilated incubator in an atmosphere practically saturated with moisture. Glass-covered plates should be inverted in the incubator.

Colonies developing after incubation should be counted with a lens of $2\frac{1}{2}$ diameters' magnification, $3\frac{1}{2}$ X. The engraver's lens No. 146 made by the Bausch & Lomb Optical Company fills the requirements.

In order to avoid fictitious accuracy, and yet to express the results by a method consistent with the precision of the work, ciphers should be used for all except the first two or three places in the numerical figure representing the total count, *e. g.*, a count of 1,325,763 would be reported 1,300,000.

In making tests for the presence of the colon group in samples of drinking water, make tests on five 10 c.c. portions, one 1 c.c. and one 0.1 c.c. portion. This is in accordance with the United States Public Health Service standard.

SUMMARY OF STEPS INVOLVED IN MAKING PRESUMPTIVE, CONFIRMED AND COMPLETED TESTS FOR B. COLI.

Steps in procedure.	Further procedure required.
I. Inoculate lactose broth fermentation tubes; incubate twenty-four hours at 37° C.; observe gas-formation in each tube.	
1. Gas-formation, 10 per cent. or more; constitutes positive presumptive test.	

Steps in procedure.

(*a*) For other than smallest portion of any sample showing gas at this time, and for all portions, including smallest, of sewage and raw water this test is sufficient . . . . None

(*b*) For smallest gas-forming portion, except in examinations of sewage and raw water III

2. Gas-formation less than 10 per cent. in twenty-four hours; inconclusive . . II

II. Incubate an additional twenty-four hours, making a total of forty-eight hours' incubation; observe gas-formation.

1. Gas-formation, any amount; constitutes doubtful test, which must always be carried further III

2. No gas-formation in forty-eight hours; constitutes final negative test . . . None

III. Make Endo plate from smallest gas-forming portion of sample under examination; incubate eighteen to twenty-four hours; observe colonies.

1. One or more colonies typical in appearance.

(*a*) If only "confirmed" test is required None

(*b*) If completed test is required, select two typical colonies for identification . V

2. No typical colonies IV

IV. Replace plate in incubator for an additional eighteen to twenty-four hours; then, whether colonies appear typical or not, select at least two of those which most nearly resemble B. coli V

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Steps in procedure.	Further procedure required.

V. Transfer each colony fished to:

1. Lactose-broth fermentation tube; incubate not more than forty-eight hours at 37° C.; observe gas-formation . . . **None**

2. Agar slant; incubate forty-eight hours at 37° C.

  (*a*) If gas formed in lactose-broth tube inoculated with corresponding culture **VI**

  (*b*) If no gas formed in corresponding lactose broth tube, test is completed and negative . . . . **None**

VI. Make stained cover-slip or slide preparation, and examine microscopically.

1. If preparation shows non-spore-forming bacilli in apparently pure culture, demonstration of B. coli is completed . . . **None**

2. If preparation fails to show non-spore-forming bacilli or shows them mixed with spore-bearing forms or bacteria of other morphology. . . . . **VII**

VII. Replate, to obtain assuredly pure culture, select several colonies of bacilli and repeat steps V and VI.

Routine Procedure.—

First Day:

1. Prepare dilutions as required.

2. Make two (2) gelatin plates from each dilution and incubate at 20° C.

3. Make two (2) agar plates from each dilution and incubate at 37° C.

4. Inoculate lactose-broth fermentation tubes with appropriate amounts for B. coli tests, inoculating two (2) tubes with each amount.

Second Day:
1. Count the agar plates made on the first day.
2. Record the number of lactose-broth fermentation tubes which show 10 per cent. or more of gas.

Third Day:
1. Count gelatin plates made on first day.
2. Record the number of additional fermentation tubes which show 10 per cent. or more of gas.
3. Make a lactose-litmus-agar or Endo medium plate from the smallest portion of each sample showing gas. Incubate plate at 37° C.

NOTE.—In case the smallest portion in which gas has been formed shows less than 10 per cent. of gas, it is well to make a plate also from the next larger portion, so that in case the smallest portion gives a negative end-result it may still be possible to demonstrate B. coli in the next dilution.

Fourth Day:
1. Examine Endo's medium or lactose-litmus-agar plates. If typical colonies have developed, select two and transfer each to a lactose-broth fermentation tube and an agar slant, both of which are to be incubated at 37° C.
2. If no typical B. coli colonies are found, incubate the plates another twenty-four hours.

Fifth Day:
1. Select at least two colonies, whether typical or not, from the Endo medium or lactose-litmus-agar plates, which have been incubated an additional twenty-four hours; transfer each to a lactose-broth fermentation tube and an agar slant and complete the test as for typical colonies.
2. Examine lactose-broth-fermentation tubes inoculated from plates on the previous day. Tubes in which gas has been formed may be discarded after the result has been recorded. Those in which no gas

has formed should be incubated an additional twenty-four hours.

Sixth Day:

1. Examine lactose-broth-fermentation tubes reincubated the previous day.

2. Examine microscopically agar slants corresponding to lactose-fermentation tubes inoculated from plate colonies and showing gas-formation.

INTERPRETATION OF BACTERIOLOGICAL RESULTS.—The number of bacteria is not as important as the kind; however, the number corresponds to the amount of organic pollution. Different temperatures are used for growing the bacteria, as they do not all grow at the same temperatures. The pathogenic bacteria do not grow at 20° C. A water containing great numbers of bacteria when counted upon gelatin at 20° C., and but few colonies upon agar at 37° C., has little sanitary significance, while the reverse would be looked upon as suspicious. The distinction between polluted waters and waters of good quality is more sharply marked by counts at 37° C. than is the case which counts at 20° C. Also, the results from the plates grown at a higher temperature are available in a much shorter time.

The determination of the number of bacteria in water is of great value when studying surface waters, such as lakes and rivers. As a rule, the number of bacteria is proportional to the pollution of the river, not necessarily fecal matter but pollution from any dead organic matter. A river contains more bacteria in winter than in summer, or we might say that the number of bacteria in a stream is an index of its turbidity. The numerical determination is also useful in determining leaks in a water supply. It is also useful in determining the efficiency of a filter.

In the routine bacteriological analysis of a water we do not attempt to isolate the specific typhoid organisms but

only those organisms which have a fecal origin, and some of the reasons for this follow.

Water is not a natural habitat for typhoid bacilli and the majority of them probably die off in a short time. As there is a period of incubation between the infection and the recognition of the disease, it is possible in water-borne cases for the typhoid bacilli to have disappeared from the water before the disease has been recognized.

If the source of the water was a flowing stream and the infection was occasional and not constant, the search for the organism would, of course, be useless. Under such conditions, even if our laboratory technique were perfect, we should not be able to prove our case.

The typhoid organism has been occasionally isolated from water supplies, but the laboratory technique is not simple and the practical difficulties are such that we know of no laboratory which attempts it as a routine procedure.

COLON GROUP.

Defined as all organisms which are lactose fermenters, aërobic and non-spore formers. Presumptive tests merely differentiate organisms which are capable of fermenting lactose.

The United States Public Health Service has adopted a standard for the bacteriological quality of the drinking waters supplied to common carriers in interstate commerce as follows: B. coli shall not be present in more than one out of five 10 c.c. portions and shall be absent in 1 c.c. and 0.1 c.c. portions.

This standard has also been adopted by the Medical Department, and is in use at the Army Medical School and the Departmental Laboratories. It is the standard aimed at in the quality of water supplied to all army camps and cantonments.

If large numbers of B. coli are present, gas often forms

in a few hours. Small numbers of somewhat attenuated B. coli may require three days to form gas. So-called attenuated B. coli does not represent recent contamination, and all B. coli not attenuated grows readily in lactose broth. No other organisms except B. Welchii gives such a test in lactose broth.

B. Welchii is of rather rare occurrence in water. If of fecal origin it is almost invariably accompanied by B. coli, and while the sanitary significance is the same it may, if desired, be distinguished from B. coli by a microscopic examination of the broth solution when long strings of much larger bacilli than B. coli are seen as well as spores.

If the laboratory examinations show organisms of the B. coli type to be absent we can definitely say that the water is safe; but if the examinations show that they are not entirely absent we could not as definitely say that the water was dangerous. As we are not able to differentiate between the organisms of human and of animal origin the mere presence of a few bacteria of the B. coli type does not necessarily indicate pollution from human beings, as they might be entirely of animal origin, coming from pasture lands or fertilized fields; but if the organisms are persistently present in small volumes of the water, say in 1 c.c. or less, the water should be considered unsatisfactory, for even though most of the organisms may be of animal origin they are generally accompanied by those from human wastes.

The following group reactions indicate the source of the culture with a high degree of probability.

Methyl red+
Vosges-Proskauer—
Gelatin—
Adonite—
Indol, usually+
Saccharose, usually— } B. coli of fecal origin.

Methyl red—
Voges-Proskauer+
Gelatin—
Adonite+
Indol, usually—
Saccharose+
} **B. aërogenes of fecal origin.**

Methyl red—
Voges-Proskauer+
Gelatin—
Adonite—
Indol, usually
Saccharose+
} **B. aërogenes, probably not of fecal origin.**

Methyl red—
Voges-Proskauer+
Gelatin+
Adonite+
Indol, usually
Saccharose+
} **B. cloacæ may or may not be of fecal origin.**

SANITARY SURVEY.

Except in those cases in which fecal pollution is entirely absent a sanitary analysis can seldom definitely establish the fact that a given sample of water is from a supply which is either entirely safe or absolutely dangerous. It can point out probable danger, and as such is an aid to be used in connection with other sources of information.

A sanitary survey means the obtaining of actual and accurate knowledge concerning the physical conditions of the terrain surrounding the source and distribution of the supply and the ways and means of conveying the water for use.

This is accomplished by gaining a knowledge of the geology of the water-shed; a study of a contour map to gain a thorough

knowledge of the slopes and elevations of the terrain and the general surface conditions regarding farm lands, pasture lands, buildings, etc. In this way only will one be able to gain a definite idea of sources of pollution and contamination. The information gained from such an investigation should be a basis of request for further information that is to be gained from the laboratory workers. In order to make as accurate determinations as possible it is absolutely necessary to have this knowledge. Samples that are sent into the laboratory should always be accompanied by all possible information as to the history and source of the water.

Single or occasional determinations of either the chemical or bacterial constituents of a water are of little value. In fact it is often misleading, especially in surface waters.

A river water may require repeated examination extending over long periods of time. A routine bacteriological analysis shows pollution but does not prove infection. However, the bacteriological analysis tells more of the present state of the water while the chemical refers more to the past state. A sanitary survey of the catchment area is frequently of much greater practical importance than all the information furnished by the laboratory. By a sanitary survey we are able to discover the sources of contamination, the kinds of pollution and the degree.

There should be added to the above statements that clinical skill, knowledge, and experience are frequently baffled unless the interpreter has accurate detailed information as outlined.

MICROSCOPIC EXAMINATION.

The microscopic examination of water consists of the enumeration of the kinds of microscopic organisms (Plankton) and an estimation of their quantity.

The term "microscopic organisms" shall include all

organisms microscopic or barely visible to the naked eye, with the exception of the bacteria. It includes the diatonaceæ, chlorophyceæ, cyanophyceæ, fungi, protozoa, rotifera, crustacea, bryophyta and spongidæ found in water.

Fragments of organic matter, silt, mineral matter, zoöglea, etc., shall be considered as amorphous matter. The recording of amorphous matter usually serves no useful purpose and shall not be considered a part of the standard method.

Apparatus.—1. A cylindrical funnel about two inches in diameter at the top, with a straight side for nine inches, narrowed over a distance of three inches to a bore of one-half inch in diameter, and terminating in a straight portion of this diameter two and one-half inches in length. The capacity of this funnel is 500 c.c. It shall be provided at the bottom with a tightly fitting rubber stopper with a single perforation and a disk of silk bolting cloth over the hole, about three-eighths of an inch in diameter.

2. A counting cell consisting of a brass rim closely cemented to a plate of optical glass. The shape and size of this cell are not essential but its depth shall be 1 mm. A convenient capacity is about 1 c.c.

3. An ocular micrometer ruled as follows: The ocular micrometer is commonly of such a size that with a 16 mm. objective and a suitable tube length the largest square cuts off one square millimeter on the stage.

Procedure. — Filter 250 c.c. of the water (more or less according to the clearness of the sample) through a one-half inch layer of quartz sand (washed and screened between 60 and 120 mesh sieves), supported by the disk of bolting cloth and rubber stopper at the bottom of the funnel. Suction may be applied to hasten the filtration.

Remove the stopper and catch the plug of sand and its entrained organisms in a small beaker or test-tube, washing down the inside of the funnel into the beaker with 5 c.c. of

clean (preferably distilled) water. Agitate the mixture of sand, water and organisms to detach the latter from the sand grains, and quickly decant the water and the organisms in suspension to a test-tube. If desired the same may then be again washed with 5 c.c. water and the wash water added to the first portion.

Cover the cell partially with a cover-glass, and by means of a pipette run the concentrate under the cover-glass until the cell is completely filled.

Cover and place on the microscope stage in a horizontal position for examination.

Count the organisms in twenty fields, *i. e.*, 20 c.mm., estimating their areas in terms of standard units.

The standard unit is the smallest square in the ocular micrometer and represents an area 20 microns x 20 microns, or 400 square microns on the stage.

Results shall be expressed in the number of standard units of each kind of microörganism per cubic centimeters and also the total number of standard units of all kinds per cubic centimeters.

1. PRELIMINARY EXAMINATION.—For the preliminary and rapid testing of waters, the following tests should be conducted simultaneously. They should be viewed as presumptive tests only.

(*a*) *Cyanides*.—To 50 c.c. of the water add 5 c.c. of 1 to 20 solution of ferrous sulphate, 2 c.c. of a 1 to 100 solution of ferric chloride, 5 c.c. of a 1 to 10 solution of sodium hydroxide, and after a few minutes, acidify faintly with hydrochloric acid. A blue precipitate indicates the presence of cyanides. If the precipitate is very slight, or of greenish appearance, recover it on a filter paper and wash with a 1 to 10 solution of oxalic acid, after which the blue color should be distinct. In case of doubtful reaction, acidify 500 c.c. of the water

with 2 c.c. of 1 to 10 sulphuric acid, distil, and make the test on the first 500 c.c. of distillate.

(*b*) *Heavy Metals.*—Acidify 500 c.c. of the water with hydrochloric acid, and treat with 10 c.c. of a solution of sodium or potassium sulphide. Allow to stand for at least an hour or longer if practicable. A black precipitate indicates the presence of lead, mercury, or copper, and a yellow or brown precipitate, the presence of arsenic or antimony. A positive test is sufficient to condemn. After separating any precipitate obtained in acid solution, make the filtrate alkaline; the formation of a sulphide precipitate, either directly or on standing, should be taken as presumptive grounds for the condemnation of the water.

(*c*) *Oxalic Acid.*—Acidify 500 c.c. of the water with sulphuric acid until strongly acid to litmus and heat. To the hot water add standard $\frac{N}{10}$ potassium permanganate solution until a permanent pink color is produced. If more than 2 c.c. of the permanganate solution is required, the presence of oxalic acid should be suspected.

(*d*) *Barium.*—If a white precipitate is produced upon acidifying 200 c.c. of the water with sulphuric acid, the presence of barium may be assumed. The same may apply in the case of lead. In case barium be present, hydrochloric acid should be used on another portion to demonstrate lead.

(*e*) *Alkaloids.*—100 c.c. of the water, acidified with 2 c.c. of 10 per cent. sulphuric acid, is treated, drop by drop, with iodin-potassium iodide reagent. A second portion is similarly treated with potassium mercuric iodid reagent. The production of a precipitate in either case should be taken as presumptive evidence of the presence of alkaloids.

2. SYSTEMIC EXAMINATIONS.—Where more time is available, the following systematic examination for alkaloids should be made, according to the scheme of M. Pierre Breteau. The operations involved can be carried out in three hours, and 2 liters of water are required.

(*a*) *Alkaloids.*—One liter of water, made slightly alkaline with sodium carbonate, is extracted in a separatory funnel with 20 c.c. of chloroform. Dry the chloroform extract with anhydrous sodium sulphate, filter, and divide among three watch-glasses. Evaporate each portion and treat as follows:

1. To residue add 2 c.c. of 1 to 10 sulphuric acid, and add, drop by drop, a solution of iodin potassium iodide. Production of a precipitate is taken as a general reaction for alkaloids.

2. To residue add 2 c.c. of 1 to 10 sulphuric acid, and add, drop by drop, a solution of potassium mercuric iodide. The production of a precipitate is a confirmatory test for alkaloids.

3. Dissolve in a few drops of concentrated sulphuric acid, and add a small crystal of potassium bichromate. The production of an intense blue or blue-violet coloration, together with positive tests in "1" and "2," indicates the presence of strychnin.

(*b*) *Copper.*—The alkaline water separated from the chloroform is faintly acidified with acetic acid and treated with 10 to 15 drops of ferric chloride solution. Then gradually, drop by drop, and with agitation, 10 per cent. ammonia is added to a very distinct alkaline reaction. The precipitate of ferric hydroxide carries down the arsenic and antimony. Boil the solution for a few minutes, and filter boiling hot. If the filtrate is colored blue, the presence of copper is indicated.

Minimal traces of copper are carried down by the ferric hydroxide and escape detection; but only appreciable quantities of copper, such as are indicated by the ammonia, are important.

(*c*) *Antimony.*—The precipitate of ferric hydroxide, drained, is washed once with boiling water and is then dissolved on the filter with 1 to 5 sulphuric acid.

The sulphuric acid solution is poured very gradually, drop by drop, into a hydrogen apparatus, which is held in a cold water bath, and is operated with 5 grams of pure zinc and 10 c.c. of dilute sulphuric acid (1 to 5). The gas current

is conducted into 20 c.c. of a 2 per cent. silver nitrate solution acidified with 2 drops of nitric acid.

If at the end of a half-hour no black precipitate of silver is produced, absence of antimony and arsenic is indicated.[1]

If a black precipitate has been produced, let the apparatus work for two hours. In that case, filter to separate the black precipitate, wash it once with water, treat it on the filter with a 1 to 5 solution of tartaric acid, and then submit the hot solution, acidified with 2 drops of HCl, to the action of hydrogen sulphide. A yellow-orange precipitate indicates the presence of antimony.

(*d*) *Arsenic.*—The liquid separated from the black silver precipitate is treated with HCl, added drop by drop, to remove the excess of silver nitrate. Filtering off the silver chloride, and treat the liquid with an excess of Bougault reagent and with 2 drops of an approximately 0.1 N iodin solution. A black precipitate or a brown coloration produced in the cold or on boiling water-bath indicates the presence of arsenic.

(*e*) *Barium.*—Add 2 c.c. of sulphuric acid to a half-liter of the water to be examined. If no precipitate has been formed at the end of five minutes, absence of barium is indicated.

If a precipitate forms, collect it, wash it once with boiling water, and extract it with a saturated solution of ammonium tartrate in order to remove any lead sulphate that may be present.

Bring a portion of the precipitate, upon a platinum wire, cautiously into a colorless flame. A persistent green coloration of the flame indicates barium.

Boil the precipitate freed from lead with a concentrated solution of sodium carbonate, recover, and wash the precipi-

[1] In case a black precipitate *is* obtained, antimony or arsenic is indicated. As the presence of either is enough to condemn the water, it is generally unnecessary to differentiate between them.

tate of the carbonate. Treat it with dilute acetic acid. The formation in the acetic acid solution of a yellow precipitate upon the addition of a few drops of potassium bichromate solution indicates the presence of barium.

Note.—The solution of ammonium tartrate, treated with sodium sulphide, gives a black precipitate if there be any lead present. The separated lead sulphide is characterized as described under (*i*).

(*f*) *Cyanides.*—The liquid, separated if need be from the precipitate produced by the sulphuric acid, is placed in a Kjeldahl flask with 50 cm. of copper wire rolled in a spiral, oxidized in a flame, and cleaned in nitric acid. Distil and collect 50 c.c. of the distillate.

Add to the distillate 5 c.c. of a 1 to 20 solution of ferrous sulphate, 2 c.c. of a 1 to 100 solution of ferric chloride, 5 c.c. of a 1 to 10 solution of sodium hydroxide, and after a few minutes acidify faintly with HCl. A blue precipitate indicates the presence of cyanides. If the precipitate is very slight, or of a greenish appearance, recover it on a small filter without wrinkles, and wash with a 1 to 10 solution of oxalic acid. The blue coloration of precipitate will then be apparent.

(*g*) *Mercury.*—If the spiral of copper is whitened, this indicates mercury, the presence of which is confirmed as follows:

Wash the spiral with water, alcohol and ether successively. Introduce the dry spiral into a dry test-tube, surround the upper two-thirds of the test-tube with a filter paper moistened with water, and heat the lower part of the tube. The mercury condenses in the middle part of the tube. Allow to cool, remove the copper spiral and the filter paper, introduce into the tube a very minute fragment of iodin, and sublime at a low temperature. The appearance of a red coating of mercuric iodide is characteristic.

(*h*) The acid solution after the removal of the copper

spiral is submitted hot to the action of hydrogen sulphide or of thio-acetic acid.

The trace of copper dissolved from the spiral gives a black sulphide precipitate that carries down the sulphides of lead and mercury, if they be present. The filtration of the hot solution is very rapid.

The black precipitate, separated from the filter, is washed three times with boiling water, and then treated with hot, 1 to 4 nitric acid. The nitric acid solution is recovered and examined as directed in (*i*). A residue of black sulphide, insoluble in nitric acid, indicates mercury.

To confirm this, dissolve this residue in a few drops of dilute aqua regia, add an excess of a saturated solution of sodium acetate, and then add, drop by drop, a 1 to 10 solution of potassium iodide. A red precipitate of mercuric iodide indicates mercury.

(*i*) *Lead.*—The nitric acid solution, separated if need be from the black mercuric sulphide, is treated with an excess of ammonia and with 2 drops of hydrogen peroxide or with an alkaline persulphate solution. After a few minutes, filter, wash the filter twice with water, exhaust the filter and its contents with a few c.c. of 1 to 4 nitric acid, heat, and add two drops of alcohol. The filtered solution, treated with an excess of saturated sodium acetate solution, and a few drops of bichromate solution, gives a yellow precipitate of lead chromate.

(*j*) *Zinc.*—The liquid separated from the black sulphides is treated with an excess of saturated sodium acetate solution, and is again heated and submitted to the action of hydrogen sulphide. The formation of a white sulphide precipitate indicates the presence of zinc.

If the color of the precipitate is doubtful, collect it on a filter, and after washing, dissolve it in dilute HCl (a few drops). The hydrochloric acid solution is treated with a little sulphurous acid, and put on a boiling water-bath for ten

minutes. Add an excess of soda followed by a few drops of a ·1 to 10 potassium cyanide solution. Sodium sulphide is then added, whereupon a white precipitate of zinc sulphide is formed.

The zinc sulphide, heated and soaked in a very dilute solution of cobalt nitrate, gives upon calcination a characteristic green lake, the so-called green of Rumiam.

METHOD OF CHLORINATING WATER IN THE FIELD.[1]

1. The official method of sterilizing water is by means of calcium hypochlorite. The powder is issued in 1 gram tubes. One tube is usually enough to sterilize one Lyster bagful of water. Break a tube of calcium hypochlorite into a clean ordnance cup, moisten the powder with a few drops of water and mix into a smooth paste. Now fill the cup with water to within one inch of the top and mix thoroughly by stirring with a clean spoon. Add this solution to a Lyster bag filled with clear water, stir thoroughly, *and allow to stand thirty minutes before using*. After thirty minutes test a cupful by adding ten drops of a solution containing 10 per cent. potassium iodide and 1 per cent. soluble starch and 0.5 per cent. zinc sulphate. The appearance of a blue color is indication that sufficient chlorin has been added to the water. If no color appears the water is highly polluted and should be boiled if used. Water showing a high degree of pollution should be reported immediately to the medical officer having water supplies under his supervision.

2. In emergency when a Lyster bag is not available, the hypochlorite method can be applied to smaller containers of known volumes by calculations based on the knowledge that a Lyster bag contains about 36 gallons of water. Thus

[1] From Memorandum No. 7, Division of Laboratories and Infectious Diseases, American Expeditionary Forces.

if a 10-gallon container is available one-quarter of the concentrated solution prepared in the ordnance cup as above can be added, etc. When smaller containers, such as 2-gallon petrol tins are used the original concentrated solution in the ordnance cup can be diluted by one-half, this dilution again diluted by one-half in another ordance cup and one-quarter of this second dilution added to the petrol tin. By using a little ingenuity the hypochlorite method can thus be applied to any container of known capacity.

3. When tubes of calcium hypochlorite are not available and the powder is available in bulk the following procedure should be adopted:

(*a*) An empty shell used in Colt's 45-automatic pistol will hold 1 gram of powdered calcium hypochlorite when filled level with the top. Always use this empty shell as a measure. Add one shellful of powdered calcium hypochlorite to an ordnance cup and make a solution as described in paragraph 1, filling the cup with water to one inch from the top. Part of this solution is used in titrating the water to be sterilized and the remainder is used for sterilizing the water.

(*b*) Rinse 4 ordnance cups with the water to be tested and fill all 4 cups to one inch from the top (500 c.c.) with the water to be tested. From a medicine dropper or pipette add 4 drops of the calcium hypochlorite solution to the first cup; 8 drops to the second cup; 12 drops to the third cup and 16 drops to the fourth cup. Mix the solutions in each cup thoroughly and allow the cups to stand thirty minutes.

Note.—20 drops delivered from a medicine dropper or a glass tube of 2 or 3 mm. bore is equal to 1 c.c.

(*c*) After thirty minutes add 10 drops of potassium iodide-starch solution from a clean medicine dropper or pipette to each of the four cups and mix thoroughly. Some of the cups will show no color, some will show a blue color. The cup that contains the smallest amount of a hypochlorite solution capable of giving a blue color with the potassium iodide-

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starch solution contains the proportion of chlorin necessary to sterilize the water being tested. Thus suppose the cup of water to which 8 drops (0.4 c.c.) of the hypochlorite solution was added gives a color, with potassium iodide-starch solution, and the sample to which 4 drops (0.2 c.c.) of the solution was added gives no color. The cup to which 8 drops (0.4 c.c.) of the hypochlorite solution was added contains the right amount of chlorin to sterilize the water being tested.

4. There are 36 gallons, or 288 pints, in the water-bag when filled to the white mark on the inside. Since 8 drops (0.4 c.c.) of the hypochlorite solution were sufficient to sterilize 1 pint, 115 c.c. of the same solution will be sufficient to sterilize the 288 pints in the Lyster bag. In practice it is believed to be safer to use twice the amount indicated by the titration, so that in the example quoted 230 c.c. of the hypochlorite solution would actually be added to the water to be treated, or one-half of the concentrated solution, in the cup to which the 1 gram of calcium hypochlorite has been added, could be added to the water in one bag and the solution prepared from the measure of hypochlorite would be sufficient to sterilize two bags of water.

5. The following table shows the amount of hypochlorite solution to add to a bag of water corresponding to the number of drops used in the titration:

Number of drops . . .	4	8	12	16	20	24	28	32
Amount of hypochlorite solution (cup measure)	$\frac{1}{4}$	$\frac{1}{2}$	$\frac{3}{4}$	1	$1\frac{1}{4}$	$1\frac{1}{2}$	$1\frac{3}{4}$	2

Note.—In the titration if the first series of drops do not show a blue color the water requires more than one measure of hypochlorite. The second series of drops will indicate the amount of a second measure of hypochlorite dissolved in a cup of water to be added to the bag in addition to the first cup.

COLORIMETER ESTIMATION OF SMALL QUANTITIES OF FREE CHLORIN.—*Reagent.*—1. Hydrochloric acid solution of ortho-toluidin[1] (0.1 per cent. orthotoluidin in a 10 per cent. solution of HCl).

Method.—Add 1 c.c. of the 0.1 per cent. orthotoluidin solution in 10 per cent. HCl to 100 c.c. of the water to be tested for free chlorin. When the free chlorin exceeds 3 p.p.m. it is necessary to use more of the reagent. The appearance of a green to orange color indicates an excess of free chlorin.

ANALYSES OF SEWAGE.

SAMPLING.—Sewage varies in strength and volume through the day and is also subject to weekly and monthly variations. In order to interpret an analysis it is necessary to have a complete record of the conditions under which the plant was running at the time of sampling. Samples for analysis should be collected at thirty-minute intervals during a twenty-four-hour period and at the same time the quantity of flow should be recorded. A part of each of these samples, in porportion to the quantity of flow, should be poured into a bottle and the result will be a representative flow. Samples should be sterilized which cannot be analyzed immediately, by the addition of chloroform, mercuric chloride, formaldehyde or a strong acid.

The tests usually made in the complete analysis of sewage are:

1. Physical tests.
   (a) Sewage flow.
   (b) Turbidity.
   (c) Settling solids.

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[1] Ellms, J. W., and Hauser, S. J.: Orthotoluidin as a Reagent for the Colorimetric Estimation of Small Quantities of Free Chlorin, Journal of Industrial and Engineering Chemistry, November, 1913, No. 11, v, 915.

(*d*) Solids (by weighing).
    (1) Suspended.
        (*a*) Volatile.
        (*b*) Non-volatile.
        (*c*) Total.
    (2) Dissolved.
        (*a*) Volatile.
        (*b*) Non-volatile.
        (*c*) Total.
    (3) Total.
        (*a*) Volatile.
        (*b*) Non-volatile.
        (*c*) Total.
2. Chemical tests.
    (*a*) Nitrogen.
        (1) Total organic.
        (2) Free ammonia.
        (3) Albuminoid nitrogen.
        (4) Nitrites.
        (5) Nitrates.
    (*b*) Oxygen.
        (1) Dissolved.
        (2) Consumed.
        (3) Biochemical demand.
    (*c*) Relative stability.
    (*d*) Chlorin.
    (*e*) Fats.
    (*f*) Miscellaneous tests for special conditions.
3. Bacterial and biological.
    (*a*) Total count on agar at 37° C.
    (*b*) Red colonies on lactose-litmus agar at 37° C.
    (*c*) Colonies on gelatine at 20° C.
    (*d*) Microscopical analysis for plant life, etc.

The procedures for chemical tests made with sewage are identical with the similarly named tests made on water.

For particular determination see Sanitary Examination of Water. However, there are several additional tests which are made on sewage only.

SETTLING SOLIDS.—An approximation of the amount of solid matter capable of being settled out of sewage is an
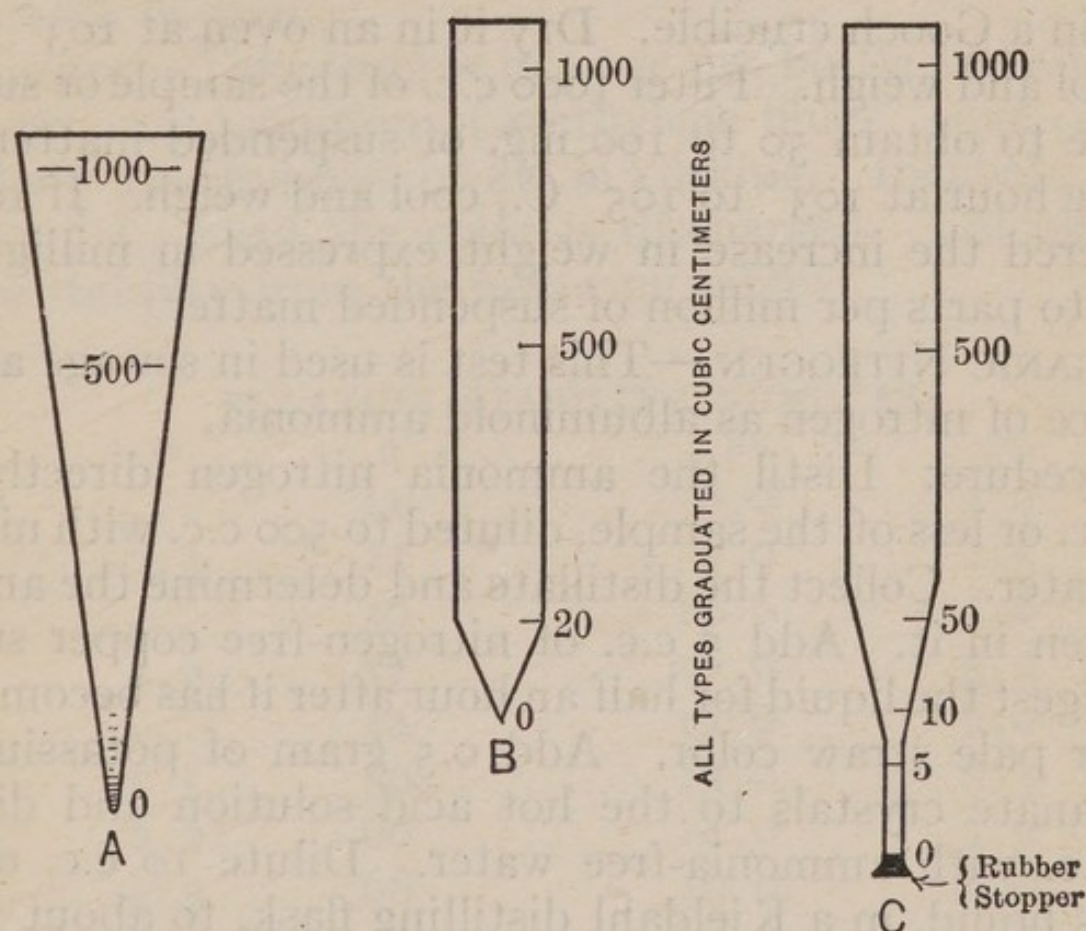
FIG. 4.—Illustration of three types of settling glasses for sewage. *A*, Imhoff cone, developed by Dr. Imhoff and largely used at German disposal works; *B*, settling glass, type used at Worcester, Mass.; *C*, settling glass, designed for use with Kansas sewages and effluents.

arbitrary period of time, usually about two hours. It is measured in special conical glasses. Glasses of several different shapes are shown in Fig. 4.

To use any of these glasses, fill to the 1000 c.c. mark with sewage, allow to settle and record the amount of solid matter in the bottom of the glass at twenty-minute intervals for two hours.

SUSPENDED SOLIDS.—*Reagents.*—Prepare a dilute cream of asbestos fiber which has been finely shredded, thoroughly ignited, treated with strong hydrochloric acid for at least twelve hours and washed with distilled water until free from acid.

Procedure: 1. Prepare a mat of the asbestos fiber $\frac{1}{16}$ inch thick in a Gooch crucible. Dry it in an oven at 103° to 105° C., cool and weigh. Filter 1000 c.c. of the sample or sufficient sewage to obtain 50 to 100 mg. of suspended matter. Dry for one hour at 103° to 105° C., cool and weigh. If 1000 c.c. is filtered the increase in weight expressed in milligrams is equal to parts per million of suspended matter.

ORGANIC NITROGEN.—This test is used in sewage analyses in place of nitrogen as albuminoid ammonia.

Procedure: Distil the ammonia nitrogen directly from 100 c.c. or less of the sample, diluted to 500 c.c. with nitrogen-free water. Collect the distillate and determine the ammonia nitrogen in it. Add 5 c.c. of nitrogen-free copper sulphate and digest the liquid for half an hour after it has become colorless or pale straw color. Add 0.5 gram of potassium permanganate crystals to the hot acid solution and dilute to 500 c.c. with ammonia-free water. Dilute 10 c.c. or more of this liquid, in a Kjeldahl distilling flask, to about 300 c.c. with ammonia-free water. Make alkaline with 10 per cent. sodium hydroxide, distill, and Nesslerize. With some samples direct Nesslerization may be used. For the ammonia determinations, in sewage analyses, direct Nesslerization is recommended rather than Nesslerization after distillation.

RELATIVE STABILITY.—If the organic matter of an effluent has been transformed to such a form that it is incapable of offensive putrefaction, the effluent is said to be stable. Or, if it has sufficient dissolved oxygen and oxidized products present to decompose the organic matter under aërobic conditions, *i. e.*, the available oxygen exceeds the required oxygen, it is stable. A perfectly stable effluent has a relative

stability of 100. If the available oxygen is 75 per cent. of the required oxygen, the effluent has a relative stability figure of 75. In determining the relative stability, advantage is taken of the fact that a sewage to which a little methylene blue has been added retains its blue color if there is any available oxygen present, but becomes colorless in the absence of available oxygen.

Procedure.—Take sample in a 150 c.c. bottle and add 1 c.c. of a 2 per cent. solution of methylene blue. Take care not to include any bubbles of air in corking. Incubate at 20° C. or 37° C. until the color is discharged. Calculate the relative stability from the following table:

RELATIVE STABILITY NUMBERS.

t^{20}	t^{37}	S	t^{20}	t^{37}	S
0.5	...	11	8.0	4.0	84
1.0	0.5	21	9.0	4.5	87
1.5	...	30	10.0	5.0	90
2.0	1.0	37	11.0	5.5	92
2.5	...	44	12.0	6.0	94
3.0	1.5	50	13.0	6.5	95
4.0	2.0	60	14.0	7.0	96
5.0	2.5	68	16.0	8.0	97
6.0	3.0	75	18.0	9.0	98
7.0	3.5	80	20.0	10.0	99

S = Relative stability or ratio of available oxygen required for equilibrium. Expressed in percentage.

t^{20} = time in days to decolorize methylene blue at 20° C.

t^{37} = time to decolorize at 37° C.

Theoretical relation: $S = 100 (1 = 0.794 t^{20})$; $S = 100 (1-620 t^{37})$.

In general effluents having a relative stability greater than 90 may be discharged into any stream without danger of their consuming any of the oxygen of the water, because

effluents of such high stability will retain oxygen indefinitely on exposure to air.

For sewages and effluents which have a very low relative stability this test may be employed to determine the actual strength of the putrescible material present. For this purpose dilutions with fully aërated tap water are made. The amount of dilution will depend on the strength of the sewage, and will probably range from 1 part of sewage to 3 parts of water to 1 to 10. A stability of 100 would indicate that at the dilution used in a fairly good stream, putrefaction would not take place. A relative stability of 50 would indicate twice the volume of diluting water employed in the test would be necessary in the stream in order to render the mixture stable.

BIOCHEMICAL OXYGEN DEMAND.

This test has the same basis as the relative stability, but while the relative stability test is only comparative, the oxygen demand test attempts actually to ascertain the amount of oxygen necessary to obtain a stable effluent. It is made under conditions closely approximating those existing in the receiving water course and so affords valuable data regarding the danger of pollution. The method is based on the biochemical consumption of available oxygen in sodium nitrate by a sewage during an incubation period of ten days at 20° C. The amount of available oxygen added as nitrate, minus the final amount as determined, indicates the amount that has been used up in keeping the sewage stable. This figure divided by 4 (the number of parts per million of oxygen that can be furnished by a stream without danger of pollution) gives the size of the stream required to dilute the sewage or effluent. For example, if 200 parts per million of oxygen are added, and at the end of ten days 100 parts per million are found the biochemical oxygen demand is 100 parts per million. A stream twenty-five times the

volume of the sewage to be added is necessary for its dilution.

Procedure.—Fill a 250 c.c. bottle with sample, add 2 c.c. of sodium nitrate solution (25.56 gm. per liter, 1 c.c. of which in 250 c.c. of water represents 50 parts per million of available oxygen) and 1 c.c. of a 2 per cent. solution of methylene blue. Incubate for ten days at 20° C., adding sodium nitrate from time to time as necessary to retain the blue color. At the end of ten days determine the amount of nitrites and nitrates. Multiply the nitrite nitrogen by 1.7 and the nitrate nitrogen by 2.9, in order to convert the nitrogen into terms of available oxygen. The differences between the available oxygen added as sodium nitrate and that found as nitrite and nitrate at the end of the incubation period gives the biochemical oxygen demand in parts per million.

FATS.

Evaporate a definite quantity of sewage to dryness according to the quality of the sewage, and heat in hot air oven at 100° C. two or three hours. Extract the dry residue with boiling ether, rubbing the sides and bottom of dish to ensure complete solution of the fat. Three extractions with ether are usually sufficient. Filter the ether solution through a 5 cm. filter paper into a small flask. Evaporate the ether slowly, dry the fatty extract for half an hour at 100° C., cool in a desiccator and weigh. If it is desirable to determine the quantity saponified fat, determine the fats with and without acid treatments, *i. e.*, acidify the sewage with $\frac{N}{50}$ sulphuric acid before evaporation. The difference in the amount of the two determinations is the content of saponified fat.

APPENDIX.

A SELECTIVE MEDIUM FOR B. INFLUENZÆ (AVERY)

Oleate-hemoglobin Agar.

PREPARATION OF MEDIUM.—A 2 per cent. meat infusion agar having a reaction of 0.3 to 0.5 acid to phenolphthalein is used. To 1000 c.c. of this is added 5 c.c. of a 2 per cent. solution of neutral sodium oleate in distilled water. This last solution when made up is sterilized in the autoclave and can be kept in stock. A sterile, serum-free suspension of rabbit or human red blood cells is prepared by centrifuging the defibrinated blood, pipetting off the supernatant serum and making the corpuscles up to the original volume by the addition of broth. One c.c. of this suspension is added to each 100 c.c. of oleate agar just before use and while the medium is hot. The resulting formula is therefore

Agar	94 c.c.
2 per cent. solution of sodium oleate	5 c.c.
Suspension of red blood cells	1 c.c.

Oleate-hemoglobin bouillon may be prepared in the same way by substituting broth for agar. Cultures are made in the usual way and incubated for forty-eight hours at 37° C.

On this medium a more luxuriant growth of B. influenzæ is obtained, with larger and less translucent colonies. The other Gram-negative cocci of the M. catarrhalis group, staphylococci and occasionally diphtheroid bacilli, grow while pneumococci and streptococci of the hemolytic and S. viridans variety fail to develop.

METHOD FOR STAINING INFLUENZA BACILLI (GOODPASTURE).

1. Steam three to five minutes in:

30 per cent. alcohol	100.0 c.c.
Basic fuchsin	0.5 grams
Anilin oil	1.0 c.c.
Phenol (crystals)	1.0 c.c.

2. Differentiate until no color comes away in U. S. P. Formaldehyde solution.

3. Wash.

4. Counter stain in saturated aqueous picric acid.

5. Dehydrate rapidly and mount.

METHOD FOR DIFFERENTIATING GRAM-POSITIVE AND NEGATIVE BACTERIA IN TISSUES (MAC CALLUM).

1. Stain for ten minutes to one-half hour or more in Goodpasture's stain, which is:

30 per cent. alcohol	100.00 c.c.
Basic fuchsin	0.59
Anilin oil	1.0
Phenol crystals	1.0

2. Wash in water.

3. Differentiate in 40 per cent. formaldehyde solution (pure formalin). This requires only a few seconds. The bright red color washes away and gives place to a clear rose color.

4. Wash.

5. Counter stain in saturated aqueous picric acid. The section remains until it assumes a purplish yellow color, about three to five minutes or less.

6. Wash in water.

7. Differentiate in 95 per cent. alcohol. The red color reappears and some of it is washed out. Some of the yellow of the picric acid is also washed out.

8. Wash in water.

9. Stain in Stirling's gentian violet five minutes or more.

10. Wash in water.

11. Gram's iodin solution.

12. Blot dry without washing.

13. Anilin oil and xylol (equal parts) until no more color comes away.

14. Two changes of xylol.

15. Balsam.

Gram-negative organisms are red, Gram-positive blue.

INDEX.

(351)

Lashman Method
of Staining Spirochaetes
in films (Vici Dobell
30.4.1916)

1. Dry in air

2. Fix in Abs Sp - 30"

3. Dry in air

4. 5% ... permanganate
for a few . (up to 5') minutes

5. Wash in water
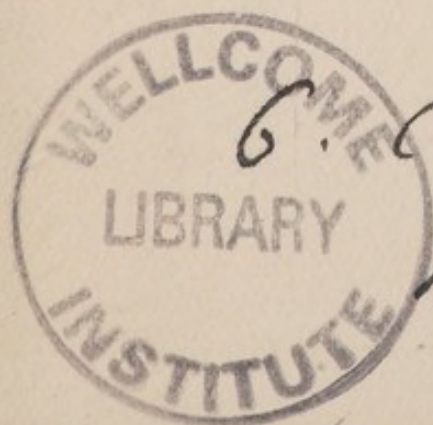
6. Carbol fuchsin at
Room T for 5' min

Van Gieson
(from Lapon)
23·XI·26

Acid Fuchsine 0·2% 2 Vols ⎫
                                    ⎬
Sat aqueous Soln q   1 Vol ⎭
    Picric Acid

Do not filter.

Stain Section first in Haematoxylin
then in this 10 - 20 secs

Then wash Rapidly in Water.
· · · · · · · · - - -   Alc
· · · · · · · · - - -   Xylol Balsam

Anaerobic Table 174

Cohltypthal do 189

Section 66

Trypi g pneumo 135

Saturation strengths of Dyes 24

Congo Red (Bernais) for Spiro

Congo Red 2 %

HCl 10% in absolute
alcohol

~~Dry in air~~

Bornis pretection
Sat. picric 6 c c
Formal. 2 c c
Acetic 0.4 c c

P.325
Langer