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MEDICAL WAR MANUAL No. 6

Authorized by the Secretary of War
and under the Supervision of the Surgeon-General
and the Council of National Defense

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.

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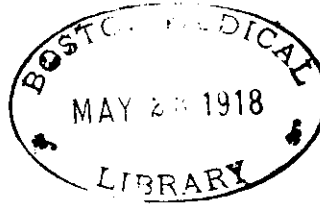

FOREWORD.

THIS manual has been compiled largely by members of the Surgeon-General's Staff. Standard books have been freely drawn upon. In addition a number of individuals from the Medical Reserve Corps outside of this office have contributed minor sections and descriptions of special methods. These men have given their work freely, without thought or desire for individual credit. Special acknowledgment is hereby made of the service rendered by Dr. Donald D. Van Slyke in preparing the section on Quantitative Analytical Methods.

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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 will give the laboratory staff a tentative conception of the types of examinations for which they must be prepared. It is intended that the simpler clinical tests, such as blood counts, differential counts, simple staining of sputum, the collection of blood for Wassermanns, etc., will be done by the clinical staffs, thus freeing the laboratory force for those clinical tests which require a more elaborate equipment and for those examinations which may be spoken of as public health functions, in that they are a part of the control of infectious disease of the camps.

1. Bacteriological water examinations for supervision of camp water supply.
2. Similar milk and food examinations.
3. Clinical diagnostic bacteriology for ward services.
4. Diphtheria diagnosis and Schick reactions.
5. Pneumococcus type determinations.
6. Meningococcus diagnosis and carrier examinations.
7. Typhoid, paratyphoid, and dysentery stool and urine examinations both of patients and for carrier detection.
8. Serological tests, agglutinations for diagnostic and differential purposes, Wassermann reactions and other complement-fixations except in camps in close proximity to department laboratories.
9. Clinical pathological tests which cannot be done by clinical staffs.
10. Autopsies and histopathology.

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

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.

Remove the swab from its container and pass it under the suspected diphtheric membrane. Then remove cotton plug from the culture tube, flame the neck of the tube, and smear the surface of the culture medium with the inoculated swab.

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 labeled 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 labeled 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.

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 labeled 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 tubes in each case should be plainly labeled with the name or identification mark of the animal, the nature of the material and 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 TEST.—A positive complement-fixation test may be obtained, as a rule, from the seventh to the tenth day of the infection, and it persists during the entire course of the disease. All blood-serum tests—of which this is one—are influenced by the injection of mallein or glanders vaccines, and these should be withheld until after the blood for the test is collected. A simple technic for collecting the blood is 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 upon it with the thumb from below, insert the needle into the vein and permit the blood to flow into the sterile bottle furnished for the purpose.

(*d*) Tightly cork the bottle, label it plainly with the name or identification mark of the animal, the nature of the contents, the test desired, and the name, rank, and station of the veterinarian to whom the report is to be returned. Pack the bottle securely in cotton to avoid breakage in the mails. 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.)

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.

Bichromate of potassium	2.5 grams
Corrosive sublimate	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 "

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 hydrate in water	100.0 c.c.

ZIEHL CARBOL-FUCHSIN SOLUTION.

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

To make up this staining solution by another method, 90 c.c. of a 5 per cent. aqueous solution of carbolic acid 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 advantageous. 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.

GABBET STAIN.—Gabbet stain for the combined decolorization and counterstaining of acid-fast bacilli is made up as follows:

Methylene blue	2.0 grams
Twenty-five per cent. sulphuric acid (sp. gr. 1018)	100.0 c.c.

The specimens are stained with hot carbol-fuchsin. As usual, however, the carbol-fuchsin is drained off and the slide immersed in the methylene blue sulphuric acid mixture.

PAPPENHEIM SOLUTION FOR STAINING TUBERCLE BACILLI.—Make a 1 per cent. solution of corallin or rosolic acid in absolute alcohol and saturate with methylene blue. Add 20 per cent. of glycerin.

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.

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.

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.

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.

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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. carbolic acid 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
Tannic acid	5.0
Twenty-five per cent. solution of silver nitrate	5.0 c.c.

III.

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.

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

This preparation is essentially a solution of sodium hypochlorite containing 0.40 to 0.5 per cent. NaClO made in such a way that it is, and remains, substantially neutral. Ordinarily commercial hypochlorite is very variable in composition and commonly contains much free alkali and occasionally free chlorin. Such solutions are very irritating and should not be used for surgical purposes. The original formula[1] for making the neutral solution requires the use of boric acid for neutralization. The reason for this may perhaps be briefly referred to. It is well known that blood and some other body fluids and also certain artificial salt solutions containing mixtures of the salts of polybasic acids—*e. g.*, phosphoric or carbonic acid—are able to retain

[1] Comp. rend., 1915, clxi, p. 150; British Med. Jour., August 28, 1915.

their essential neutrality even after the addition of limited quantities of acid or alkali. This is due to the fact that the addition of acid or alkali simply changes the relative proportion of two or more salts of the polybasic acid present in the solution. Such solutions are often referred to as "balanced," and the salts in them are called "buffer salts." Utilizing the same principle, and employing the feeble polybasic boric acid, a simple balanced hypochlorite mixture was prepared which maintains essential neutrality under all conditions. It should be understood that the insignificant antiseptic action of boric acid has nothing to do with the employment of this acid, nor is the boric acid employed for the purpose of liberating free hypochlorous acids, as in Lumier's or Lorrain Smith's preparations.

Sodium hypochlorite, whether prepared according to the following formulæ or according to other methods that will occur to the chemist, *e. g.*, from salt by electrolysis, when used in neutral solution at 0.5 per cent. concentration, is found to be a valuable antiseptic for the treatment of infected wounds. Its action is extremely rapid and then ceases as soon as all the hypochlorite is decomposed, hence the methods for using the solution efficiently must provide for its frequent renewal. For further details concerning surgical technic, reference may be made to the book by Carrel and Dehelly and to the papers noted below.[1]

The hypochlorite solutions possess the valuable property of assisting in the rapid dissolution of necrotic tissue, doubtless owing to their ability to react with proteins with the formation of soluble products. They possess a slight but definite hemostatic action, but are actively hemolytic and should not

[1] A. Carrel and G. Dehelly: Le traitement des plaies infectés, Masson et Cie., Paris, 1917. Carrel, Dakin, Daufresne, Dehelly and Dumas: Presse Méd., October 11, 1915. Tuffier: Bull. de l'Acad. de Méd., 1915, lxxiv, No. 38. Dépage, A.: Bull. et mém. Soc. de chir. de Paris, 1916. xlii, p. 1987. Lyle, H. H. M.: Jour. Am. Med. Assn., January 13, 191[illegible]

be injected intravenously. The hypochlorites are extremely reactive substances chemically and should neither be heated above 37° C. nor used with other antiseptics, nor with alcohol or ether, and should be protected from strong light.

PREPARATION OF NEUTRAL SODIUM HYPOCHLORITE.

One hundred and forty grams of *dry* sodium carbonate (Na_2CO_3) or 400 grams of the crystallized salt (washing soda) are dissolved in 10 liters of tap water and 200 grams of bleaching powder containing 24 to 28 per cent. of "available chlorin" are added. (Bleaching powder varies considerably in its available chlorin content, though when bought in bulk the fresh product is fairly constant in composition. It is advisable to determine the "available chlorin" in each large batch of bleaching powder purchased. Bleaching powder with less than 23 per cent. of available chlorin should be rejected. Exceptional samples may contain as high as 35 per cent. available chlorin, and in such cases it is well to reduce correspondingly the ingredients taken in the above formula. For purposes of rough calculation one may assume that using 200 grams of bleaching powder for 10 liters of solution the resulting product will contain as much sodium hypochlorite as is represented by the available chlorin of the bleaching powder divided by 50. Thus 25 per cent. "available chlorin" bleaching powder will give 0.5 per cent. sodium hypochlorite solution.) 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 a cotton plug or paper. Forty grams of boric acid are added to the clear filtrate and the resulting solution is ready for use. The boric acid must not be added before filtering but only afterward. The exact strength should be determined

from time to time, by titration, with $\frac{N}{10}$ thiosulphate solution. It is important that the solution should not be stronger than 0.5 per cent. sodium hypochlorite, or irritation of the skin may be frequent. On the other hand it should not be less than 0.4 per cent. or its germicidal action is materially diminished. The solution should also be tested for neutrality by adding a little of it to a trace of solid phenolphthalein suspended in water. No red color indicating free alkali should develop or else more boric acid must be added; this is, however, rarely necessary with the above proportions. The solution should not be kept longer than one week. (A stronger solution may be prepared by decomposing bleaching powder with dry sodium carbonate in the proportion of 150 grams to 105 grams dissolved in 1 liter of water. The mixture is filtered and a measured portion of it (20 c.c.) rapidly titrated with a boric acid solution of known strength (31 grams per liter, one-half normal), using phenolphthalein suspended in water as an indicator (the usual alcoholic solution of phenolphthalein will not serve, because the alcohol is at once attacked), in order to determine the amount of boric acid to be added to the rest of the filtrate. (Each cubic centimeter of $\frac{N}{2}$ boric acid calls for 3 grams of boric acid to be added.) 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 should be mixed with 7 parts of water before use. It can be kept for a month without serious decomposition.

From Chlorin and Sodium Carbonate.[1] A solution is prepared containing 14 grams of dry sodium carbonate per liter (= 16.6 grams monohydrate or 38 grams washing soda). A measured quantity, 4.8 grams per liter (or about

[1] Cullen, G. E., and Austin, J. H.: Proc. Soc. Ex. Biol. and Med., December 19, 1917.

1600 c.c.) of chlorin gas is allowed to run into the solution. Chlorin may be obtained in liquid form in steel cylinders and is easily measured by a chlorin meter manufactured for the purpose. This is a stable, economical, and convenient source of chlorin. Ten c.c. of the solution is then titrated. If the solution is too strong it should be diluted to 0.5 per cent. NaOCl with 1 per cent. sodium carbonate, which serves to correct the unduly diminished alkalinity caused by the excess of chlorin introduced into the solution. However, the designated amount of carbonate is planned to give, at a concentration of 0.5 per cent. NaOCl, the minimum degree of akalinity consistent with stability, and if chlorin has been introduced in such excess that the titer exceeds the desired amount by more than 3 or 4 c.c. of $\frac{N}{10}$ thiosulphate or if the solution fails to give a momentary flash of color with *alcoholic solution of phenolphthalein* it should be rejected. The solution must, of course, show no color with powdered phenolphthalein. The solution should be titrated for hypochlorite concentration every twenty-four to forty-eight hours.

Preparation of $\frac{N}{10}$ *Thiosulphate Solution.*—Dissolve 24.82 grams of pure crystalline sodium thiosulphate in water and make up to 1 liter. One cubic centimeter of this standard solution is equivalent to 0.003723 of sodium hypochlorite.

Method of Titration.—To 10 c.c. of the hypochlorite solution, add approximately 5 c.c. of a 10 per cent. solution of potassium iodide and 2 c.c. of glacial acetic acid. Iodin is liberated and dissolves in the excess of iodide present. The thiosulphate 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 0.03723 gives the percentage of sodium hypochlorite present.

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

"*Chloramin-T*,[1] the abbreviated name for sodium toluene sulphon chloramin. Chloramin-T can be used in stronger solution (up to 2 per cent.) than the hypochlorites. It is more stable and exerts more prolonged antiseptic action and is considerably more effective than hypochlorite when acting in the presence of much blood. It is not toxic and is less irritating than the hypochlorites, and has but little solvent action on necrosed tissue. It is well suited for use on wounds previously cleansed with hypochlorites or dichloramin-T, and in suitably dilute solutions may be used in the eye and on other sensitive parts. It may be applied in solution, as an impregnation of gauze, or in a sodium stearate cream."

"*Dichloramin-T*,[1] the abbreviated name for toluene sulphon dichloramin. Dissolved in oily media it may be sprayed upon wound surfaces or poured into accessible parts of deep wounds. It yields moderate amounts of antiseptic to watery media, such as secretions from wounds or mucous membranes. 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" is a heavy oil prepared from paraffin wax by replacing part of the hydrogen in the compounds contained in the wax with chlorin. Those hydrocarbons which predominate in solid paraffin are designated by names ending in "cosane," *e. g.*, tetracosane ($C_{24}H_{50}$), so that the term chlorcosane indicates a chlorin derivative of this series without specifying any definite chemical constitution. It is prepared from any clean paraffin wax melting at 50° C. or over by treating it with chlorin gas at a temperature ranging between 120° and 140° C. until an increase in

[1] Dakin and Dunham: Handbook of Antiseptics.

weight amounting to from 45 to 55 per cent. of the wax has taken place. During this process hydrochloric acid is evolved. The small quantity of this acid remaining in the product is removed by shaking with dry sodium carbonate (about 5 per cent.), and the hot oil is then filtered through a dry fluted paper. It is then ready for use as a solvent for dichloramin-T.

Chlorcosane is a bland, almost tasteless oil, with a viscosity between that of olive and castor oils. The chlorin it contains is attached to carbon and is therefore inert, like the chlorin in common salt.

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 effected, 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 adequate. The solution may be applied directly to the surfaces of a wound by any convenient method, among which 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 such use it can, however, 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 prac-

ticable, 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 gently warming the solutions will, in such cases, become clear, and are 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 chlorin is more rapidly consumed.

Chlorcosane possesses so many advantages over eucalyptol, which was first used as a solvent for dichloramin-T, that there is now no longer any occasion for using eucalyptol for that purpose.

PREPARATION OF CHLORAMIN-T PASTE.—Prepare in hot water a 7.5 per cent. solution of sodium stearate. This may be prepared either from 750 grams of pure sodium stearate or by neutralizing 696 grams of stearic acid with 100 grams of sodium hydroxide (for a 10-liter lot). 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 container of which is surrounded by hot water. The container should not be more than half-full. A hole should be bored at the bottom of the wooden bucket, in order to allow cold water to run through and gradually reduce the temperature. Let the cold water run as soon as the machine starts, and regulate the flow so that the complete cooling takes about one hour. Then open the container and add chloramin-T (chlorazene) dissolved in a little water to make 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.

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 36 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,

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 Na tartrate . . .	173.0 grams
KOH	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.

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 Thoma, or Zappert-Ewing; 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, 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.

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 tapeworms, 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.

CHEMICAL TEST FOR OCCULT BLOOD.[1]—*Procedure for the Examination of Stools for Occult Blood.*—Approximately 10 grams of the stool are transferred to a beaker, 25 c.c. of distilled water are added, and the mixture stirred until of uniform consistency. Over a low flame the mixture is heated with constant stirring to boiling and kept at the boiling temperature for several minutes. After cooling, one-half of the mixture is transferred to a glass-stoppered bottle, 5 c.c. Merck's reagent glacial acetic acid and 25 c.c. ether are added and the mixture thoroughly shaken. Two cubic centimeters of the ether extract contained in a test-tube are treated with 0.5 c.c. (of a $\frac{1}{60}$ solution) of either Merck's guiaconic acid or the preparation described above, and finally 1 to 5 drops of 30 per cent. Merck's reagent perhydrol are added slowly from a pipette. A decided green or light or dark blue color indicates the presence of blood in quantity to be of clinical significance. The above procedure is capable of detecting 0.0001 c.c. of blood in 1 gram of stool. (When 1 c.c. of 3 per cent. H_2O_2 (medicinal) is used in place of the 5 drops of 30 per cent. perhydrol, very faint, and in some cases negative results are obtained in extracts which give unmistakable positive results with the usual quantity of perhydrol). Before carrying out this test for occult blood 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. Bacterial examination is considered elsewhere.

[1] Guaiaconic acid is prepared by extracting powdered guaiac gum with a solution of KOH, acidifying the diluted extract with acetic acid, filtering off the precipitate, drying, extracting with 95 per cent. alcohol, filtering from a dark brown flocculent material, and removing the alcohol from the filtrate by distillation. The residue in the flask is again dissolved in KOH, precipitated with acetic acid, filtered, and dried. The yield is about 60 per cent. of the crude gum. (Lyle and Curtman.)

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.

No operculum	Ova with a terminal spine			150 x 60	Schistbeomum 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.
		Ova do not contain an embryo .		125 x 72	Echinostomum malayanum.
				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.
	Small ova less than 50 m.	Operculum prominent; prolongation at the non-operculated pole		30 x 16	Clonorchis sinensis.
		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.

Nematodes	Ova with a single envelope.	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.
				8 to 32 blastomeres	80 x 48	Hemonchus contortus.
					80 x 45	Trichostrongylus probolurus
					83 x 45	Trichostrongylus instabilis.
				Thick embryo, folded in two	87 x 48	Trichostrongylus vitrinus.
					54 x 32	Strongyloides intestinalis.
				Thin, twisted embryo	80 x 55	Metastrongylus apri.
				Mammillated, brown	60 x 44	Ascaris lumbricoides.
		Wall ornamented		Regularly cribbed with depressions, yellow	75 x 65	Ascaris canis.
				Cribbed with depressions, except at the poles, brown	66 x 42	Eustrongylus visceralis.
Acanthocephalus	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.

PREPARATION OF SALVARSANIZED SERUM FOR INTRASPINOUS 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 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.

ISO-AGGLUTINATION TESTS FOR TRANSFUSION.

The blood serum of human beings and of some other animals may, under certain conditions, contain antibodies

against the red cells of the individuals of the same species. This fact is important in connection with transfusion tests, and it is necessary before using a donor to make sure that his serum does not agglutinate or cause hemolysis of the corpuscles of the patient, and *vice versa*. Serious results may otherwise ensue. Curiously enough, according to their possession of these so-called iso-antibodies, human beings fall into four groups which are inherited by Mendelian laws. The following table illustrates this:

GROUPING OF HUMAN BEINGS ON THE BASIS OF ISO-AGGLUTININS.

Cells.		SERA. I. 1	2	3	4	II. 5	6	7	III. 8	9	IV. 10
I	1	o	o	o	o	o	o	o	o	o	o
	2	o	o	o	o	o	o	o	o	o	o
	3	o	o	o	o	o	o	o	o	o	o
	4	o	o	o	o	o	o	o	o	o	o
II	5	+	+	+	+	o	o	o	+	+	o
	6	+	+	+	+	o	o	o	+	+	o
	7	+	+	+	+	o	o	o	+	+	o
III	8	+	+	+	+	+	+	+	o	o	o
	9	+	+	+	+	+	+	+	o	o	o
IV	10	+	+	+	+	+	+	+	+	+	o

Thus the serum of Group I acts on cells of II, III and IV.
Thus the serum of Group II acts on cells of III and IV.
Thus the serum of Group III acts on cells of II and IV.
Thus the serum of Group IV acts on cells of none of the others.
Thus the cells of I are agglutinated by none of the others.
Thus the cells of II are agglutinated by Sera I and III.
Thus the cells of III are agglutinated by Sera I and II.
Thus the cells of IV are agglutinated by Sera I, II and III.

To carry out the tests, it is necessary to collect serum and cells from donors and patients. The blood for serum is taken either into Wright capsules or small test-tubes, and is allowed to clot and stand until the serum is expelled. It is extremely important in these tests that the serum should be taken carefully, so that it may not be tinged with hemoglobin. The cells are collected by taking about 10 drops of blood into straight salt solution containing 9½ grams of salt and 10 grams of sodium citrate to the liter of distilled water. The blood cells may be washed, although this is not absolutely necessary, and should be made up into a 5 per cent. suspension in the usual way. Some observers prefer a 2 per cent. suspension for this work. The test can then be done in a number of different ways.

One very excellent method consists in making the mixtures in small Wassermann tubes in the following way:

1. Pt. serum .	0.25 c.c.	Pt. cells. . .	0.25 c.c.
2. Donor's serum	0.25 c.c.	Pt. cells . .	0.25 c.c.
3. Pt. serum .	0.25 c.c.	Donor's cells .	0.25 c.c.
4. Donor's serum	0.25 c.c.	Donor's cells .	0.25 c.c.

Another convenient method, as described by Ottenberg, is as follows: "Make 12 Wright capillary pipettes of strong glass tubing, 4 or 5 mm. in diameter. These pipettes are made by cutting the tubing into pieces about 4 inches long, heating the center of each piece to melting and drawing out to a capillary of about 1 mm. diameter and 4 or 5 inches long. The capillary is then broken at the middle, giving two pipettes. Adjust nipple to the pipettes. Make a mark on the capillary end of each pipette near the hilt. Draw up one volume of serum to the mark, then a small bubble of air, then another volume of serum, and another bubble of air, and then a volume of the cells which are to be mixed with the serum (the serum is used in excess because in this way

it is sometimes possible to detect a weak hemolytic action which would otherwise be overlooked on account of the low complement activity of human blood). Draw the mixture into the body of the pipette and seal the capillary tip in the flame. Mix the fluid by rotating the pipette. Remove the nipple. Throughout these steps keep the pipette approximately horizontal so that the fluid remains in the body of it and does not flow to either end. Seal the open end of the pipette by dipping in melted paraffin. Mark the pipette with a glass pencil, using the number assigned to the serum as the numerator, that assigned to the cells as the denominator, of a fraction. For illustration of results we may insert the following example of an actual test. The letters Y, H, T and E represent the patients. + marks indicate agglutination. O indicates absence agglutination and hemolysis. h indicates hemolysis. The vertical column under "Serum" indicates that the serum of the patient is mixed with the cells of each of the donors whose numbers are in the column to the left. The vertical column under "Cells" indicates that the cells of the patient are mixed with the serum of each of the donors whose numbers are in the column to the left.

It will be noted that for patient Y donors 3 and 5 are available. For patients H, T and E donors, 1, 2, 4, 6 and 11 are available.

It is obvious also, if one wishes to study the results a little more closely, that the patients H, T and E belong to Landsteiner's group 1, as their red cells are not agglutinable. To the same group, of course, belong donors 1, 2, 4, 6 and 11.

Patient Y and the rest of the donors must belong to the other groups, and it is obvious, of course, that patient Y belongs to the same group as donors 3 and 5 because their bloods do not interagglutinate.

If patient and donors 3 and 5 belong to group 2, then donors 7, 8, 9 and 10 must belong to group 3. It is interesting to

note that of the two patients who have hemolysins in their blood, patient T has hemolysins only affecting the one group (presumptive group 3—Nos. 7, 8, 9 and 10) and agglutinins affecting the other group (Nos. 3 and 5), while patient E has not only agglutinins but hemolysins affecting both groups. This illustrates the fact that there are two hemolysins as well as two agglutinins and that the groups of the hemolysins parallel those of the agglutinins.

Y.			H.			T.			E.		
Donor.	Serum.	Cells.	Donor.	Serum.	Cells.	Donor.	Serum.	Cells.	Donor.	Serum.	Cells.
1	0	+	1	0	0	1	0	0	1	0	0
2	0	+	2	0	0	2	0	0	2	0	0
3	0	0	3	+	0	3	+	0	3	+h	0
4	0	+	4	0	0	4	0	0	4	0	0
5	0	0	5	+	0	5	+	0	5	+h	0
6	0	+	6	0	0	6	0	0	6	0	0
7	0	+	7	+	0	7	+h	0	7	+h	0
8	0	+	8	+	0	8	+h	0	8	+h	0
9	0	+	9	+	0	9	+h	0	9	+h	0
10	0	+	10	+	0	10	+h	0	10	+h	0
11	0	+	11	0	0	11	0	0	11	0	0
Y	0	..	H	0	..	T	0	0	E	0	

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 2 or 3 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.

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 paraffin 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 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

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. 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 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.

EXAMINATION OF TUBERCULOUS MATERIAL.

SPUTUM.—In making ordinary sputum examinations for the tubercle bacilli the particle to be examined should be carefully chosen, the little caseous bits should be looked for

[1] Unna's alkaline methylene blue:

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

and picked out for smearing on a slide. Often it may be of help to take a particle of sputum and press it between two glass slides to flatten it out, and in this way the little caseous pieces can be more easily found.

It is of much help sometimes to use a proprietary preparation spoken of as Antiformin. The stock bottle of antiformin should be kept in the dark in a refrigerator and well stoppered. A 15 per cent. solution with water is made up for use. It is well to remember that distilled water or other water that has stood around in the laboratory for some time, especially if there have been animals confined in the same room, may contain non-pathogenic acid-fast bacilli. It is well, therefore, to pay attention to the source of water used for the dilution of the antiformin. The sputum is well mixed in a high graduate or urine glass with two or three volumes of the 15 per cent. antiformin and allowed to stand for several hours in the incubator or at room temperature. The solid parts of the sputum and the mucus will go into solution, vigorous centrifugalization can then be resorted to, and the sediment several times washed with water or salt solution. The sediment in which the acid-fast bacilli, which are insoluble in the antiformin, are concentrated can then be smeared on slides and examined. It is well to smear it in a drop of serum or egg albumen, since it may easily wash off the slide in the process of staining unless this is done. The antiformin does not kill acid-fast bacilli. Therefore, if desired, sediment so obtained can be injected into guinea-pigs or may be planted on Petroff's medium or Dorsett's egg.

TUBERCULOUS URINE.—Famulener advises the dilution of the urine with an equal volume of 95 per cent. alcohol before high-speed centrifugalization. The sediment so obtained can be smeared for morphological examination. It is often necessary to resort to guinea-pig inoculation to differentiate between tubercle bacilli and smegma bacilli.

TUBERCULOUS STOOL EXAMINATION.—Small parts of the stool are well emulsified in salt solution and may be treated with antiformin, as above described, or as advised by Famulener, diluted with an equal volume of 95 per cent. alcohol. The sediment is smeared as indicated above.

EXAMINATION OF PLEURAL OR PERITONEAL EXUDATES.—It is not often that tubercle bacilli can be found in such exudates morphologically. It is best to allow the fluids to stand until they have clotted, then break up a bit of the clot representing 2 or 3 c.c. until it can be sucked into a syringe and injected into guinea-pigs.

EXAMINATION OF MILK FOR TUBERCULOSIS.—Samples of the milk are centrifugalized in large tubes at high speed. The upper layers are pipetted off and the lower 2 or 3 c.c. injected into guinea-pigs.

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 Peppenhein-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 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.

TUBERCULAR SPINAL FLUID.—Tubercular 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 highest limits. 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. 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 actions 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.

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.

[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.

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

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

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.

TABLE OF INDICATORS.

Indicator.		Color.		pH at which color changes.	Form in which indicator is prepared for use.	Special use in titration.
Chemical name.	Common name	Acid.	Alkaline.			
*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.	
*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.	Titration of organic acids with mineral alkali, and of mineral acids with mineral alkali in presence of weak organic base (benzidine sulphate method).
*Thymol-sulphon-phthalein (alk. range)	Thymol blue	Yellow	Blue	8.0–9.6	Water sol. Na salt 0.1 per cent.	Titration of organic acids with mineral 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 pH of biological fluids. These indicators are but little affected by protein. Journal of Bacteriology, 1917, vol. ii, p. 33.

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 $D_{\frac{25}{25}}$. 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 $D_{\frac{25}{4}}$.

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 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 determination 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.

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 quan-

titatively 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

[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

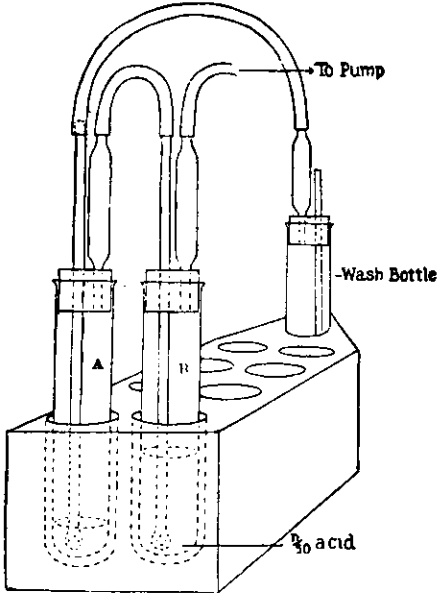

FIG. 1.—Apparatus for determining urea content by means of urease.

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*
   - 0.5 c.c. urine.
   - 5.0 c.c. phosphate solution.
   - 1.0 c.c. 10 per cent. urease.
   - 2 drops caprylic alcohol.

Place stopper as shown in Fig. 1 and let stand fifteen minutes.

2. Meanwhile measure into *B*
   - 25 c.c. $\frac{N}{50}$ acid.
   - 1 drop 1 per cent. sodium alizarin sulphonate indicator or methyl red.
   - 1 drop caprylic alcohol.

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 × 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.

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.

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.

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.

[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.

Technic.—Into a tall beaker or cylinder 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 $\frac{N}{10}$ potassium permanganate solution. 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.

Technic.—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.

$$\textit{Calculation.}— 10 \times \frac{8.1}{\text{Reading}} = \text{mg. creatinin.}$$

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 creatin 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]

[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 78. 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_2SO_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_2O 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 gram $BaSO_4$ = 0.3430 gram SO_3.
1 gram $BaSO_4$ = 0.4201 gram H_2SO_4.
1 gram $BaSO_4$ = 0.1374 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.

Result.—Multiply the number of c.c. of $\frac{N}{10}$ alkali used by 0.004 to obtain the number of grams of SO_3 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.

Technic. — 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. $\frac{N}{10}$ Disodium phosphate.
2. $\frac{N}{10}$ Monopotassium phosphate.
3. $\frac{N}{5}$ Sodium acetate.
4. $\frac{N}{5}$ Acetic acid.
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 para-nitrophenol.
9. Saturated solution of methyl red, 50 per cent. alcohol.
10. Toluol.

Apparatus.—250 c.c. flasks of glass yielding little or no alkali to water are preferable. Medium-sized test-tubes of good, clear glass.

The disodium phosphate, $Na_2HPO_4 + 12H_2O$, is exposed to the air for a week, whereby it loses 10 molecules of water

[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.

of crystallization, becoming $Na_2HPO_4 + 2H_2O$, which is stable. 17.8 grams to 1 liter makes a $\frac{N}{10}$ solution; 13.6 grams of KH_2PO_4 to 1 liter makes a $\frac{N}{10}$ solution; 27.34 grams of CH_3COONa, $3H_2O$ to 1 liter makes a $\frac{N}{5}$ solution; $\frac{N}{5}$ CH_3COOH is made in the usual way by titration against a known alkali solution.

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 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 para-nitrophenol (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.

STANDARD SOLUTIONS OF KNOWN HYDROGEN ION CONCENTRATION.

	Solution.	pH.[1]
No. 1.	0.50 c.c. N/10 KH_2PO_4 + 240.0 c.c. N/10 Na_2HPO_4 made up to 500 c.c. with H_2O	8.7[2]
No. 2.	0.50 c.c. N/10 KH_2PO_4 + 60.0 c.c. N/10 Na_2HPO_4 made up to 500 c.c. with H_2O	8.0[2]
No. 3.	5.00 c.c. N/10 KH_2PO_4 + 25.0 c.c. N/10 Na_2HPO_4 made up to 500 c.c. with H_2O	7.4[3]
No. 4.	5.00 c.c. N/10 KH_2PO_4 + 11.5 c.c. N/10 Na_2HPO_4 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," page 74.
[2] Phenolphthalein.
[3] Neutral red and sodium alizarin sulphate.
[4] This and remainder of table sodium alizarin sulphate.

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.

Technic.—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

[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.

chalk-white precipitate forms and the blue color of the mixture begins to lessen perceptibly, 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 DETERMINATION OF THE ALBUMIN.—Take 100 c.c. of urine. If necessary make it faintly acid with dilute acetic acid and heat on the water-bath until the albumin begins to separate in flakes. After drying the outside of the beaker boil for two minutes over a free flame.

If the albumin does not coagulate well, carefully add a drop or two of dilute acetic acid. Excess of acid may cause some of the albumin to stay in solution.

Filter while still hot through a Schleicher and Schüll nitrogen-free filter (No. 589, 9 cm.). If further quantitative determinations are to be made on the urine, filter into a measuring flask, wash the beaker and filter with small amounts of distilled water until the volume at room temperature is brought up to its original amount. Wash precipitate on the filter with more warm water and then determine the nitrogen of filter paper and its contents by the Kjeldahl method.

The albumin may also be removed before doing a nitrogen partition by adding an equal volume of alumina cream[1] and filtering. The nitrogen removed is determined by Kjeldahling 10 c.c. of the filtrate.

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' precipitation of acetone as a basic mercuric sulphate compound. Oxidation of hydroxybutyric acid and precipitation of the acetone are carried out simultaneously in the same solution, so that the technic 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.

[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).

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 Merck's fine light "reagent" $Ca(OH)_2$ 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 HYDROXYBUTYRIC 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 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 collected, 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 hydroxybutyric 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

[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.

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[1] is equivalent to 13 mg. of precipitate in titration of the latter.

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[2] . . .	24.8	12.4
β-hydroxybutyric acid . . .	26.4	13.2 (14.0)[3]
Acetone plus aceto-acetic acid	20.0	10.0

[1] "M" signifies the molecular weight in grams dissolved in 1 liter of water.

[2] 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 hydroxybutric 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.

[3] The factor in parentheses is the usual factor $\times \frac{170}{160}$ 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.

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.

PHENOSULPHONEPHTHALEIN TEST FOR KIDNEY FUNCTION.—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 6 mg. of the phthalein intramuscularly in the deltoid or lumbar muscles.

Exactly one hour and ten minutes from the time of injection, the patient passes water, and again at the end of two hours and ten minutes from the time of injection. The two specimens are kept separate. If a long period before examination is to elapse, they are rendered acid with phosphoric acid.

The next step is to determine how much of the drug has been excreted during the two periods of collection. Both lots (*i. e.*, that of the first and second hours) are separately rendered alkaline with enough caustic soda solution of 25 per cent. strength to bring out a deep reddish-purple color. Each specimen so colored is then diluted separately to 1000 c.c. with water.

The amount of phthalein is then determined by the colorimetric method. The Duboscq instrument or the Rowntree and Geraghty modification of the Hellige colorimeter may be used. If the Duboscq instrument is used, the standard solution should be one-half strength; that is, 3 mg. to the liter, alkalinized with 1 or 2 drops of a 25 per cent. solution NaOH. The left-hand cup of the instrument is half-filled with this solution and set at 10, or slightly above or below 10 if the zero point requires correction as described in the next paragraph. The right cup is similarly filled with the solution to

be tested, the color equalized, and the reading made, including the fraction indicated by the vernier.

The equality of the two Duboscq cups should be tested by placing standard solution in both cups. If, with the right cup at 10, the reading of the left is 10.5, the left, containing the standard, should be placed at 10.5 instead of 10 when analyses are made.

The calculation is: per cent. excretion = $50 \times \frac{10}{R}$ where R is the reading of the right-hand cup containing the urine, 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-

[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.

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 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. 85, 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. 113).

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.

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 appa-

[1] See Determination of Urea in Urine.

ratus 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.

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 AND VAN SLYKE.—*Principle.*—The proteins are removed from the blood plasma by coagulation by heat in acid solution in the presence of an excess of magnesium sulphate.[2] The fluid is clarified by the addition of animal charcoal and in the clear filtrate the chlorides are determined by titration with potassium iodide. For this purpose an

[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.

[2] See end of description for other methods of removing protein. Since carbonic acid concentration affects the distribution of chloride between plasma corpuscles, blood for chloride determination should be drawn and centrifuged with the same precaution as blood for plasma bicarbonate determination (page 123).

excess of standard silver nitrate solution is added, the resulting precipitate of silver chloride is removed by filtration, and the amount of silver remaining uncombined is determined by titration with potassium iodide in the presence of nitrous acid and starch. As soon as the iodine has combined with all the silver, any additional amount added gives a blue color with the starch.

Reagents.—

10 per cent. magnesium sulphate solution.

Blood charcoal (Merck's reagent; chloride-free).

Silver nitrate solution (1 c.c. equivalent to 2 mg. of NaCl).

Silver nitrate	5.812	grams
Nitric acid (sp. gr. 1.42)	250.0	c.c.
Water to	1000.0	c.c.

Potassium iodide solution (1 c.c. equivalent to 1 mg. of NaCl):

Potassium iodide	3.0	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
Sodium nitrite	20.0	grams
Soluble starch	2.5	grams
Water to	1000.0	c.c.

Dissolve the starch in 500 c.c. of warm water, add the salts, and heat until dissolved. While still hot filter through cotton (paper might remove starch), wash the filter with hot water, and after cooling, make the 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, and the nitrite to liberate the iodine 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 solution to the first appearance of a blue color. The iodide solution is then diluted so that 10 c.c. are exactly equivalent to 5 c.c. of the silver solution.

Technic. — Centrifuge 5 c.c. or more of oxalated blood and with an Ostwald pipette, calibrated to *contain* (not deliver) 2 c.c., measure 2 c.c. of the plasma so obtained into a 20 c.c. stoppered volumetric flask containing 10 c.c. of 10 per cent. magnesium sulphate solution, rinsing the pipette twice by drawing up the solution. Add 2 drops of 50 per cent. acetic acid, add water to the 20 c.c. mark, mix, stopper lightly, and heat in the water or steam-bath to 100° C. for ten minutes. Allow to cool and mix with about 0.3 gram of blood charcoal (Merck's reagent) in a beaker. Filter through a dry, folded filter until a water-clear filtrate is obtained. Measure 10 c.c. of the filtrate into a 25 c.c. volumetric flask, add 5 c.c. of the silver nitrate solution (equivalent to 10 mg. of NaCl), and make up to 25 c.c. Add 2 drops of caprylic alcohol, stopper the flask, and invert several times. Allow to stand five minutes, and filter through a dry, folded filter, obtaining a clear, colorless filtrate. To 20 c.c. of the filtrate add an amount of the "starch solution" equal to the amount of silver solution present in the volume of filtrate used; *e. g.*, in the present instance (5 c.c. of silver solution diluted to 25 c.c. and 20 c.c. of filtrate taken) 4 c.c. of silver solution is present and 4 c.c. of starch solution must be added. Then titrate with the potassium iodide solution to the appearance of the first definite blue color.

Result.—Using 20 c.c. of filtrate from the silver chloride,

$$\text{Gm. NaCl per liter} = \frac{12.5\ (8 - \text{c.c. KI})}{\text{c.c. plasma filtrate taken}}$$

The chlorides in human plasma are normally from 5.62 to 6.25 grams of sodium chloride per liter, according to the amount ingested.

In view of the present difficulty of obtaining chloride-free blood charcoal, Harding and Mason have devised the following means of removing the proteins: "2 c.c. of oxalated plasma are pipetted into a 20 c.c. volumetric flask containing 10 c.c. of distilled water; 0.5 c.c. of a 7 per cent. copper sulphate solution is added, together with 2 c.c. of $\frac{N}{50}$ NaOH. The contents of the flask are well mixed, 2 drops of caprylic alcohol added, and the solution diluted up to the mark. The flask is loosely stoppered and heated ten minutes in a boiling water-bath. Two or three times during the heating the flask is removed from the bath, the stopper tightened, and the precipitate and fluid are mixed by inverting three or four times. At the end of the period the flask is cooled and the liquid filtered through a folded filter into a dry flask. Ten cubic centimeters of the filtrate is pipetted into a 25 c.c. volumetric flask, to it is added 5 c.c. of 10 per cent. magnesium sulphate, and 5 c.c. of the standard silver nitrate solution. From this point the procedure is as described by McLean and Van Slyke."

Foster, in Folin's laboratory, obviates the use of charcoal by precipitating the proteins with metaphosphoric acid. Take 2 c.c. of plasma (or whole blood) in a 20 c.c. flask nearly full of water. Slowly and with stirring add 1 c.c. of a 25 per cent. solution of metaphosphoric acid prepared within the last forty-eight hours. Fill to the mark, shake well, and let stand ten minutes, with occasional shaking. Filter and use 10 c.c. of the filtrate for the titration.

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 stagnant 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 drawn the stopper is loosened and the blood stirred with the inlet tube in order to assure distribution of the oxalate. The tube should not be shaken or inverted. The blood is centrifuged in it within a half-hour. In place of the tube illustrated 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

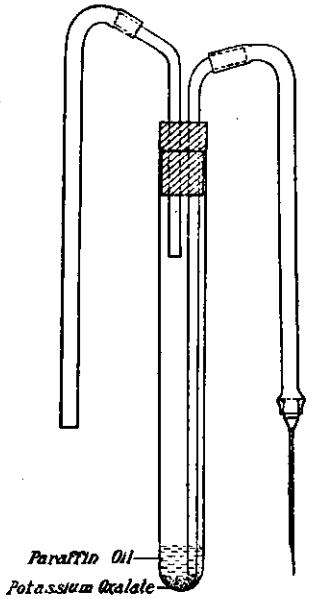

FIG. 2

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

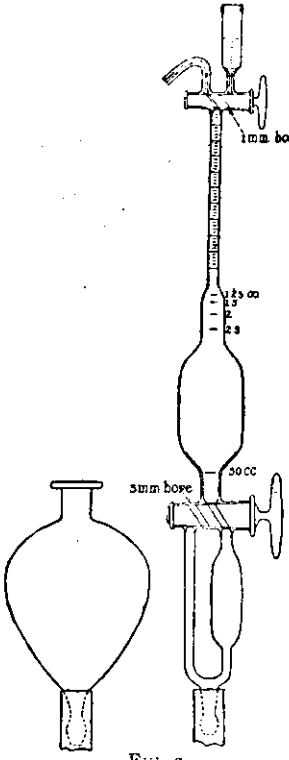

FIG. 3

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 leveling 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 allowed 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 apparatus 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 128 and 129 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

TABLE FOR CALCULATION OF CARBON DIOXIDE COMBINING POWER OF PLASMA.[1]

Observed vol. gas × $\frac{B^1}{760}$	C.c of CO_2, reduced to 0°, 760 mm., bound as bicarbonate by 100 c.c. of plasma.				Observed vol. gas × $\frac{B}{760}$	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°
0.20	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] $\frac{B}{760} = \frac{\text{Barometric pressure}}{760}$

TABLE (*continued*).

Observed vol. gas × B/760	C.c. of CO_2, reduced to 0°, 760 mm., bound as bicarbonate by 100 c.c. of plasma.				Observed vol. gas × 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

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 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 15 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 5 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 apparatus. 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.

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. Co.	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 "

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 $(0.999 - 0.0046t) \times \frac{\text{barometer}}{760}$, 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 on p. 131, 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.

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]

[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 133.

Technic.—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 volumetric 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 solution.

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,

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_2O_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*).

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 autoclave 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

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.)

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 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.

Break four eggs into a 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 the Arnold. 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.

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 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 184.

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.

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 repeated for five successive days. If haste is desired the medium can be heated in the Arnold after the first or 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.

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.

SODIUM GLYCOCHOLATE AGAR (OTHERWISE KNOWN AS MACCONKEY'S MEDIUM).—This medium consists of a 1.5 or 2 per cent. agar to which 1 per cent. of lactose and 0.5 per cent. of sodium glycocholate are added. It contains the usual amount of peptone, from 1 to 2 per cent., and is titrated to about 1±.

Special media useful for examinations of carriers, etc., will be described in the sections dealing with the individual subjects.

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 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.

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 recom-

mended 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: Use glucose ascitic agar or Loeffler's.

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

For meningococci: Use glucose sheep serum agar in 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 glucose agar or glycerin agar.

Suspected tetanus material can be planted in deep anaërobic glucose agar tubes, either before or after heating to 80° C. by Kitasato's method, and a little of it can be macerated and injected into mice.

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, fitted with a nipple drawn to a capillary point at the tip, 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 and a few plates of sodium-glycocholate agar 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.

SPECIAL BACTERIOLOGICAL METHODS.

TYPHOID WORK (PARATYPHOID AND DYSENTERY).

There should be in the laboratory several stock cultures of typhoid bacilli for Widals; also agglutinating sera for B. typhosus, B. paratyphosus A and B, paratyphosus B, and the dysentery bacilli.

TYPHOID STOOLS AND CARRIERS.—Stools are collected on swabs within test-tubes. Swabs consist of a little cotton on a stick, thrust into a test-tube plugged with cotton, and so dry sterilized. Label on each tube to be marked with name, rank, company, regiment, and whether or not kitchen personnel.

When the swabs are received, the feces are rubbed up in sterile salt solution or broth—pieces about the size of a split pea are rubbed up in about 10 c.c. of solution—allowed to stand and settle for fifteen minutes, and then a loopful is taken from top and placed on the surface of a hardened plate of Endo's medium. This is then spread carefully over the entire surface with a sterile glass rod or capillary pipette bent in the shape of a hockey stick.

ENDO'S MEDIUM.—Into container, 1 liter tap water, marking level of fluid. Add 30 grams of thread agar, 10 grams of peptone, 5 grams of NaCl, 5 grams of beef extract. Cook until dissolved, best autoclave thirty minutes, 15 pounds; filter through sterile gauze or cotton. If necessary, clear with egg. For this purpose, for each liter beat up white of one egg in tin dipper with 10 c.c. of warm water, until egg is well mixed. Add this to agar cooled to 55° C.,

mix thoroughly, heat for thirty minutes or autoclave, and filter through cotton.

To Filter through Cotton.—Small wire spiral placed in bottom of large funnel; over this place cotton as follows: Small square of absorbent cotton split into two sheets; the upper rotated through an angle of 180 degrees until the fibers lie at right angles to those of the lower sheet. A somewhat larger square of gauze can be placed over the cotton, and the whole inverted over a glass funnel, with the gauze layer out. This may be heated in a steam sterilizer for half an hour to sterilize further and improve its sterilizing power. After the larger part of the agar has run through, the four corners of the square of gauze can be gathered up and twisted.

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.

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 exactly hit 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.

Preliminary agglutination slide method: A 1 to 100 dilution is the proportion of potent antityphoid serum and also of antisera for paratyphoid A and B and dysentery. A large loopful of each serum and of salt solution are placed in a row on a slide, and a bit of the suspected colony is picked up with a platinum needle and emulsified in each drop. Organisms will agglutinate within two or three minutes in their corresponding serum. This identification must be confirmed by fermentation test and agglutination reaction on the pure culture.

FERMENTATION TEST.—*Russell Double Sugar Medium.*—To plain agar, preferably 3 per cent. agar of a reaction of 0.8 per cent. acid to phenolphthalein, 5 per cent. aqueous litmus is added to give a distinct purple. The reaction is then adjusted by adding NaOH until the mixture is neutral to litmus. To this, 1 per cent. of lactose and 0.1 per cent. of glucose is added after being dissolved in a small amount of hot water. The medium is tubed and slanted after sterilization in the Arnold (not more than fifteen minutes in the Arnold each time should be used to avoid breaking down the lactose). On this medium, the suspicious colonies are inoculated by surface streak and stabbing into the bottom of the medium. The typhoid bacillus gives a colorless growth on a blue background on the surface, with

a red butt. The colon bacillus produces acid throughout and bubbles; paratyphoid forms gas, acid in the bottom, surface blue; dysenteries look like typhoid.

Agglutinations of pure cultures are made in test-tubes, $\frac{3}{8}$ by 4, or can be done in regular Wassermann tubes.

BRILLIANT GREEN AGAR FOR TYPHOID ISOLATION (KRUMWIEDE).—Krumwiede has recently devised a brilliant green agar with which he has had excellent results.

The basis is an extract agar like that used for Endo's medium:

Beef extract	0.3 per cent.
Salt	0.5 "
Peptone	1.0 "
Agar	1.5 "

Domestic peptones are satisfactory.

Dissolve in autoclave; clear and filter. A clear agar is essential. The final reaction of the medium is to be neutral to Andrade's[1] indicator, which in terms of phenolphthalein is 0.6 to 0.7 per cent. acid (normal HCl). It is more convenient to have the reaction set slightly alkaline to litmus at the time of preparation and to acidify each bottle as used. The agar is bottled in 100 c.c. amounts and autoclaved. When needed, the bottles are melted and the volume of each corrected (if necessary) to an approximate 100 c.c. Add to each bottle:

One per cent. Andrade's indicator.

Acid to bring to neutral point of the indicator.[2]

[1] Andrade's indicator:

0.5 per cent. aqueous acid fuchsin	100 c.c.
Normal NaOH	16 c.c.

The dye is slowly (two hours) alkalinized to the color-base; the red tint is restored by acids.

[2] An agar is neutral to Andrade when, hot, the color is a deep red, but fades completely on cooling. This is determined by cooling 3 or 4 c.c. of acidified hot agar in a serum tube under the tap and adjusting accordingly.

One per cent. lactose.[1]
One-tenth per cent. glucose.[1]
Brilliant green in 0.1 per cent. aqueous solution.

Two dilutions of dye are used in routine plating, corresponding to 1 to 500,000 and 1 to 330,000 in terms of solid dye (0.2 c.c. and 0.3 c.c. of 0.1 per cent. solution per 100 c.c. of agar). The sample of dye which Krumwiede has used is from Bayer, but he has also tested and found equally satisfactory samples from Grubler and Höchst; 0.1 gram of dye is accurately weighed on a foil, washed with boiling H_2O into a 100 c.c. volumetric flask and made up to the mark when cool. The flask should be clean and neutral (by test). Fresh solutions vary in activity (see Standardization Tests); they keep about a month.

Each bottle is mixed and poured into six plates only (a thick layer of agar gives the most characteristic colonies). Plates are left uncovered until agar has "jellied;" porous tops are used; dry plates are essential to avoid diffusion.

Standardization.—The agar must have proper "balance." The reaction is important; sediment reduces the activity of the dye, and light colored media are better than darker ones. Different lots of agar with the same dye solution act uniformly; a new batch or a new solution must be tested.

Any variation in the composition of the media necessitates a readjustment of dye concentration; this statement cannot be overemphasized.

Serum dilutions are made as follows:

Add 0.1 c.c. of serum to 9.9 c.c. salt solution in test-tube = 1 to 100; 1 c.c. of this to 4 c.c. salt solution = 1 to 500; 1 c.c. of 1 to 500 to 1 c.c. salt solution = 1 to 1000.

The 1 to 100 dilution can be kept in ice-chest for several days and used over again for higher dilutions.

[1] These are conveniently added from one sterile solution containing 20 per cent. lactose and 2 per cent. dextrose; 5 c.c. to 100 of agar gives the requisite concentration.

AGGLUTINATION TEST.—Into four agglutination tubes put: (1) 0.5 c.c. of 1 to 100, (2) 0.5 c.c. of 1 to 500, (3) 0.5 c.c. of 1 to 1000, and (4) 0.5 c.c. salt solution. To each of these add 0.5 c.c. of a suspension of the suspected bacilli, made as follows:

The salt solution is gently poured over the culture in the double-sugar medium and emulsified with a soft platinum loop, not breaking agar, until suspension has turbidity of about twenty-four-hour broth culture.

This can be carefully poured into another test-tube and gently shaken, then allowed to settle and measured off with nipple pipette. Or a standard loopful of the suspected culture may be emulsified in 1 c.c. of the serum dilution directly. These tubes are incubated or put on a water-bath.

Motility for observation and Gram-staining of these cultures should be carried out, and if typhoid agglutination is unsuccessful a rough orientation agglutination, as described above, for paratyphoid and dysentery, should be made, followed up by specific macro-agglutination as described for typhoid. It is necessary for accuracy in this work to use an agglutinating serum of relatively high titer to rule out group reactions common with the paratyphoids.

TYPHOID DIAGNOSTIC AGGLUTINATION (WIDAL).—Stock cultures can be used, daily transplanted into broth tubes with meat extract broth of an acidity not exceeding 0.5. It is suggested that several laboratories may perhaps be able to furnish standard suspensions of formalinized typhoid cultures.

The patient's serum is taken in Wright capsules. Serum is centrifugalized from clot and taken up in capillary pipette. Dilutions are made in capillary pipettes as for opsonic tests, 1 to 10, 1 to 20, 1 to 40. Hollow-ground slide preparations are made in which a loopful of each of these dilutions is mixed with a loopful of culture, giving preparations of 1 to 20, 1 to 40, 1 to 80.

Original serum dilutions can be made by drop method: 1 drop to 9 of salt solution = 1 to 10, etc. A control preparation of salt solution and culture should be made in each case. Preparations are set away and looked at every fifteen minutes for one hour.

If formalinized suspension from such a laboratory is available, macroscopic tests in $\frac{3}{8}$ by 4 test-tubes can be made of same dilutions as described for stool diagnosis.

TYPHOID BLOOD CULTURES.—Blood is taken from cubital vein and placed at bedside in a sterile syringe, and place immediately 1 c.c. into a tube of glycerin-peptone bile made as follows:

Ox bile	9 parts
Glycerin	1 part

To which add:

Peptone	2 per cent.

Or sterile filtered ox bile can be used without additions.

Distribute 10 c.c. to a tube and sterilize.

If sterile Petri dishes are available, place 1 c.c. of blood in a tube of melted agar containing 1 per cent. of sodium glycocholate, cooled to 45° C. The blood is mixed and poured immediately into Petri dishes. Bile tubes and bile plates are placed immediately in the incubator, and transfers made to slant agar from bile tubes in twenty-four hours.

If the specimen cannot be carried directly to a laboratory, the blood should be taken in the fluid bile medium in test-tubes, the cotton stopper removed and replaced by cork freshly sterilized in melted paraffin. For ease of taking typhoid blood cultures at a distance from the laboratory, bile medium may be stored 5 c.c. in test-tube 4 by $\frac{5}{8}$ inches with a sterile paraffin cork.

While bile media are very useful for typhoid-blood culture work, they are not indispensable. If bile is not available,

excellent results can be attained by taking the blood into small flasks containing 100 c.c. of plain broth or oxalate broth. Success is also achieved by plating in glucose agar.

TYPHOID URINE.—Urine for the isolation of typhoid bacilli should be taken aseptically in a sterile container at bedside and urine transferred with nipple pipettes to broth in flasks or tubes. If motile, Gram-negative bacilli are present in the broth after twenty-four hours' incubation, isolation may be done in the usual way and identification by agglutination and Russell double sugar.

SIMPLE DIAGNOSTIC TABLE FOR GRAM-NEGATIVE BACILLI.

FERMENTATION.

	Dextrose.	Mannit.	Lactose.	Saccharose.	Dulcit.	
B. fecalis alkaligenes	o	o	o	o		Do not liquefy gelatin.
B. dysentery (Shiga)	ac	o	o	o	..	
B. dysentery (Flexner)	ac	ac	o	ac	..	
B. dysentery (Hiss-Russell)	ac	ac	o	o	..	
B. typhosus	ac	o	o	o	..	
B. paratyphosus	+	o	o	o	..	
B. coli communis	+	+	+	o	+	Liquefy gelatin.
B. coli communior	+	+	+	+	+	
B. lactis aërogenes	+	+	+	+	o	
B. acidi lactici	+	+	+	o	o	
B. of proteus group	+	+	o	+	?	

\+ = gas.
ac = acid, no gas.
o = neither acid nor gas.

DIPHTHERIA THROAT CULTURES.

SWAB TUBES.—Swab tubes should be put up in small test-tubes made up with a stick, about five or six inches long, on

the end of which a piece of absorbent cotton is tightly wound. This is thrust into a test-tube, passing by the side of the cotton plug which plugs the tube. Label should be on swab tubes.

Medium.—Should be Loeffler's medium, preferably made at "base" laboratory. The preparation is described elsewhere.

TAKING OF THROAT CULTURES.—In taking throat cultures, no blind jab at the throat should be made, but pharynx and tonsils carefully illuminated, tongue depressor used, and swab rubbed over area of inflammation or exudation, if possible dislodging a portion of the membrane. The swab should then be used to inoculate the surface of medium and put back into the swab tube so that a direct smear from swab may also be made. No time should be unnecesarily lost between inoculation and incubation of medium, since at room temperature other organisms may outgrow Klebs-Loeffler.

After twelve to twenty-four hours' incubation, smears are made on slides or cover-slips of culture and stained with Loeffler's alkaline methylene blue. This consists of:

Saturated alcoholic methylene blue .	30 c.c.
1 to 10,000 aqueous KOH	100 c.c.

The stain is best after some preservation. It is left on the fixed slides for one to three minutes.

Usually this stain is enough for a skilled worker for diagnosis. In doubtful cases, if it is desirable to bring out polar bodies, the following stain can be used (two solutions):

Acid Methylene Blue.—

Methylene blue	1 gram.
Absolute alcohol	20 c.c.
Glacial acetic acid	50 c.c.
Distilled water	1000 c.c.

Dissolve and filter. Smears covered for two to five seconds, washed in water, counterstained with Bismarck brown.

THE SCHICK REACTION.—The purpose of the Schick reaction is to determine whether or not an individual has such natural immunity against diphtheria to be, for all practical purposes, unsusceptible to infection. 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 Schick 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 a human being containing a sufficient amount of 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.

The usefulness of the reaction depends entirely upon the fact that the proper dose of toxin for such tests 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 this amount is contained in 0.2 c.c. volume.

Such dilutions are made as follows: Of the toxin furnished by the health department or other laboratory, make a small amount of stock solution such 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. menol or fresol 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 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 pseudo-reaction 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 pseudo-reaction 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 pseudo-reaction.

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 some others, but can be easily prepared if toxin of which the M. L. D. is noted is procured and proper dilution made in such a way that 0.2 c.c. contains $\frac{1}{50}$ M. L. D.

MENINGOCOCCUS WORK.

MAKING MEDIA FOR MENINGOCOCCI.—*Sheep Serum Agar.*—Veal infusion agar, using Fairchild's peptone.

Reaction 0.4+ to phenolphthalein.

Add 1 per cent. dextrose.

Autoclave and tube, 5 c.c. to each tube, for plates, use 10 c.c. in each tube.

Sheep serum water, 1 part serum to 3 parts distilled water, is sterilized by placing in the autoclave for forty minutes at fifteen pounds' pressure.

Add 1 c.c. of sheep serum water to each 5 c.c. of melted agar, which has been cooled to 50° C.

Slant the tubes and incubate for seventy-two hours.

Keep at room temperature forty-eight hours before using.

Defibrinated human or rabbit's blood, laked with the smallest amount of sterile distilled water that will lake it, may be added to glucose agar in quantities sufficient to give a deep transparent pink color. This is perhaps the best plate and slant medium for meningococcus.

GROWING MENINGOCOCCUS CULTURES FOR STOCK USE.—Sear the slant surface of the sheep serum agar with a hot, heavy platinum loop.

Inoculate heavily with same loop.

Incubate tube for sixteen hours only.

Keep at room temperature for thirty-six hours.

NOTE.—Do not put tubes in the ice-box at any time. Do not leave tubes in the incubator more than twenty hours. If cultures have been recently isolated, they should be transplanted more frequently than thirty-six hours.

METHOD OF CULTURE.—The postpharyngeal space is swabbed with a West tube.

The swab should be inoculated on the center of the plate. This inoculated area is then spread over the entire surface of the plate by means of a platinum loop.

FIG. 4.—West swab tube.

The medium for plate cultures is given above. An added advantage in detection of meningococcus colonies may be obtained by adding 2 c.c. of a 10 per cent. litmus solution (in distilled water), which is sterilized separately, to each 10 c.c. of the medium. Meningococci will have their usual characteristics and besides will appear pink, whereas M. catarrhalis will appear blue or gray blue, while M. flavus will show up in yellow or green opaque colonies.

The plates should be incubated at 37° C. for eighteen to twenty hours, not longer.

Suspicious colonies are fished from the plate and stained for morphological study, then transplanted to sheep-serum agar for stock and plain agar for agglutination tests. If the plain agar cultures show no growth, the agglutination tests may be made with the sheep-serum agar cultures, but plain agar is preferable.

Litmus is not a great advantage as an addition to the plating media. It is a little more useful in indicating acid colonies if 2 per cent. of glucose is added, to the agar instead of 1 per cent.

METHOD OF AGGLUTINATION.—The agglutination is prepared by washing the growth of an agar slant (preferably plain agar) in 2 c.c. normal salt solution. Of this emulsion of live bacteria, 0.2 c.c. is used for each dilution of serum.

For diagnosis, polyvalent antimeningitis horse serum (immunized to numerous cultures of all types) is used. The dilutions are made up as follows: 1 to 50, 1 to 100, 1 to 500, 1 to 1000, 1 to 2000. Of each of these dilutions, 0.8 c.c. is placed in small tubes (9 x 1 cm.).

Controls of the bacteria in salt solution should always be included.

The tubes are then placed in an oven at 55° C. for overnight (sixteen hours), after which they are read.

A meningococcus is completely agglutinated in a dilution of at least 1 to 100 by the polyvalent serum. Other organisms are not agglutinated by this serum in 1 to 50 dilution.

For routine diagnostic agglutinations on larger numbers of carriers, it should be remembered that polyvalent meningococcus serum will not agglutinate all meningococcus strains more highly than 1 to 200, and a standard dilution of 1 to 100 or 1 to 200 should be used—as indicated by preliminary tests on spinal-fluid cultures isolated at the particular camp. It must not be forgotten that Micrococcus flavus agglutinates in normal horse serum and the agglutination results should therefore be carefully coördinated with the cultures. M. flavus grows at room temperature and turns yellow.

A further separation of the meningococcus into its various types is possible by the use of monovalent sera. These sera are usually prepared by immunizing rabbits to the various types of meningococci, as the normal (or regular) and the paranormal (or parameningococcus) types. If either type serum fails to agglutinate, and the organism is agglutinated by the polyvalent serum, the meningococcus is then regarded as irregular. A further subdivision of the irregular types is possible, into those which are irregularly normal, or irregularly para, etc., by immunizing rabbits to such groups of meningococci.

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 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.

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 suspension. 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 antipneumococcus 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 subgroups—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 large 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 water-bath 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 176.

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..

CHOLERA.

ISOLATION OF CHOLERA SPIRILLA FROM STOOLS.—The isolation of the cholera organisms from feces is more easily accomplished than is the isolation of typhoid bacilli, since in well-developed cases of cholera the organisms are present in relatively large quantities. A number of agar and gelatin plates can be directly inoculated with dilutions of the stools. Stools are also inoculated into tubes containing Dunham's peptone broth. If no suspicious colonies are obtained directly from the plates, smears can be made from the upper layers of the Dunham broth tubes and examined morphologically for spirilla.

A good agglutinating serum is essential in cholera diagnosis, since agglutinations are made at each stage of the work; even with the rice-water stool a positive agglutination may be obtained with a serum of high titer.

The cholera colony on agar is opalescent, and direct microscopic agglutinations and smears from the plates may estab-

lish the diagnosis. If the plates are negative, reliance must be placed on enrichment in peptone salt media (Dunham's broth).

Agglutinations as well as smears may be made from specimens taken near the surface, where there is more oxygen, at intervals of a few hours. It is advisable to make a second series of agar plates from the Dunham tubes to fall back upon when necessary.

Of the selective media described, probably the most favorable one is the medium of Dieudonné.—This consists of seventy parts of ordinary 3 per cent. agar, neutralized to litmus, to which thirty parts of a sterile mixture of defibrinated beef blood and normal sodium hydrate solution are added. This mixture is sterilized before being added to agar.

This alkaline agar is poured into plates and allowed to dry for a day or so in the incubator, and the material to be examined is smeared on the surface with a glass rod, as in typhoid isolation.

Identification is carried out by means of the indol reaction, agglutination in specified serum, and by morphology and liquefication of gelatins. The true cholera spirilla do not hemolyze blood, but do liquefy coagulated blood serum.

ISOLATION OF CHOLERA SPIRILLA FROM WATER.—Since cholera spirilla grow excellently in Dunham's peptone broth, a large quantity of the suspected water can be taken, that is, quantities of 1 liter, and to these added to each liter 10 grams of peptone and 5 grams of sodium chloride. In this way, the water itself is made into Dunham's broth, and further examination of these cultures can be done after incubation, as described above.

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.

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 added by 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.

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.

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. Then by focussing up and down concentric rings of light can be seen and brought to center on the preparation. This centering of the light rings is accomplished by shifting the condenser from side to side by means of the lateral screws, and up and down by means of the vertical condenser screw. By gently moving the condenser up and down, adjustment is made 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's-eye lens on the source of light is unsatisfactory, a 2-liter flask full of clear water just in front of it is useful.

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 indistinguish-

able from pallidum. In such cases, the findings of the dark field must be tentatively interpreted, together with the clinical appearance and the Wassermann reaction.

EXAMINATION FOR GAS BACILLUS.

The gas or Bacillus aërogenes capsulatus is normally found in the soil and in the normal intestinal canal of man and mammals. In infections, it is found chiefly at the site of trauma and, late in the disease before death, may be found in the circulating blood. It is a group rather than a single species, and is an obligatory anaërobe, though small quantities of oxygen do not absolutely inhibit its growth as they do the growth of the tetanus and some other anaërobes. It is a large Gram-positive, non-motile bacillus with rounded ends. It rarely forms chains. Spore formation is inconstant. Capsules are seen when the smears are made directly from the lesions but are rarely formed in culture.

Pure cultures can be obtained either by anaërobic plating or by the Nuttall method, which consists in injecting a small amount of exudate into the ear vein of a rabbit and killing the rabbit after a few minutes and incubating it for four or five hours. Gas bubbles on the liver will often contain pure cultures of the organism.

CULTURAL ISOLATION OF TUBERCLE BACILLI.

The principles on which Petroff's method rests are, first of all, the bactericidal power of 3 per cent. sodium hydroxide on non-acid-fast bacteria, and the selective action of dyes like gentian violet on bacterial growth, as first practically utilized by Churchman.

The medium used by Petroff is made as follows:

1. MEAT JUICE.—500 grams of beef or veal are infused in 500 c.c. of a 15 per cent. solution of glycerin in water, in a cool place. After twenty-four hours the meat is squeezed in a sterile press and the infusion collected in a sterile beaker.

2. EGGS.—The shells of the eggs are sterilized by ten-minute immersion in 70 per cent. alcohol. They are broken into a sterile beaker, well mixed and filtered through sterile gauze. One part of meat juice is added to two parts of egg by volume.

3. GENTIAN VIOLET.—One per cent. alcoholic solution of gentian violet is added to make a final proportion of 1 to 10,000.

The three ingredients are well mixed. The medium is tubed and inspissated as usual.

Equal parts of sputum and 3 per cent. sodium hydroxide are shaken and incubated at 38° 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 is 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.

RABIES.

Smears are made by pressing between a glass slide and a cover-glass a small, thin section of the gray matter from (*a*) Ammon's horn, (*b*) the cerebellum, or (*c*) the cerebral cortex;

the material is spread along the slide by moving the cover-glass along with the finger.

Without allowing to dry, the smears are fixed for about ten seconds in neutralized[1] methyl alcohol to which 0.1 per cent. of picric acid has been added. The excess of the fixative is removed by blotting with fine filter paper.

The fixed smears are stained in the following solution:

Saturated alcoholic solution of fuchsin	0.3 part
Saturated alcoholic solution of methylene blue	2.0 parts
Distilled water	30.0 parts

NOTE.—This solution, which is a modification of the one proposed by Van Gieson for staining the Negri bodies in smears, changes rather quickly at room temperature; but, kept in the ice-box it gives good results for an indefinite time.

The stain is poured on the smear and held over the flame until it steams. The smear is then washed in tap water and blotted with fine filter paper. With this stain the Negri bodies appear magenta, the nerve cells blue, and the red blood cells yellow or salmon in color.

For embedding blocks of suspected material in paraffin, Zenker fixation is preferred. Sections are cut from the paraffin block, 3 or 4 micra in thickness. The paraffin is removed from the sections, which are then run down through the alcohols to water. They are then placed in Gram's iodin solution to remove the bichloride of mercury. Wash in water. Remove iodin in 95 per cent. alcohol. Wash in water. Place in 5 per cent. aqueous solution of eosin for twenty minutes. Wash in water. Place in Loeffler's methylene blue for fifteen minutes. Wash in water. Place in saturated solution of colophonium (resin) in absolute alcohol until the sections turn to a pink color. Place in

[1] The wood alcohol is neutralized by adding sodium carbonate (Na_2CO_3) about 0.25 gm. to 500 c.c. of the alcohol.

absolute alcohol, then in xylene, Canada balsam, cover-glass. Examine in oil. With this stain, the Negri bodies are pink to a magenta in color, the nerve cells blue, and red blood cells are yellowish in color.

The following is the routine method of handling material:

If the material is fresh, smears are made from the three parts mentioned and according to the method described, and blocks are embedded in paraffin for sections. If Negri bodies are not found, an emulsion is made of good-sized pieces from the different parts of the brain, and intracerebral inoculations are made into three rabbits; about 0.25 c.c. of the emulsion is inoculated. An emulsion is also made of the different parts of the brain in glycerin for later inoculations, if for any reason the first should fail. Sterilized, neutral glycerin is used. These emulsions remain active in the ice-box for over three months.

Contaminated, doubtful material is made into a weaker emulsion in order to lessen the number of bacteria; and rabbits are inoculated in the muscles of the back.

The glycerinated emulsion made from part of the same material is inoculated after two weeks, unless positive results have been gotten from the weak emulsion.

LENTZ'S METHOD OF STAINING NEGRI BODIES.—With Lentz's method it is possible to prepare stained sections of the brain within four hours, and the picture is beautiful and clear cut. But the method is somewhat complicated, and may fail where one uses it only occasionally.

The method is as follows:

For Sections.—(*a*) Fix 1 mm. thick pieces of brain in acetone in the incubator for one hour.

(*b*) Place in the paraffin bath for one hour, and embed in paraffin.

(*c*) Cut thin sections 2 or 3 micra thick.

(*d*) Remove paraffin with xylol and place in absolute alcohol.

For Smears.—(*a*) Make smears as on page 185.

(*b*) Without allowing to dry, fix the smears one minute in methyl alcohol and put in absolute alcohol.

Proceed with sections or smears as follows:

1. Stain one minute in an eosin solution (0.5 gm. water-soluble eosin in 60 per cent. alcohol, 100 c.c.).
2. Wash in water.
3. Stain in Loeffler's methylene blue one minute.
4. Wash in water.
5. One minute in Lugol's solution.

I	1	Fresh each time.
KI	2	
Aq. dest. ad.	300	

6. Wash off in water.
7. Differentiate in methyl alcohol until no more blue is visible and the preparation appears entirely red.
8. Wash in water.
9. Stain half minute in Loeffler's methylene blue.
10. Wash in water.
11. *Blot dry with filter paper.*
12. Differentiate in alkaline alcohol (30 c.c. absolute alcohol plus 5 drops of 1 per cent. solution of sodium hydrate in absolute alcohol) until the preparation has only a weak eosin color left. It is very necessary that the alkaline alcohol contains no water.
13. Differentiate in acid alcohol (30 c.c. absolute alcohol plus 1 drop of 50 per cent. acetic acid) until the ganglion fibers are still to be seen as weak blue-stained lines.
14. Wash off quickly in absolute alcohol.
15. Clear in xylol and mount in balsam.

Negri bodies magenta in color; inner bodies black; nerve cells blue; red blood cells pink.

PREPARATION OF ANTIRABIC VACCINE FOR INJECTION.—Antirabic vaccine may be obtained by telegraphing the Hygienic Laboratory, Twenty-fifth and E Streets, N. W.,

Washington, D. C. (or, when urgent, from the local State Board of Health).

Directions for the Use of Rabies Virus Shipped from the Hygienic Laboratory, Public Health Service, Washington, D. C.—The virus should be kept in an ice-box or other cold place. This material is perishable and must not be kept on hand for future use. Each bottle contains the number of doses required, until further shipments are made, of cord dried for the number of days indicated on the label. Further shipments of cord to complete treatment already begun are made without further request.

Dose.—Each small section of cord (about 1 to 8 cm.) constitutes one dose.

The following equipment is necessary for making and using the emulsion: Physiological salt solution; alcohol; absorbent cotton or gauze; glass or porcelain mortar and pestle (capacity 10 to 20 c.c.); thumb forceps; hypodermic syringe (at least 3 c.c. capacity), with large needle; glass pipette, 5 c.c., graduated at least to 0.5 c.c.; small conical test-glass or beaker or other small container. These must be sterilized and then the instruments, etc., rinsed in sterile salt solution. The technic must be aseptic throughout.

To Make the Emulsion.—Remove one section of cord from the bottle with the thumb forceps and rinse it free of glycerin with sterile salt solution in the small glass container; place it in the empty mortar, and, without the addition of any fluid, rub up as finely as possible. Then 2.5 c.c. of the salt solution are gradually added by means of the pipette, taking care between the additions of the salt solution to rub to a uniform consistency. Draw all of the emulsion into the syringe.

To Use.—Scrub the skin at the site of inoculation with alcohol and inject the emulsion into the subcutaneous tissue, being careful not to injure muscular layers or visible

veins. Alternate successive injections on the two sides of the anterior abdominal wall.

For the schemes of injection see following table:

SCHEME FOR INTENSIVE TREATMENT.

Twenty-five doses.

Day of treatment.	Cord marked.	Number of injections.	Amount injected.		
			Adult, c.c.	5 to 10 years, c.c.	1 to 5 years, c.c.
1	8–7–6	3 injections, interval 3 hours	2.5	2.5	2.5
2	4–3	2 injections, interval 6 hours	2.5	2.5	2.0
3	5–4	2 injections, interval 6 hours	2.5	2.5	2.5
4	3	1 injection	2.5	2.5	2.0
5	3	1 "	2.5	2.5	2.0
6	2	1 "	2.5	2.0	1.5
7	2	1 "	2.5	2.5	2.0
8	1	1 "	2.5	1.5	1.0
9	5	1 "	2.5	2.5	2.5
10	4	1 "	2.5	2.5	2.5
11	4	1 "	2.5	2.5	2.5
12	3	1 "	2.5	2.5	2.0
13	3	1 "	2.5	2.5	2.0
14	2	1 "	2.5	2.5	2.0
15	2	1 "	2.5	2.5	2.0
16	4	1 "	2.5	2.5	2.5
17	3	1 "	2.5	2.5	2.5
18	2	1 "	2.5	2.5	2.0
19	3	1 "	2.5	2.5	2.0
20	2	1 "	2.5	2.5	2.5
21	1	1 "	2.5	2.5	2.0

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 is 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.

SANITARY EXAMINATION OF MILK.

SANITARY BACTERIOLOGICAL EXAMINATION OF MILK.
A. TOTAL COUNT.

I. PLATE METHOD.—For preparation of apparatus and media see p. 225, 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. 225.) 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 $\frac{56{,}000y}{11d}$, 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.

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 vertical column headed by this number with the horizontal column corresponding to the percentage of fat.

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

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.

In classifying water from a sanitary stand-point, it is good or bad, or a potable or non-potable water. We might go further and divide waters into three classes—good, polluted, and infected.

A *good water* is one of good sanitary quality as determined by physical inspection, bacteriological and chemical analyses, a sanitary survey of watersheds, and by clinical experience.

A *polluted* or *contaminated water* is one containing organic waste of either animal or vegetable origin. We would call this a suspicious water.

An *infected water* contains the specific microörganisms of human diseases.

Water Used and Wasted.—In considering this subject it is necessary to keep in mind the importance of encouraging a generous use of water for sanitary purposes and, at the same time, the conservation of pure water and the economic value of a purified water. The average amount of water required for domestic use is about 17 to 18 gallons per capita. Few persons realize the amount of water that is wasted in almost every town. It is often stated that about one-half of the water supply of American cities is wasted. The three principal causes are: leakage from faulty mains and service pipes, waste from defective house-fittings, and waste resulting from an unmetered or unmeasured service.

Dual Water Supplies.—The use of two water supplies, one cheap for general purposes and the other high class for personal

use, has often been discussed. There are arguments for and against such a system and even in the most intelligently served community the danger is very great. Professor Sedgwick has suggested that cities may be given a double water supply provided the one for general use be disinfected in such a way as to discourage its being used for drinking purposes.

Sources of water supply may be divided as follows: (1) Rain or snow water; (2) surface water, including ponds, lakes, streams and rivers; and (3) ground water, including springs and wells.

Rain water, when collected from a clean, impervious surface in the open country is the purest of natural waters. The use of rain water for drinking purposes has met with little favor by sanitarians, despite its exceptional purity, because it is so frequently collected and stored in such a careless manner. It is advisable to filter rain water, especially in towns near dusty roads, the first flow of rain water being discarded, as it would contain the grossest impurities. The storage of rain water is very important. As rain water attacks iron, lead, zinc, and other metals, storage tanks or delivery pipes should not be made of these materials.

Surface Waters.—Streams are the natural sewers of the regions they drain. Most of our large rivers flow through more than one State, therefore the interstate pollution of streams becomes a national problem. No stream draining an inhabited region can be considered safe without some method of purification.

Lakes and ponds are excellent sources of water when kept free from pollution with wastes of human life and industry. Lake water is generally apt to be soft and free from serious organic impurities. Even these supplies should be disinfected with chlorin for added safety.

Impounding reservoirs are artificial ponds or lakes, usually made by damming across a narrow valley.

Ground water is taken from the ground by means of wells or springs. In passing through the soil the water takes up a rather large amount of carbon dioxide, which is set free from organic decomposition. The water, thus acidulated, has a greater solvent action for lime and other mineral constituents, so that ground water is apt to be harder than surface water and to contain a larger amount of inorganic substances, such as lime, iron, common salt, etc. Ground water in any quantity is found in sandy, gravelly, or sandstone formation. It is only in the limestone region that ground water exists as flowing rivers or in large bodies. Filter galleries or excavations in sandy materials near river banks have been used in the past. Such water corresponds to that taken from sand and gravel deposits.

Wells may be shallow or deep, dug or bored. A shallow well usually means one that is dug and lined with stone or brick. Deep wells generally mean drilled or the so-called artesian wells. If the water is drawn from a depth of 100 feet or more without passing an impervious stratum, the well is usually called a deep well. If the well passes through an impervious stratum into a pervious one beneath, in which the water rests upon another impervious stratum, it is called an artesian well.

All wells should be carefully constructed. One of the most important points in the case of shallow wells is to extend the casing at least eighteen inches above the surface of the ground. The wall floor should rest upon casing in order to prevent frogs, mice or bugs from crawling in.

Springs may be regarded as natural wells outcropping where geological formation is favorable. Springs may be polluted from various sources. The overlying layer of porous soil may be too thin to remove the contamination of surface washings from privies, stables, etc. They may also be contaminated by surface washing, and to prevent this there should be a cement casing around the spring and high enough to ward off the washings.

RELATION OF POLLUTION OF WATER TO THE TRANSMISSION OF WATER-BORNE DISEASES.

Water supplies may be said to be contaminated by three classes of materials: Vegetable and animal refuse, wastes from human beings, wastes from industrial operations.

A water contaminated by the latter, unmixed with either of the other two, cannot cause infectious diseases, and can only be of danger to the human beings using it in proportion to the deleterious physical or chemical action which these wastes may have on the human body.

A water contaminated with vegetable or animal refuse, or both, but not containing any human excrement or other human waste, will not be the means of transmitting any infectious disease to human beings except possibly, under some circumstances, those communicable from animals to man, of which anthrax and foot-and-mouth disease are examples. Whether the tubercle bacilli of bovine character which may be present in the discharges of animals when washed into a water supply can cause tuberculous infection in the human beings using that water has not been satisfactorily determined.

Likewise, it is as yet more or less uncertain whether a water containing a large amount of organic matter of vegetable or of animal origin, excluding that from human sources, is liable to cause pathological or diseased conditions, not transmissible in character, in those using it. For example, it is not known whether a water containing a large amount of organic matter of this character may not be the means of stimulating a gastro-enteric and diarrhea in infants, young children, or invalids.

We may say of these two groups of contaminating materials that they are non-specific in any disease-producing property which they may have. They do not produce any definite infectious diseases except in the cases mentioned.

When we come to consider the second group—that is, of pollutions with wastes from human beings—we find the really important contaminations from the public health stand-point. However, here we can make certain differentiations with profit. It is possible to use for potable purposes a water polluted with human excrement from perfectly healthy individuals who are not harboring any disease-producing bacteria and yet not have any resulting disease in the users of the supply, for the reason that no disease-producing bacteria gain access to the water. This pollution may be of considerable volume and yet cause no specific disease. The chief danger in such cases is the possibility that a case, or cases, of typhoid fever may at any time occur among the group of healthy persons polluting the water. The liability of this polluted water to produce disease will depend on two factors—chiefly the amount of the pollution and the directness with which it reaches the consumer. If the infectious material is large in amount but a relatively long period is taken for the water to reach the user, the danger is less than if the amount is small and the avenue of travel and the rate of passage are brief, because the bacteria of disease are relatively short-lived in water.

All these facts must be kept clearly in mind, and must be considered in determining official action regarding a water supply upon receipt of reports of the chemical or bacteriological examination of samples.

These examinations cannot give results which enable the interpreter to state that the pollution is either specific or not specific. Therefore, the most critical dangers to a water supply cannot be determined solely from the results of laboratory examinations. They can only be determined by a personal investigation or thorough inspection of the whole system, with full knowledge at hand of the presence or absence of cases of water-borne diseases on the watershed.

The laboratory examinations are often, but not always,

of value in determining to some extent, but rarely to the fullest degree desirable, the following points:

1. The vegetable origin of contaminations.
2. The animal, including human, origin of pollutions.
3. The relative amounts of such contaminations and pollutions.
4. The directness of such contaminations and pollutions when the latter are or have been present in considerable amounts.

They can therefore furnish data of a general character regarding a water, but this information alone will not generally be sufficient in amount or definite enough in character to warrant basing upon it alone a decision as to present and future potability of that supply.

It should also be stated that a single examination of a series of samples collected at one time furnishes information only of conditions at that time; and if the supply is liable to any fluctuations in either amount or character, then new conditions may be created and the possibility of such fluctuations or changes cannot be determined from a single, or always even from a number, of examinations.

A water supply subject to pollution, direct or indirect, from human sources should be under constant, scientific, technical supervision. If a surface water, then this supervision should consist first of personal inspection carried on more or less continuously, and, second, of laboratory examinations at very frequent intervals. If a ground water, the laboratory examinations should be conducted frequently.

EXAMINATION OF WATER—SANITARY ANALYSIS.

When a complete sanitary analysis of a water is made, it 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 importance.

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.

The object of a sanitary chemical analysis is not to determine the amount of certain compounds of carbon, hydrogen, and nitrogen in the water, because these compounds are in themselves dangerous, but to determine the absence or presence 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.

For this purpose we determine the amount of organic matter, living or dead, that is suspended or dissolved in the water, the amount of certain of the products of decomposition of organic matter, and the amount of certain minerals dissolved in the water.

Each class of waters has its own characteristics. The significance of any given factor must be judged separately for each particular case.

In making an analysis of water, standard methods are used. The methods have been prepared by a competent committee of the American Public Health Association. The advantages of using a standard method are self-evident: It, at least, gives results that are fairly comparable with the work of others.

PHYSICAL EXAMINATION.—Odors in water are very objectionable. As a rule the most objectionable ones develop in surface waters and are caused by the growth of algæ, diatomes, protozoa, and other microscopic beings. 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 or uroglena, aromatic odor of asterionella and pig-pen odor of anabæna, which is one of 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.

Removal of tastes and odors may be accomplished through various means of aëration and by treatment with copper sulphate.

Color in water is usually from a vegetable origin, such as dead leaves, bark, and roots. If the water contains iron it will be perfectly clear on coming from the ground, but will soon turn a rusty yellow color, caused by oxidation of soluble ferrous salts to insoluble ferric salts.

Removal of color may be brought about through storage, because of bleaching action and precipitation and through oxidation by aëration. Coagulants, such as aluminum sulphate, iron, and lime, are added to water containing color. The chemicals combine with the coloring matter and form a precipitate which drops out of suspension, leaving a clear supernatant liquid.

Turbidity is synonymous with muddiness, usually due to clay or silt. This may also be removed by the use of a coagulant, or, if the turbidity is sufficiently coarse, it may be filtered out without previous chemical treatment.

Reaction.—The alkaline reaction of natural waters ordinarily depends upon carbonate and bicarbonate of calcium and magnesium. The incrustants are caused by the sulphates, chlorides, nitrates, and silicates. Acid constituents are represented by aluminum and iron sulphate.

MICROSCOPIC EXAMINATION.—The procedure recommended in the "Standard Methods for the Examination

of Water and Sewage," by the American Public Health Association, should be followed in this work.

The main object of the microscopic examination of water is to determine the presence or absence of those microörganisms which produce objectionable tastes and odors. The determination is also of value as an index of pollution. The organisms comprise the diatomaceæ, chlorophyceæ, cyanophyceæ, fungi, protozoa, rotifera, crustaceæ, and other minute organisms not including bacteria.

Microscopic examination may be considered in five parts:

1. Indicating sewage contamination when such organisms as beggiatoa and miscellaneous objects, as yeast cells, starch grains, fibers of wood and paper, threads of silk, etc., are noted.

2. As indicating progress of self-purification of streams by noting changes in the character of microscopic organisms.

3. Explaining chemical analysis. Large amount of albuminoid ammonia might show a large amount of living sewage pollution. Possibly the sudden decrease in nitrates may be caused by some microscopic organisms using the nitrogenous food.

4. As explaining the cause of turbidity, odors, etc. This has been explained under the heading of Odors earlier in the chapter.

5. As a means of identifying the source of a water (in special cases). The presence of certain microscopic organisms in water gives a clue to its origin. In this way the presence of a surface water may be detected.

6. As a method of studying the food of fishes, oysters, and other aquatic organisms. Where there are no plankton there are no fish. Plankton refers to minute floating animals and plants that are drifted about by waves and currents.

Limnology is that branch of science that treats of lakes and ponds, their geology, their geography, their physics, their chemistry, their biology, and relations of these to each other.

Temperature determined by means of thermometer and thermophone and the study of dissolved gases are important matters.

High excess of chlorin seems to accompany heavy growths of organisms.

Seasonal Distribution.—During the winter the lakes will contain very few organisms.

Horizontal Distribution.—Under this heading we have *Littoral* organisms, which include all those forms that are attached to the shore or to plants on the shore. *Limnetic* organisms are those that make their home in the open water.

Vertical Distribution.—The protozoa as a class seek the upper strata of water.

Removal of growths was taken up under the heading of odors.

CHEMICAL ANALYSIS.—The procedure recommended in the "Standard Methods for the Examination of Water and Sewage" for the American Public Health Association should be followed in this work.

According to standard methods, results are now expressed in parts per 1,000,000. One milligram in 1000 c.c. equals 1 part per 1,000,000. One part per 1,000,000 equals 0.068 grains per United States gallon.

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 substance remaining represents the inorganic matter.

Organic Matter.—Organic matter in the soil and that passing through it with the ground water is of two main kinds, carbonaceous and nitrogenous. These may become oxidized to CO_2, CH_4, and H_2. These end-products are rarely reached in nature as the result of decomposition of

organic matter in the soil. Carbonaceous matter comprises the cellulose, lignin, and similar bodies. These may be oxidized to carbon dioxide. The nitrogenous matter comprises albumins, the waste of animal life, and proteids. These may be oxidized to nitrates. The organic matter in itself is not dangerous to health, but is undesirable because it putrefies and thus gives the water disagreeable tastes and odors; besides this, it offers food for bacterial growth.

Nitrogen is determined as: (1) total nitrogen; (2) nitrogen as free ammonia; (3) nitrogen as albuminoid ammonia; (4) nitrogen as nitrites; (5) nitrogen as nitrates.

Nitrogen as albuminoid ammonia is an approximate measure of nitrogenous organic matter from two sources, vegetable and animal. Proteids and amino bodies from vegetables are much more stable than from sewage and evolve nitrogen less rapidly. The amount is therefore an index of pollution. Organic matter of animal origin yields a larger amount than of vegetable origin. As a rule the albuminoid ammonia in a surface water should not exceed 0.3 p.p.m. and ground water, as a rule, not more than 0.15 p.p.m.

Nitrogen as free ammonia is the result not only of decomposition of nitrogenous organic matter, but is also formed during the process of denitrification, by which nitrates are again reduced to nitrites and nitrites to ammonia.

In a surface or ground water free ammonia represents one of the latter stages of putrefaction of organic matter; thus, the bacterial decomposition of sewage yields large amounts of ammonia. The ammonia itself ordinarily found in drinking water is harmless; its significance lies in the fact that it indicates the presence of putrefying organisms.

Its presence in clean and properly stored rain water has much less significance than in a surface or ground water.

Deep well waters of exceptional purity and practically sterile, may contain a relatively high percentage of free

ammonia. This is supposed to come from a chemical reduction under high pressure and perhaps temperature of the geological nitrogenous matter in coal and alluvial deposits. In general, free ammonia is less of a danger signal than the albuminoid ammonia. More than 0.15 p.p.m. must be regarded as suspicious.

Nitrogen as nitrites is an indication that either oxidation of organic nitrogen or decomposition of nitrates is taking place. It represents the transitional stage of oxidation of organic matter between ammonia and nitrates, and therefore indicates incomplete oxidation of the protein and the active growth of bacteria. As a rule, pure water contains no nitrites or traces, only; however, nitrites may be absent from an impure water owing to the fact that the oxidation has not reached this stage or, perhaps, has entirely passed it. The absence of nitrites therefore does not mean that the water is necessarily safe, while their presence in any but the smallest measurable amounts shows pollution. Usually high free ammonia values and high nitrite values go hand in hand, as both processes are usually going on simultaneously. Nitrites are a danger signal in the same sense that the colon bacillus is a danger signal, indicating pollution but not necessarily infection, for they do not tell the source of nature of the organic matter.

Nitrites are not only formed by the nitrifying bacteria in the soil from the ammonia, but are also formed from the denitrification of nitrates by a variety of microörganisms. The typhoid bacillus, the colon bacillus and many other bacteria have the power of producing nitrites in culture media.

Nitrates.—The final step in the mineralization or nitrification process is the conversion of nitrites into nitric acid, which combines with a base to form nitrates. In the form of nitrates, the nitrogen is completely mineralized and Nature's cycle of conversion has been completed. Their

presence, therefore, signifies past or distant pollution. While the absence of nitrates does not necessarily mean purity, their presence, on the other hand, does not necessarily indicate immediate danger. If the water contains an appreciable quantity of nitrates and no nitrites, it shows that the source of pollution has been distant, and that the organic matter has been completely oxidized. In waters considered pure the nitrates are rarely less than 0.3 part, or may run as high as 1.6 parts per 1,000,000, according to some authorities. A water may contain a large amount of nitrates and also a considerable amount of free ammonia and nitrites. Such a water has been incompletely purified and usually contains a considerable number of bacteria, and if some of these are of human origin the water is, of course, unsafe.

On the other hand there may be a large amount of nitrates and the free ammonia and nitrites be practically absent. Such a water would be one that had at one time been badly polluted with organic matter, but this material had been mineralized by the purification processes. Waters in this condition generally show low bacterial counts and the absence of fecal organisms (bacilli of the B. coli type).

Chlorin.—The organic matter found in water is not stable, but is in a state of transition until it is completely mineralized; but the compounds of chlorin are very stable, and when they once gain access to water they remain to bear witness against it and to serve as a tell-tale of past bad associations. It is because of this fact that so much significance is given to the chlorin content of a water.

The legitimate sources of chlorin in natural waters are from the sea and from the natural deposits of salt that are the remains of prehistoric seas. Storms break up the waves into spray, which is carried inland by the wind currents and with it small particles of salt. This spray is washed down by the rain, so that all surface water contains some salt. The quantity is quite proportional to the distance from the

seacoast, so that several hundred miles inland it is only present in small quantities, while near the seacoast the quantity is considerable, so that where it is not a natural constituent of the earth, as in the salt regions of this State, the normal amount of chlorin for a surface water from any given locality can be quite accurately determined, and any excess above this amount is an indication of pollution by animal or human wastes.

Salt being used in the domestic animal diet and to a much larger extent in the human kitchen, is therefore found in the animal and human wastes; and when such wastes reach a water the presence of this excess chlorin will indicate animal or human pollution which has reached the water at some time in its history. The pollution may have been completely mineralized, yet the chlorin remains to show what has been.

The mixture of even a small proportion of sea water renders the water hard and salty and undesirable for domestic use. The writer had quite a good deal of experience with the mixture of sea water and fresh water at the Miraflores, Canal Zone. It was found there that a water containing 60 to 70 parts per 1,000,000 of chlorin caused trouble with the boilers, and this particular water supply was abandoned.

Oxygen consumed is synonymous with oxygen required and oxygen absorbed. This is a measure of carbonaceous organic matter which is partly oxidized by $KMnO_4$ solution. Water which oxidizes rapidly usually contains unstable carbonaceous matter. Oxygen consumed is closely related to organic color.

Dissolved oxygen represents the degree of aëration or oxygenation of water. The amount of oxygen in solution is fairly constant in waters of uniform composition freely exposed to the air. Water containing sewage and other oxidizable matters uses up the dissolved oxygen.

The absence of dissolved oxygen permits the growth of

anaërobic organisms that cause putrefaction and impart putrid tastes and odors to waters.

The amount of oxygen found in the water of a running stream taken at different points may furnish valuable information as to the rapidity with which the process of self-purification is taking place from a chemical stand-point. When the dissolved oxygen is used up the fish die off. Gasch, Marsson, and Hofer have found undoubted evidences of a close relation between an insufficient amount of dissolved oxygen and fish diseases of both a parasitic and bacterial nature. If dissolved oxygen is absent from ground water, sulphates may be reduced to sulphides and hydrogen sulphide set free.

Carbonic acid may exist in water in three forms: free carbonic acid, bicarbonate, and carbonate. The carbonic acid of the carbonate plus half that of bicarbonate is known as the "bound carbonic acid."

Carbon dioxide from decomposition of organic matter is first of importance in ground waters. It greatly increases the solvent action of water which may cause corrosion of the water pipes.

Iron in water influences its quality from the stand-point of desirability rather than from the stand-point of health. After hardness there is no question of greater practical importance in considering the quality of a water. All natural waters contain a certain amount of iron, and ground waters are apt to contain it in objectionable amounts, which, with the presence of organic matter, promotes the growth of crenothrix. This organism is very troublesome in water pipes. The solution of the iron is brought about by the organic matter. The iron exists in the soil as ferric compounds. These are reduced by the organic matter to ferrous salts, which are soluble in water containing carbonic acid. When ground waters containing iron are first drawn they look clear, but the ferrous salts in solution are soon

oxidized on contact with the air to insoluble ferric salts, which are precipitated as red oxides.

Iron Removal.—One part per 1,000,000 of oxygen oxidizes 7 parts of iron. Acids, organic matter, and manganese interfere with the precipitation of ferric hydrate. Most of the carbon dioxide must be eliminated. Aëration for removal of iron is applied in several ways—by fountains, pre-filters, which consist of a very coarse filtering medium; sunlight also assists in the precipitation of iron.

Lead.—Chemical tests for lead should be made. No water should be used for drinking purposes containing even a trace of lead. The source of the lead in the water is almost always from lead service pipes or some other containers used in collecting, storing or delivering the water.

EXAMINATION OF WATER FOR POISONS.

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]

[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.

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 precipitate 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.

SANITARY BACTERIOLOGICAL EXAMINATION OF WATER.

STANDARD METHODS, A. P. H. A., 1917.—*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.

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 "
Lactose	10 "
Water	1 liter

AGAR.

Beef extract	3 grams
Peptone	5 "
Agar dried at 105° C.	15 "
Water	1 liter

GELATIN.

Beef extract	3 grams
Peptone	5 "
Gelatin dried at 105° C.	100 "
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 are neutral to phenolphthalein.

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.

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.	
(*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

Steps in procedure.	Further procedure required.
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
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

Steps in procedure.	Further procedure required.
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 technic were perfect, we should not be able to prove our case.

The typhoid organism has been occasionally isolated from water supplies, but the laboratory technic 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.

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.

The United States Hygienic Laboratory 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 absent in 1 c.c. and 0.1 c.c. portions.

The specific organisms that are found in water that may cause their corresponding diseases are B. typhosus, cholera vibrio, dysentery bacillus, certain forms of ameba causing amebic dysentery and tubercle bacillus.

Therefore, it is the number of B. coli found in a ground water, as in a surface water, rather than the *mere presence*, which is of sanitary importance.

SANITARY SURVEY.

The preceding discussion of sanitary water analysis makes it quite evident that 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.

When we speak of a sanitary survey we mean the obtaining of the actual knowledge of the physical conditions surrounding the source of the sample, the possible sources of pollution, the geology of the watershed, the slope of the ground, etc. 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 us 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.

We would add to the above statements that clinical skill, knowledge, and experience are frequently baffled unless the interpreter has accurate detailed information as outlined.

PURIFICATION OF WATER SUPPLY.

Most of the early efforts toward water purification had for their purpose the removal of suspended materials, which rendered the water offensive to sight or taste, and it was not until the demonstration of the Altoona, Germany, filters during the cholera epidemic in the city of Hamburg, in 1892, that the remarkable hygienic efficiency of such efforts was suspected or even appreciated. Since then the introduction of water purification plants has progressed by leaps and bounds throughout the world. In 1916 it was estimated that in the United States 30,000,000 people were being supplied with water either filtered or sterilized, or both. This progress has been greatly stimulated by the popular regard of the germ theory of disease, the published records of water-borne epidemics, and the great advance during recent years of the bacteriology of water supplies.

Among the various methods used in the purification of water supplies are the following:

1. Clarification.
   - (*a*) Aluminum sulphate.
   - (*b*) Iron and lime.
2. Filtration.
   - (*a*) Slow sand filters.
   - (*b*) Mechanical rapid gravity filters.
   - (*c*) Pressure filters.

3. Sterilization.
   (*a*) Hypochlorite of lime or chlorine gas.
   (*b*) Copper sulphate.
   (*r*) Ultraviolet ray.

In purifying waters carrying large amounts of clay particles, or waters with a large amount of coloring matter, it is customary to use a coagulant, such as aluminum sulphate alone or a combination of iron and lime. These chemicals coagulate the clay particles and also form a chemical compound with the coloring matter, which causes it to form a precipitate, or what is known commercially as a floc, which drops out of solution and falls to the bottom of the sedimentation basin, leaving a clear supernatant liquid. All waters that are very turbid and contain much coloring matter must be treated in this manner before filtration.

Slow sand filters have been popular up until a few years ago. The most elaborate plants of this type are at Albany, Washington, Philadelphia, Providence, etc., where the waters treated are relatively much less in turbidity.

The slow sand filter is of English origin and consists of a bed of selected sand $2\frac{1}{2}$ feet to 5 feet in depth, underlaid with open-jointed tile or drains. The water to be treated is applied until the bed is well covered and thereafter at substantially uniform rates of from 2,000,000 to 5,000,000 gallons per acre daily for a period of usually from three or four weeks, when the filter is taken out of service and cleaned by scraping off the film of intercepted impurities which have accumulated on the top of the filter from the water. The water which is collected from the underdrain is relatively pure, and unless previously polluted with discharges from human beings will require no further treatment. When such a previous pollution is known to have occurred it is usual to chlorinate the water. Slow sand filters are usually constructed in units of about one acre each, and are covered with masonry roofs where the winter temperature is sufficient to produce thick ice.

The latest means of filtration is that known as the rapid gravity filter, which is of American origin and has been in use since about 1880. It was primarily intended to remove the evidence of turbidity from surface waters and to accomplish this at a high rate. The rate of filtration is generally about 125,000,000 gallons per acre per day.

In the development of this type of a rapid sand filter it has been found necessary to properly prepare the water for filtration. This preparation consists of treating the water with some chemical coagulant, after which the treated water is allowed to remain in a sedimentation basin for a certain period of time, generally six to eight hours. From the sedimentation basin the supernatant water flows on to the rapid sand filters. These filters may be constructed of wood or concrete, having a special straining system, of which there are a number of types. A filtering medium consists generally of about 12 inches of gravel placed over the strainers and about 30 inches of sand, which has a uniform coefficiency and is placed on top of the gravel. A clear water well completes the filtration system. The filtered water collects in this well and is pumped from this to the city. As examples of this type of the filter we have those installed at Grand Rapids, Mich., Cincinnati, Ohio, St. Louis, Mo., New Milford, N. J., Panama and many others.

Pressure filters are useful in purifying water supplies on private estates and swimming pools. The filtering material is enclosed in a water-tight steel tank or iron case and the water is admitted under pressure and withdrawn after filtration without loss of pressure, except that which occurs through frictional resistance of the sand and strainer system. As the filter continues in operation, the accumulation of impurities intercepted gradually clogs the pores or interstices of the filtering material, so that a time eventually arrives when either the resistance becomes too great or the flow of water too small, or both, thus it is necessary to throw the filter out of commission and wash it by reversed pressure.

WATER STERILIZATION.

CALCIUM HYPOCHLORITE.—Calcium hypochlorite (commercial chloride of lime) is now available in two forms: small compressed tablets for individual use and large containers for camp use.

The small tablets, made in three sizes, composed of $CaCl_2O$ and NaCl, yield 3, 17 and 35 mg. of chlorin. If kept dry, cool, and in a dark place these tablets retain their strength several months. It is necessary in the sterilization of water that sufficient time be allowed for the action of the chlorin on the bacteria. The minimum time of action should be twenty minutes.

For waters overtreated with calcium hypochlorite or chlorin, sodium thiosulphate is used to remove the excess chlorin. This salt is available in tablets or large containers.

In the treatment of the larger bodies of water it is more common practice to make up the hypochlorite solutions of 0.5 to 2 per cent. strength. This strength would be 1 to 4 pounds of bleaching powder to 200 pounds of water. It is probable that solutions as strong as 5 per cent. may be made without material loss of oxidizing power, but the more dilute solution gives more satisfactory results.

Essential features of hypochlorite treatment:

1. Powder should be made into a smooth paste.
2. Paste should be thoroughly stirred in the beginning to dissolve all soluble parts of the chemical.
3. Further stirring not essential, but is convenient for keeping the sludge well distributed.
4. Keep solution tanks covered.
5. Solutions deteriorate but little in standing, 2 per cent. per day.
6. Period of contact should be not less than twenty minutes.
7. Point of application should be such that thorough mixture of hypochlorite and water supply is obtained.

8. Solution tanks should have two valves: sludge valve on bottom and discharge valve on side. Remove sludge daily.

9. Uniformity of hypochlorite feed necessary.

10. Strength of hypochlorite solution must be determined with each new charge (see Laboratory Procedure) and the strength of the solution adjusted.

CHLORAMIN.—The addition of ammonia to bleach or sodium hypochlorite solution very markedly increases the germicidal efficiency. This increased efficiency is not noted in similar mixtures with solutions of chlorin gas.

A concentrated solution of ammonia should be avoided. The best results are secured by diluting the ammonia in separate vessels until the solution contains 0.3 to 0.4 per cent. of anhydrous ammonia and discharging this solution through an orifice in the usual way into the hypochlorite discharge pipe just before its point of entry into the suction of the pump.

The action of the ammonia is to produce a chlorin derivative of ammonia known as chloramin (NH_2Cl).

LIQUID CHLORIN.—Essential features of operation:

1. A greater rate of feed than 6 pounds per cylinder per hour will cause the chlorin to freeze.

2. Simultaneous discharge of chlorin from two cylinders preferable.

3. Keep meter warm.

The operation of the manual control chlorinator, solution feed, is as follows:

Chlorin gas in cylinder *A*, controlled by valve *S*, is introduced through the check valve *G* and meter *J* into the chlorin absorption chamber *I* and water at the same time is introduced through the connection *V*. The resulting chlorin solution is piped through the tube *U* to the point of application. When the chlorin solution is introduced into a suction line under a negative head the water seal *N* is provided to prevent air being drawn into the suction line.

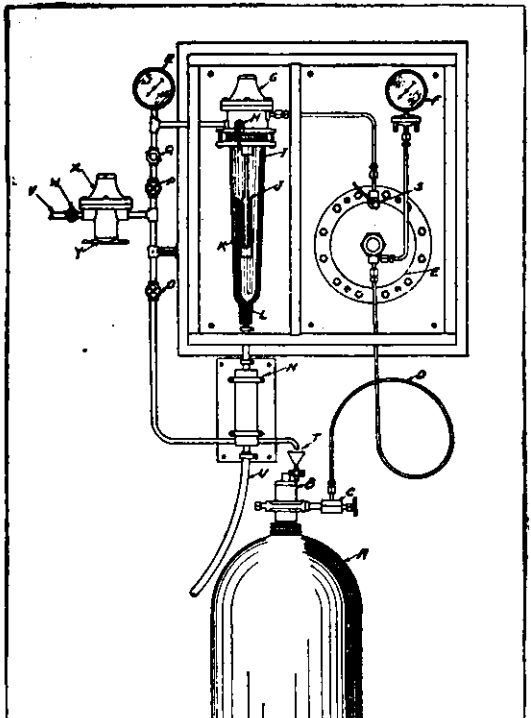

FIG. 5.—*A*, chlorin tank; *B*, valve on chlorin tank; *C*, auxiliary valve; *D*, flexible connection; *E*, pressure compensating valve for taking care of the varying pressures in the chlorin tank and also maintaining a constant drop in pressure across the valve *S*; *S*, control valve; *G*, valve to prevent moisture from getting back into control parts of apparatus; *H*, valve to control flow of water and to keep chlorin out of incoming water connections; *F*, pressure gauge showing pressure in tank; *J*, chlorin flow meter (inverted siphon type); *I*, chlorin absorption chamber; *K–L*, chlorin solution line; *V*, water connection; *P*, water valve; *R*, gauge; *O*, water valve to water seal *N*; *T*, water spill; *U*, chlorin solution line to point of application; *N*, water seal; *X*, water pressure reducing valve; *Q–Y*, strainers. Size, apparatus mounted in wall cabinet, 20 x 21 inches.

In the treatment of water a chlorin flow meter to be reliable must give a constant rate—not per day, nor per hour, nor per minute, but even less than per second—for the obvious reason that the water is flowing constantly and must receive its chlorin constantly and not intermittently.

The chlorin absorption chamber *I* and meter *J* are made of special annealed glass. They are consequently absolutely unattacked by the chlorin solution. Furthermore, the flow of chlorin can be actually seen no matter how small. A flow of one pound of chlorin per twenty-four hours (which amount will treat 300,000 gallons of water) is a flow of 0.000694 pound per minute or 0.0000115 pound per second. This quantity is entirely too minute to measure by any method but a volumetric one.

The type meter used will indicate accurately from $\frac{1}{10}$ pound of chlorin per twenty-four hours (0.00000115 pound per second) up to twelve pounds.

The operation of this meter is fully described and illustrated on page 238.

A ready solubility of chlorin is secured by the action of a water jet which thoroughly churns up the chlorin and water in the jar.

Colorimeter Estimation of Small Quantities of Free Chlorin Reagent.—1. Hydrochloric acid solution of orthotoluidin[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.

[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, v, No. 11, 915, November, 1913.

A Method of Titration to Determine the Amount of Chlorin Actually Needed for Sterilization of Water.—As adapted by Lt.-Colonel E. R. Whitmore to American service conditions. 1. Rinse a regular ordnance cup (supplied with each canteen, and holding one pint or 500 c.c.) with water, leaving a few drops of water in the cup. Break one of the tubes of calcium hypochlorite into the cup and mix it into a paste with the few drops of water. As soon as the powder is thoroughly moistened, fill the cup with water to within one inch of the top (500 c.c.) and mix well by pouring into another cup and then back. Part of this solution is used in titrating the water to be sterilized, and the remainder is used for sterilizing the water. This solution contains 0.3 gm. of available chlorin.

2. Rinse four ordnance cups with the water to be tested, and fill all four cups to one inch from the top with the water to be tested. With a pipette (a hypodermic syringe may be used) add 0.2 c.c. of the calcium hypochlorite solution to the first cup of water; add 0.4 c.c. to the second cup; add 0.6 c.c. to the third cup; add 0.8 c.c. to the fourth cup. Mix the solution with the water in each cup by pouring it back and forth into another cup, and allow the cups to stand for thirty minutes. (Fifteen minutes if time is important.)

3. Test each cup with 1 c.c. of 0.1 per cent. solution of orthotoluidin in 10 per cent. HCl solution. The cup that contains the smallest amount of the hypochlorite solution capable of giving an orange to green color, contains the proportion of chlorin necessary to sterilize the water being tested. Thus suppose the cup of water to which 0.4 c.c. of the hypochlorite solution was added gives a color with the orthotoluidin, and the sample to which 0.2 c.c. of the solution was added gives no color. The samples to which 0.4 c.c. of the hypochlorite solution was added contains the right amount of chlorin to sterilize the water being tested.

There are 36 gallons in the water bag, or 288 pints. Since 0.4 c.c. of the hypochlorite solution was sufficient to sterilize

one pint, 115 c.c. of the same solution will be sufficient to sterilize the 288 pints in the Lyster bag. The pint of hypochlorite solution already prepared would therefore be capable of sterilizing more than three bags of water. In practice, however, 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 half the solution (250 c.c.) could be added to the water in one bag and the solution prepared from the tube of calcium hypochlorite would be sufficient to sterilize two bags of water.

KI and starch solution may be used as an indicator if orthotoluidine cannot be obtained. Add 1 c.c. of an approximately 10 per cent. solution of potassium or sodium iodide and then about 5 c.c. of starch solution prepared by boiling about 1 per cent. of starch with water for two minutes.

PERMANGANATES AND INSOLUBLE COAGULANTS.—Purification by permanganates involves destruction of germs and organic matter by permanganate, destruction of residual permanganate by reducing agents capable of forming insoluble manganese oxides, and removal of the oxides by filtration. If an insoluble powder is agitated with the mixture the precipitated manganese is agglomerated and readily filtered out. After testing colocothar, alumina, kaolin, calcium phosphate, clay, powdered brick, and other substances, the conclusion is that the best insoluble coagulant is a mixture of talc, MnO_2, and precipitated $CaCO_3$. Two powders of the following composition are being used: (1) $KMnO_4$, 0.06 gram; MnO_2, 0.05 gram; precipitated $CaCO_3$, 0.02 gram; powdered talc, 0.37 gram. (2) $Na_2S_2O_3$, 0.06 gram; powdered talc in sufficient quantity to make volume equal to No. 1. They are added to 1 liter of water by a special volumetric spoon, the mixture is stirred until perfect coagulation occurs, and the precipitate is removed by filtration through cotton in a funnel. The filtrate is clear and colorless.

Copper sulphate is also used for sterilizing water. However, as a rule, copper sulphate is not used in the same way as chloride of lime, but is used more frequently as an algecide, where excellent results are obtained.

When copper sulphate is used as an algecide the water should be examined to determine the particular kind of algæ that is causing trouble in the water supply. Some algæ growths are killed easier than others. Copper sulphate applied in amounts such as 1 part of copper sulphate to 5,000,000 parts of water will kill the well-known blue and green algæ. When applied to a water supply the copper sulphate is placed in gunny sacks and towed at the back of a row-boat around the edge of a reservoir.

Sterilization may be brought about by the ultraviolet ray. This method is very efficient, but at the present time is too expensive to be used on a large scale except where electricity may be purchased for less than 1 cent. per kilowatt hour. Excellent results are obtained by its use, however, and it is especially well adapted for the sterilization of water for swimming pools. In fact it is the accepted method now of purifying swimming pools.

METHOD OF SEWAGE DISPOSAL.

The term sewage is taken to mean the solid and liquid water wastes from human habitation and manufactories, including slaughter houses, etc., diluted with the water used. It is quite obvious that sewage varies greatly for different periods of day and night.

The decomposition of organic material without the production of offensive odors is accomplished by bacterial life naturally present and when provided with sufficient quantities of oxygen. The rapidity of such decomposition depends upon whether the oxygen can be immediately brought into intimate contact with all parts of the organic matter or whether disintegration or molecular change must first

occur. This is known as aërobic decomposition. The decomposition of organic materials by bacteria naturally present and which do not require oxygen is the more rapid process of disintegration, and this is usually accompanied by the production of foul and offensive odors. This is known as anaërobic decomposition.

The easiest method of disposing of sewage is to discharge it into some body of water. This is known as the dilution method. Here the natural forces that transfer putrescible organic material into inorganic and inert material are brought into play. This change must be brought about without causing nuisance. The factors influencing the successful disposal of sewage by dilution are:

(*a*) *Suspended matter*, which subsiding forms sludge deposits, that deplete available oxygen in the overlying water, thus producing objectionable conditions. This condition is dependent upon the velocity of the current into which the sewage is discharged. Velocity less than three feet per second may permit this.

(*b*) *Sufficient Diluting Water.*—This should be approximately 6 cubic feet per second per 1000 persons contributing sewage, or a dilution approximately 1 to 50. (This figure is dependent upon velocity and oxygen content of diluting water also character of sewage discharged.)

(*c*) *Diluting Water of High Oxygen Content.*—While there is still oxygen in water containing organic matter the condition of putrefaction is not possible. The more free oxygen per unit of volume of water the greater its oxidizing power. Water saturated with oxygen contains at ordinary temperature and pressure about 10 parts per 1,000,000. If sewage contains about 500 p.p.m. of organic matter it is evident that 50 volumes of water are required for complete oxidation.

When dilution is not possible artificial purification must be resorted to. In the artificial purification of sewage different objects are sought, namely:

(*a*) To improve esthetic conditions by preventing discharge of offensive floating matter into a stream. (Mechanical or physical processes, such as screens, grit chambers or plain sedimentation basins.)

(*b*) To prevent a nuisance (oxidation process, such as filtration).

(*c*) To prevent introduction of disease organisms into a stream (sterilization, by hypochlorite or liquid chlorin).

These objects are brought about by the following methods or by combination of these methods.

1. *Screening.*
   - (*a*) Coarse.
   - (*b*) Fine.
2. *Sedimentation.*
   - (*a*) Rapid in grit chambers.
   - (*b*) Slow in settling basin.
     - Plain.
     - With chemical precipitation.
     - Septic tank (digestion of sludge in same tank).
     - With sludge digestion in separate tank (Imhoff tank).
   - (*c*) With aëration.
     - Plain.
     - With the aid of sludge from previously aërated sewage (activated sludge).
3. Contact bed treatment.
4. Trickling filter treatment.
5. Intermittent filtration.
6. Broad irrigation.
7. Disinfection.

The method or methods to be decided upon depends upon the object to be attained and the requirements of the particular situation.

In making an analysis of sewage the following determinations are usually made:

(*a*) The total solids.

(*b*) The portion of the total solids that is dissolved.

(*c*) The portion of the total solids that is suspended.

(*d*) The settling solids—that portion capable of settling out in two hours.

(*e*) The mineral matter—the portion of total solids remaining after ignition.

(*f*) The organic matter—the portion of total solids lost in ignition.

(*g*) Organic nitrogen—a measure of the amount of nitrogenous organic matter.

(*h*) Albuminoid ammonia, which is a measure of the less stable portion of the nitrogenous matter.

(*i*) Free ammonia, the first step in the "nitrogen cycle"—a measure of how far and at what rate transition is being made from organic to inorganic conditions.

(*j*) Nitrites and nitrates—conversion of ammonia products into nitrites and nitrates known as "nitrification" marks the ultimate change in the biological purification of sewage.

(*k*) Oxygen consumed—a measure of the quantity of organic matter in which nitrogen is absent.

(*l*) Putrescibility tests—to determine if there is a sufficient supply of oxygen present to bring about complete oxidation of organic matter present.

The physical condition of the principal constituents of a sewage of average strength would be (numbers are parts per 1,000,000, according to Metcalf and Eddy):

Total solids, 800	Suspended solids by filter paper, 300	Settling solids two hours, 150	Organic, 100	Settling solids, 150	Total solids. 800
			Mineral, 50		
		Suspended solids colloidal, 150	Organic, 100		
			Mineral, 50	Colloidal solids, 200	
	Dissolved solids, 500	Dissolved colloidal, 50	Organic, 40		
			Mineral, 10		
		Dissolved crystalloidal, 450	Organic, 160	Crystalloidal sol., 450	
			Mineral, 290		

It is the organic matter in sewage that causes trouble. The object of sewage treatment is to remove it or to convert

it into mineral matter. When sewage is treated to deprive it of its objectionable features, one or both of two fundamentally different processes are employed.

(*a*) Actual removal, such as through screening.

(*b*) Conversion of putrescible matter into stable substances. Purification is usually accomplished in successive steps, the degree of purification increasing with each step.

SCREENS.—Various sizes used, from the coarse rack, that merely removes heavy material, as a protection to machinery, to the very fine that supplements sedimentation basins, by removing the greater part of the suspended matter. The coarse screens are usually fixed, while the fine are operated mechanically, either on the rotary or the conveyer system. Screens are now being used where some partial method of purification is essential.

(*a*) To remove floating suspended matter that would be objectionable to the eye.

(*b*) Where after chlorination it can be safely turned into a stream without danger of nuisance.

Results Accomplished by Screens.—Coarse screens 1.0″–0.5″ mesh remove 5 to 10 p.p.m. suspended matter.

Fine screens 0.2″–0.02″ mesh remove 25 to 160 p.p.m. suspended matter.

Grit Chambers.—Grit chambers are intended to remove heavy material, such as sand, gravel, bits of coal and cinder known as "grit." It is desirable to eliminate this material as a protection to machinery and because it entails difficulty in further steps in purification. Its removal is accomplished by sedimentation in small basins by retarding the velocity of flow through the basin. Grit settles according to its size and specific gravity. In general a velocity of $2\frac{1}{2}$ to 3 feet per second is too great to allow much subsidence. If grit is to be removed it must fall below this figure.

Results Accomplished by Grit Chambers.—Removal of 10 to 40 parts per 1,000,000 of suspended matter.

Plain Sedimentation.—In this process sewage is allowed to stand or flow very slowly through tanks where the solids capable of settling under existing conditions gradually subside to the bottom. There is no aim to encourage bacterial action or to remove the colloidal matter which will not settle unaided in from two to eight hours.

Detention Period.—The heaviest matter settles first; this is followed by the lighter particles. The period may be very short, perhaps thirty minutes, but it rarely exceeds two hours.

Work Accomplished.—The suspended solids removed depend upon the detention period. Usually a detention period of two hours will permit a removal of about 60 per cent of the suspended matter. With the longest practical detention periods not much greater removal can be accomplished.

CHEMICAL PRECIPITATION.—The idea here is to remove a greater quantity of the suspended matter by mixing with the sewage one or more soluble chemicals which, reacting with themselves or with some inorganic substance in the sewage, form a precipitate which drags down with it suspended matter. The most common chemicals used are CaO, $Al_2(SO_4)_3$ and $FeSO_4$.

Results Accomplished.—The results depend upon the quantity of chemical precipitant used. In actual practice 65 to 90 per cent. of the total suspended matter is removed.

Removal of suspended matter by sedimentation and chemical precipitation involves another problem, viz., disposal of the sludge or settled material. This is the greatest difficulty in the application of sewage disposal by chemical precipitation. The attendant expense has prevented its general adoption and has even caused its abandonment where originally installed. The desire to diminish the attendant problems of sludge disposal has led to the development of various types of sedimentation tanks that aim to further sludge digestion.

SEPTIC TANK.—This process, aside from the physical sedimentation of solids, depends upon anaërobic bacterial action. This anaërobic bacterial action causes a breaking down of the complex organic substances into simpler ones. Insoluble substances are also changed into soluble ones as a result of bacterial activity through enzymes. The primary object of this tank is sedimentation, the period varying from eight to twenty-four hours. Sedimentation takes place in contact with decomposing sludge. The decomposition of the sludge is accompanied by production of gas and a reduction in the quantity of sludge.

Results Obtained from Septic Tank.—Thirty to 70 per cent. of the sludge by volume is converted into gas. Reduction in weight of solid matter is from 10 to 40 per cent. All the settling solids and a portion of the colloidal solids are removed. Violent ebullition diminishes efficiency of this process as regards sedimentation and often carries out large quantities of finely divided solids. The effluent frequently has an offensive odor, a great avidity for dissolved oxygen, and may contain substances inimical to oxidizing bacteria. Since it depends upon anaërobic bacterial action it should not be used preliminary to a process involving oxidation processes brought about by aërobic bacteria.

IMHOFF TANK.—The object here is to digest the sludge in chambers apart from the sewage in the sedimentation tank. There are two chambers: a sedimentation chamber (upper chamber) and a sludge digestion chamber (lower chamber). The function of the upper part is to remove the settling solids. The sewage is passed through as quickly as possible, avoiding the exhaustion of the supply of oxygen. The action in the sludge chamber is similar to that of the septic tank. The solids accumulate in this chamber continuously and are digested by the bacteria.

Results Accomplished.—Practical detention period is about four hours. This permits a subsidence of 45 to 65 per cent. of the total suspended matter.

CONTACT BEDS.—The failure of various tank treatments to remove fine suspended matter and the necessity of oxidizing dissolved organic matter of tank effluents before their discharge into natural waters led to the development of processes for transforming this organic matter into stable substances. The first of these was the contact or bacteria bed. This is a water-tight tank filled with broken stone, cinders, coke or other inert material. The material is about ½ inch to 1½ inches in size and the depth of the bed is about 4 feet. The voids in the bed, originally 40 to 50 per cent., gradually become filled with solid material and the contact material has to be removed and cleared after about five years' service. Beds are built in series of two or three. The effluent passes from one bed to another, being improved in quality by each successive treatment. Contact beds are filled with sewage, allowed to stand full, emptied and allowed to rest.

The following schedule illustrates such a cycle:

Time of filling	1.00 hour
Time of contact	0.75 "
Time of emptying	0.25 "
Time of resting	6.00 "

The work accomplished by the contact bed is dependent upon two main forces, physical and biological. It removes by surface attraction and absorption colloidal and dissolved substances which are later oxidized by bacterial action into stable inorganic compounds. The oxidizing power of the bed is dependent upon the supply of atmospheric oxygen absorbed during the rest period.

Results Accomplished.—General evidence shows that contact beds will give a non-putrescible effluent when they treat from 125,000 to 150,000 gallons per acre per day for each foot of depth of effective filling material. Standard practice is a rate of 600,000 gallons per day for beds with an effective

depth of 4 or 5 feet. Removes an average of 60 per cent. of applied bacteria.

SPRINKLING FILTERS (known as trickling filters, percolating filters, etc.).—These were developed from the contact filter, with the object of attaining higher rates of filtration and to eliminate serious complications from clogging. Filtering material is of broken stone, size 1 inch to 2 inches diameter; usual depth of bed 5 to 7 feet. Bottom of bed is water-tight and has an underdrain system which collects effluent. Sewage is applied to beds in fine spray by means of fixed sprinkler nozzles whereby sewage is saturated with atmospheric oxygen.

Method of Operation.—Sewage usually applied intermittently—a dosing period and a resting period. Dosing is regulated by a tank of fixed capacity which gives the nozzles an operating period of from two to five minutes. The resting period depends upon the rate of filtration, and is usually such that when the filter is operated at maximum rate it is about three times the dosing period.

Results Accomplished.—A sprinkling filter can satisfactorily treat sewage at a rate of 2,000,000 gallons per acre per day. A disadvantage of this type of filter is that it discharges suspended solid matter, which must be settled by allowing effluent from the filter to be retained for a short period in sedimentation tanks. Even with this, better results for cheaper cost can be attained than with any method thus far developed.

INTERMITTENT SAND FILTRATION.—This method is generally used without preliminary treatment other than coarse screens or grit chambers. It consists of applying small volumes of sewage to areas of porous sand, allowing the sewage to drain from the pores, then fill with air, and in repeating the dose some hours or days later. It affects a higher degree of purification than can be obtained by any of the other methods in use. The effluent from it is stable and is free from turbidity and color. It removes about 99 per cent. of the bacteria present in the raw sewage.

Filtering material is sand, either fine grained or coarse. Rate of application for raw sewage will average 60,000 gallons per acre per day; usual depth of filtering material 4 to 6 feet. When there has been preliminary treatment the rate can be increased.

Intermittency.—Intermittent dosage is essential. The cycle should be regulated so that there is always sufficient oxygen present in the pores of the material and that the oxidizing processes are not discontinued and the anaërobic processes allowed to begin.

ACTIVATED SLUDGE.—This process consists of aëration of sewage in tanks in contact with sludge from previously aërated sewage. The object of aëration is:

1. To supply sufficient oxygen to support aërobic action of bacteria.

2. To produce sufficient agitation of the tank contents to assure intimate contact of the activated sludge particles with all of the sewage. The time required for aëration increases with the strength of sewage and the degree of purification required. In practice it seems to be better to apply air at a constant rate and vary the period of aëration rather than to attempt to regulate the air discharge to meet the needs of the sewage at the moment. Four or five hours' aëration with 20 per cent. sludge has given sufficient purification at Milwaukee, Wis. Approximately 2 cubic feet of air per gallon of sewage is required. Twenty to 40 per cent. of activated sludge is considered adequate. There is intimate relationship between the period of aëration, the quantity of air required, and the proportion of activated sludge. It is necessary at the outset to secure a sufficient quantity of activated sludge. This is accomplished by continuous aëration to activate sludge until nitrification is complete. It is in this condition that sludge is most active. The degree of purification effected by this method is dependent upon the maintenance of the activity of the nitrifying organisms. The activated sludge treatment appears to

accomplish work comparable with an efficient intermittent sand filter. This process is still in an experimental stage, but has many points of promise, particularly a possible commercial value to the sludge.

STERILIZATION OF SEWAGE.—This treatment is for the purpose of destroying objectionable bacteria, particularly germs of intestinal disease.

Two methods of chlorination:

1. Hypochlorite of lime.
2. Liquid chlorin.

Required doses:

Crude sewage, 4 to 12 parts per 1,000,000 of chlorin.
Septic effluent, 10 to 15 parts per 1,000,000 of chlorin.
Sprinkling filter, 3 or 4 parts per 1,000,000 of chlorin.

The required dose depends upon the amount of unstable organic matter present in the liquid to be treated.

Efficiency.—The dose stated should remove 95 to 99 per cent. of the bacteria present. The time of contact has an influence in the efficiency of the process. In practice this is usually about fifteen minutes.

BROAD IRRIGATION.—In this process sewage is applied intermittently to land at a rate so low that it does not interfere with the raising and harvesting of crops. Rate varies from 3000 to 12,000 gallons per acre per day. Objections to its use relate to odors, prejudice to use of sewage in growing of vegetables, also to transmitting of disease germs by flies and other insects.

SLUDGE DISPOSAL.—Accompanying all sedimentation processes there is always the attendant necessity for disposal of sludge. This is a bothersome problem:

(*a*) Because of the unstable nature of the material.

(*b*) Because of the expense due to the large percentage of water in sludge (85 to 98 per cent.).

Sludge has a limited commercial utilization on account of its fertilizing properties, also on account of the grease that may be extracted from it.

Methods of disposal:

1. Digestion, then air drying.
2. Sludge pressing, usually confined to chemical precipitation plants.
3. Application to land.
4. Dispersion into water (large cities with harbors).
5. Lagooning—construction of dykes to permit of application of wet sludge to soil.
6. Filling upon low lands.
7. Incineration.
8. Destructive distillation.
9. Mechanical drying.
10. Use as filler for fertilizers.

Sewage purification is effected in successive steps, the degree to be dependent upon the conditions to be satisfied. Examples of methods to be followed to secure an effluent that is thoroughly stable and will not cause putrefaction would involve the following:

I. (*a*) Grit chamber for removal of sand.

(*b*) Imhoff tank and sludge drying beds (tank to remove suspended matter and permit digestion of sludge so that it can be dried on beds without causing nuisance).

(*c*) Sprinkling filters—for oxidation of organic matter in solution, with accompanying basins for sedimenting the filter effluent.

II. (*a*) Removal of suspended matter with fine screens.

(*b*) Intermittent sand filtration.

The essentials of any process are securing the best results with least cost. Since the carrying out of the above described processes are largely chemical and biological in their nature it is essential that the operation of all disposal plants be under the direction of someone thoroughly understanding the processes involved.

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APPENDIX.

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

[1] Manufactured by J. M. Ney Co., Hartford, Conn. Sold by dentists' supply firms in $\frac{1}{10}$-ounce vials under the trade designation No. $\frac{1}{4}$ velvet cohesive gold cylinders.

volumes of water, and require more of the oxidizing materials. The excess of acid and chlorin 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, previously 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 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 chlorin 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 0.01 mg. of mercury in the amount of material taken for analysis. Calomel in therapeutic doses occasionally gives a positive reaction in the urine.