CLINICAL LABORATORY METHODS

PLATE I.—BENEDICT'S TEST FOR SUGAR.

1. Green—Showing only a Trace of Sugar.
2. Red—Showing a Large Amount of Sugar.
3. Yellow—Showing a Small Amount of Sugar. (*From Gradwohl and Blaivas.*)

CLINICAL

LABORATORY METHODS

BY

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WITH 69 ILLUSTRATIONS AND
5 COLOR PLATES

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PREFACE

Every physician interested in the proper utilization of clinical laboratory methods must be impressed with the large amount of laboratory work which by reason of its inaccuracy is misleading rather than helpful. This is true even of such a simple procedure as a blood count.

Accurate results in the laboratory depend first of all on the intelligent and conscientious work of the individual making the tests. Equally important, however, is the use of correct procedures.

This little volume is presented to physicians and laboratory workers as a series of procedures which have been thoroughly tried out and found to give accurate results. It represents the outgrowth of notebooks used in the development and standardization of the laboratory of a general hospital.

In the selection of the methods the first requirement has been the correctness of the underlying principle, and next the adaptability of the procedure to routine use.

Only one method is given for each quantitative determination, and only one for a qualitative test where a single one is adequate. An attempt has been made to present each procedure in a simple manner. Wherever possible tables have been inserted to aid in the understanding of the technic and to assist in the calculations.

All discussion of the interpretation of results has been intentionally avoided. The book is in no sense a textbook. However, the normal figures for the method are included in each quantitative test and occasionally types of abnormal findings are given.

In the preparation of the book free use has been made of original articles in the laboratory journals, the various manuals of laboratory technic, such as Folin's Manual of Biochemistry, and notes taken by the author of lectures on clinical microscopy at the Johns Hopkins Medical School. Credit has been given wherever possible.

I am much indebted to Dr. Ralph H. Major, professor of medicine, University of Kansas, School of Medicine, for his interest and helpful criticism throughout the work. Mr. Joseph Bobbio, a former assistant, has made numerous valuable suggestions concerning the procedures in blood chemistry.

RUSSELL L. HADEN.

Kansas City.

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CHAPTER I

QUALITATIVE EXAMINATION OF THE URINE

Routine Examination

A routine qualitative examination of the urine is made as follows:

(1) Note the patient's name, case number and the date.

(2) If single specimen mark "S.S."; if a total twenty-four hour specimen measure the amount in cubic centimeters.

(3) Determine the reaction with litmus paper. This may be conveniently done by dipping a small piece of neutral litmus paper into each specimen.

(4) Note the color and whether turbid or clear.

(5) Take the specific gravity with the urinometer. Read the urinometer at the bottom of the meniscus. If the specimen is very small, dilute the urine with an equal volume of distilled water, take the specific gravity and multiply the decimal portion of the figure obtained by two.

(6) Test for albumin with heat and acetic acid or with concentrated nitric acid. Make the test for albumin only on *clear urine.* If the specimen is not clear, shake with infusorial earth and filter.

Make a quantitative determination of the albumin (page 41) on all specimens showing as much as three plus or four plus.

(7) Test for sugar with Benedict's reagent. If the test is positive, identify the reducing substance according to Table I. If a total twenty-four hour specimen is available, determine the amount of sugar present.

(8) Centrifuge a specimen and examine the sediment microscopically.

Remarks.—All specimens marked "Catheterized" should be so noted on the report.

Test for blood, bile, urobilin, acetone, diacetic acid or indican if requested, or if indicated.

Always record relative amounts as "occasional W.B.C.", "many W.B.C.", etc.

Reagents

Benedict's Solution (Qualitative).—

Copper sulphate (crystals)	17.3 gm.
Sodium or potassium citrate	173 gm.
Sodium carbonate (crystals)	200 gm.
Distilled water to make 1000 cubic centimeters.	

The copper sulphate dissolved in about 100 c.c. is poured slowly, stirring constantly, into the citrate and carbonate, previously dissolved in about 700 c.c. of hot water. The mixture is cooled and diluted to 1 liter.

Nylander's Reagent.—Digest two grams of bismuth subnitrate and four grams of Rochelle salt in 100 c.c. of a hot 10 per cent solution of potassium hydroxide. The reagent should then be cooled and filtered.

Obermeyer's Reagent.—Add 2-4 grams of ferric chloride to a liter of hydrochloric acid (sp. gr. 1.19).

Scott-Wilson Reagent.—To 10 g. of mercuric cyanide dissolved in 600 c.c. of water add a cooled solution of 180 g. of sodium hydroxide in 600 c.c. of water. Transfer this mixture to a heavy-walled glass jar, and to it add 2.9 g. of silver nitrate dissolved in 400 c.c. of water. The silver solution should be added in a slow stream, and the addition must be accompanied by constant and exceedingly vigorous stirring with a heavy glass rod. If properly made, the silver dissolves completely, giving a clear solution which is at once available for use. If the solution is turbid, it should be set aside to settle for three or four days and the clear supernatant liquid removed by means of a siphon.

In the clear reagent a new sediment gradually forms, so that the solution deteriorates slowly and after a few months is not serviceable for quantitative work, though still good for qualitative tests.

Lugol's Solution.—Dissolve 4 grams of iodine and 6 grams of potassium iodide in 100 c.c. of distilled water.

Barfoed's Solution.—Dissolve 9 grams of neutral crystallized copper acetate in 100 c.c. water and add 1.2 c.c. 50 per cent acetic acid.

Ehrlich's Reagent.—Dissolve 4 grams paradimethyl amino benzaldehyde in 30 c.c. concentrated hydrochloric acid and add 30 c.c. distilled water.

Fehling's Solution.—

Solution 1

Copper sulphate crystals	34.65 gm.
Distilled water to	1000 c.c.

Solution 2

Rochelle salt	173.0 gm.
Sodium hydrate	125.0 gm.
Distilled water to	1000 c.c.

Dissolve the Rochelle salt in hot water, cool, add the sodium hydrate, and make up to one liter.

Equal volumes of Solutions 1 and 2 are mixed in a test tube and boiled; the deep blue fluid should remain perfectly clear. The urine is added to the hot mixture in small amounts.

Qualitative Tests

1. **Albumin.**—Heat and Acetic Acid.—The urine must be acid and clear. If alkaline or neutral, make slightly acid by adding a few drops of 3 per cent acetic acid. If cloudy, shake with infusorial earth, and filter.

Fill a test tube two-thirds full of the clear acid urine and gently heat the upper half of the fluid to boiling. A turbidity is due either to phosphates, carbonates or albumin. Acidify the urine further by the addition of 3-5 drops of 3 per cent acetic acid, adding it drop by drop to the hot solution. If the precipitate is due to phosphate or carbonate, it will disappear under these conditions; if it is due to albumin, it will become more flocculent. Very small quantities of albumin may not appear on heating, but will show after the addition of acid. Examine the tube by transmitted light against a black background.

Heller's Nitric Acid Test.—Place about 2-3 c.c. of con-

centrated nitric acid in the bottom of a test tube and run in 4-5 c.c. of urine over the acid with a pipette. There should be a distinct layering of the acid and urine. A white layer at the junction indicates the presence of albumin.

Record results as follows:

Negative	0
Faint trace	Ft. +
Trace	+
Moderate amount	++
Large amount	+++
Very large amount	++++

Make a quantitative determination of the albumin on all specimens showing as much as three or four plus.

2. **Sugar.**—To 5 c.c. of Benedict's reagent in a test tube add eight (not more) drops of urine. Boil the fluid vigorously for from one to two minutes over a free flame or place in boiling water-bath for five minutes and allow to cool spontaneously.

In the presence of glucose the entire body of the solution will be filled with a yellow or red precipitate (Plate 1). If the glucose is below 0.3 per cent, the precipitate forms only on cooling. If no sugar be present, the solution remains perfectly clear or shows a faint turbidity which is blue in color, due to urates.

Bulk and not color is the basis of the test.

Concentrated urines must be diluted before adding to the reagent. Large amounts of albumin, if present, must be removed before making the test.

If Benedict's test is positive identify the reducing body (see page 33, "Identification of Reducing Substances in Urine").

3. **Acetone.**—Test with Scott-Wilson Reagent.—In a large test tube, such as used for the determination of urea (Fig. 20), place first 5 c.c. of urine and 1-2 drops dilute acid (HCl or H_2SO_4). Then insert the rubber stopper carrying the absorption tube. Place the test tube in a beaker of lukewarm water (35-40° C.) and aspirate the volatile acetone by means of a moderately rapid air current into a test tube containing 5 c.c. of distilled water and 5 c.c. Scott-Wilson reagent. If acetone is present, even if only in minute traces, the solution becomes turbid. If the amount of acetone obtained is extremely

small, turbidity may not appear for 5 to 10 minutes. This test is specific for acetone.

Sodium Nitro Prusside Test.—To 5 c.c. of urine in a test tube add about 1 gm. of ammonium sulphate and a few drops of 5 per cent sodium nitroprusside. Shake a few times to dissolve the salt, then layer with concentrated ammonia. A violet color indicates acetone.

4. **Diacetic Acid.**—Gerhardt's Test.—Introduce about 5 c.c. of urine into a test tube and add ferric chloride (about 40 per cent) drop by drop. In the presence of large amounts of diacetic acid, a Bordeaux red color is produced.

5. **Bile.**—Foam Test.—Shake vigorously a test tube one-half full of urine; if the foam presents a distinct yellow color, bile is present.

Rosenbach Test.—Acidify the urine with hydrochloric acid and pass through a small filter several times. Touch the stain on the paper with a drop of yellow nitric acid. If bile is present a play of colors is seen at the edge of the drop. From within outward the colors are yellow, red, violet, blue and green.

Iodine Test.—Layer a very dilute alcoholic solution of iodine over urine. A green ring at the zone of contact shows bile. The iodine solution should be of a pale yellow color, (1 part of tincture of iodine plus 30 parts of 95 per cent alcohol).

6. **Blood.**—Guaiac Test.—To about 4 c.c. of urine add 1 c.c. of glacial acetic acid and 2 c.c. of ether; shake gently; pour off the ether and add to it a few drops of freshly prepared alcoholic solution of gum guaiac, (1 gm. in 60 c.c. 95 per cent alcohol), and 1 c.c. of 3 per cent hydrogen peroxide. If blood is present, a blue color develops in the ether.

7. **Indican.**—Obermeyer's Test.—Mix equal parts of Obermeyer's reagent and urine in a test tube. Add a small amount of chloroform and invert several times. If an excess of indican is present the chloroform becomes dark blue.

8. **Urobilin.**—Schlesinger's Test.—About 10 c.c. of acid urine are treated with 5 or 6 drops Lugol's solution to convert any urobilinogen present into urobilin. The urine is now mixed with an equal quantity of a freshly made saturated solution of zinc acetate in absolute alcohol and filtered. If urobilin is present, the fil-

trate shows a green fluorescence when held against a dark background and examined by transmitted light. Place filtrate in spectroscope and note characteristic absorption spectrum, a wide band between *b* & *F* (Fig. 26-*A*).

9. **Urobilinogen.**—Add 1 c.c. of Ehrlich's reagent to 10 c.c. of urine in a test tube. If an abnormally large amount of urobilinogen is present, a red color develops in the cold. A red color may develop in normal urine on heating.

10. **Bile Salts.**—Hays' Sulphur Test.—This test is based upon the principle that bile acids have the property of reducing the surface tension of fluids in which they are contained. Cool about 10 c.c. of urine in a test tube to 17° C. or lower and sprinkle a little finely pulverized sulphur upon the surface of the fluid. The presence of bile acid is indicated if the sulphur sinks to the bottom of the liquid; the rapidity with which the sulphur sinks depending upon the amount of bile acids in the urine. The test is said to react with bile acids when the latter are present in the proportion of 1:120,000.

Foam Test.—(V. Udransky). To 5 c.c. of urine in a test tube add three or four drops of a very dilute (1:1000) aqueous solution of furfurol. Place the thumb over the top of the tube and shake the tube until a thin foam is formed. With a small pipette add two or three drops of concentrated sulphuric acid to the foam and note the dark pink coloration produced in the presence of bile salts.

Microscopic Examination of Urinary Sediment

The sediment is obtained for microscopic examination by centrifuging the urine in a conical centrifuge tube. The specimen employed for examination should be perfectly fresh. A drop of the sediment is removed with a pipette and transferred to a glass slide. The preliminary examination is made with low magnification and with the light cut off as much as possible. If necessary a cover glass is placed on the drop of sediment, which is then examined under higher magnification.

In the preparation under examination look for:

(1) *Red blood cells.*

(2) *White blood cells* with polymorphous nuclei, (pus cells).

(3) *Epithelial cells:* (a) squamous, usually large with small round nucleus; (b) round or cuboidal with large vesicular nucleus.

(4) *Amorphous sediment:* urates, which are usually pink in color and dissolve on heating; phosphates, white in color, which do

Fig. 1.—Uric acid crystals. (*From Gradwohl and Blaivas, after Hawk.*)

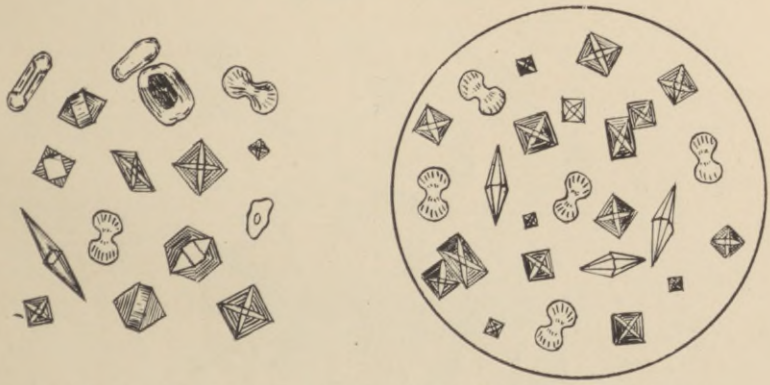


Fig. 2.—Calcium oxalate crystals. (*From Gradwohl and Blaivas.*)

not dissolve on heating, but are soluble in acids; carbonates which are soluble in acid with the evolution of carbon dioxide.

(5) *Crystals:* (a) *In acid urine:* uric acid, which are usually

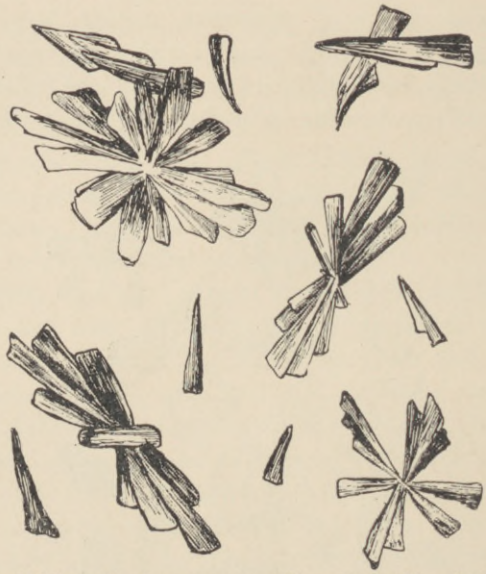


Fig. 3.—Calcium phosphate crystals. (*From Gradwohl and Blaivas.*)

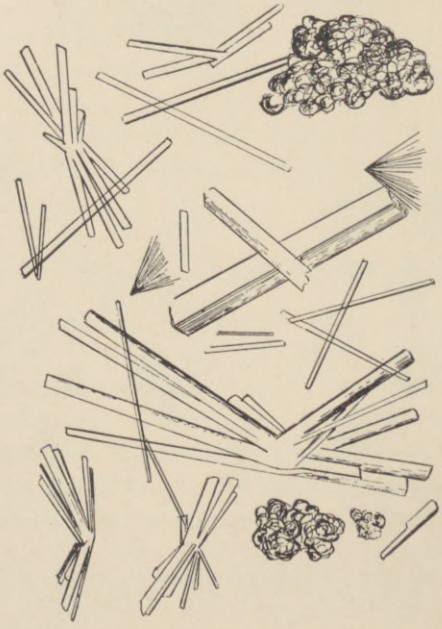


Fig. 4.—Calcium sulphate. (*After Hensel and Weil.*)

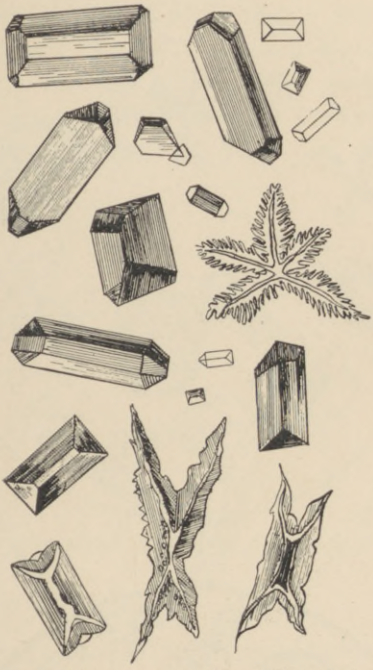


Fig. 5.—"Triple phosphate." (*After Ogden.*)

Fig. 6.—Calcium carbonate crystals. (*After Hawk.*)

Fig. 7.—Acid sodium urate. (*After Hawk.*)

Fig. 8.—Ammonium urate crystals. (*After Peyer.*)

colored reddish or yellowish brown and occur in a variety of forms, such as rhombic prisms, wedges, dumb-bells, whetstones, prismatic rosettes and irregular or hexagonal plates, (Fig. 1 and Plate II);

calcium oxalate, which are formed as highly refractive octohedra or as dumb-bell forms (Fig. 2); calcium phosphate occurring as slender, colorless, rhombic tablets, often grouped together as rosettes (Fig. 3); calcium sulphate, which crystallizes in the form of long thin needles or prisms (Fig. 4).

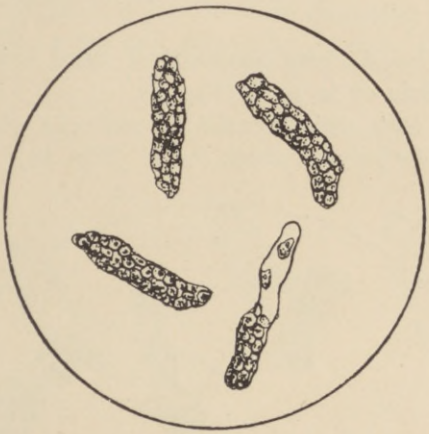


Fig. 9.—Epithelial casts. (*After Hawk.*)

Fig. 10.—(*a*) Blood casts (yellow in color); (*b*) Pus casts. (*After Hawk.*)

(b) *In alkaline urine:* ammonia magnesium phosphate, ("Triple Phosphate"), which occurs in two forms, prisms and the feathery type (Fig. 5); calcium carbonate, which appears as dumb-bells or spheres with radiating lines resembling similar forms of

calcium oxalate (Fig. 6); sodium urate, which occurs as groups of fan-shaped clusters or prismatic needles (Fig. 7); ammonium urate, found in the burr-like form of the thorn apple crystal or yellow or reddish-brown spheres covered with sharp spicules (Fig. 8).

(6) *Casts.*—These are derived from the renal tubules. The sides are parallel, the ends are rounded and fairly abrupt. Casts are classified as follows:

(a) *Epithelial Casts* composed of renal epithelial cells in whole or in part (Fig. 9).

(b) *Pus casts* which consist of pus cells, characterized by their polymorphous nuclei (Fig. 10).

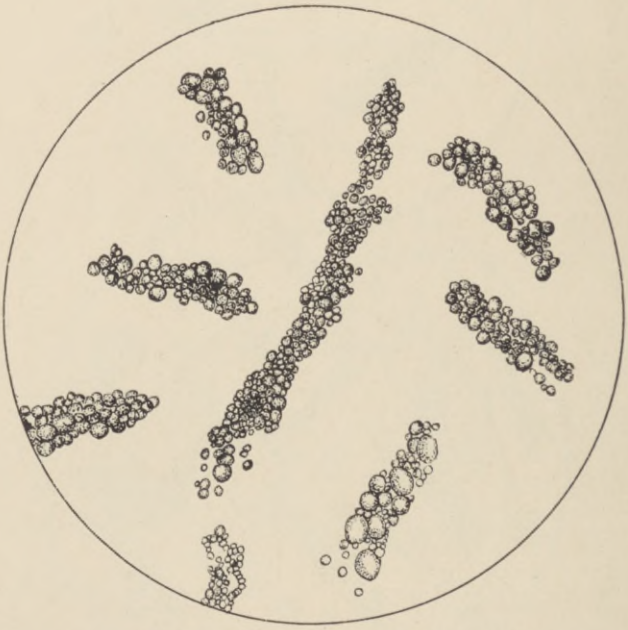


Fig. 11.—Fatty casts. (*After Peyer.*)

(c) *Blood casts* containing one or more red blood cells (Fig. 10).

(d) *Fatty casts* which result from the fatty degeneration of the cells of epithelial casts (Fig. 11).

(e) *Granular casts* which have coarse or fine granules (Fig. 12).

(f) *Waxy casts* which are opaque, very refractive, homogeneous, and white or yellowish in color.

(g) *Hyaline casts* which are very pale, have little refractivity, and are difficult to see unless the light is cut off (Fig. 13).

(h) *Cylindroids* are bodies resembling casts, but are distinguished by the fact that they have a tapering end, often going to a threadlike process (Fig. 14).

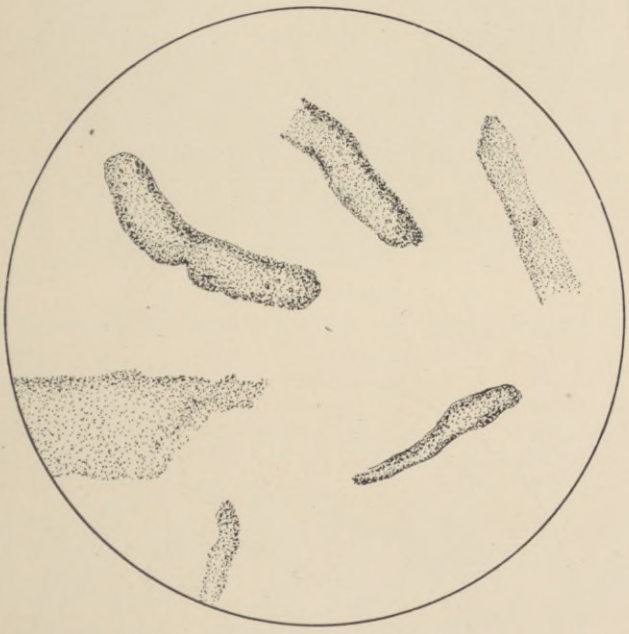


Fig. 12.—Granular casts. (*After Peyer.*)

Identification of Reducing Substances in Urine

If the test with Benedict's reagent is positive, do the following tests on the specimen in the order given.

1. **Nylander's Test.**—To one-half test tube urine add one-tenth the volume of Nylander's solution; boil for 3 to 5 minutes. A reducing substance forms a black precipitate.

2. **Phenyl-hydrazine Test.**—To 4 c.c. of albumin-free urine add ½ c.c. glacial acetic acid and 5 drops of phenyl-hydrazine. Boil

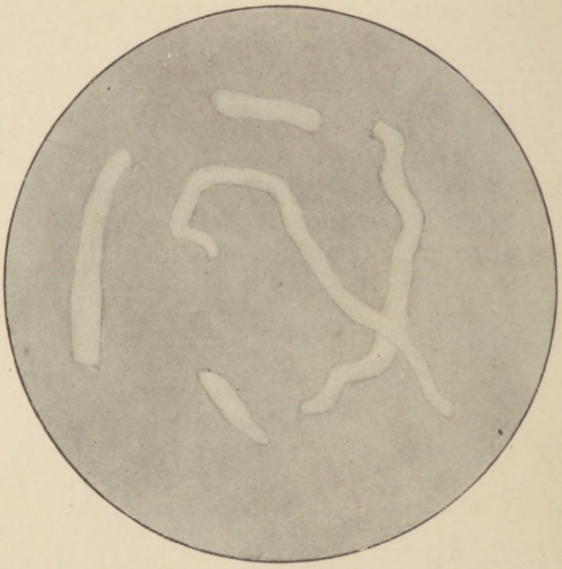


Fig. 13.—Hyaline casts. (*After Hawk.*)

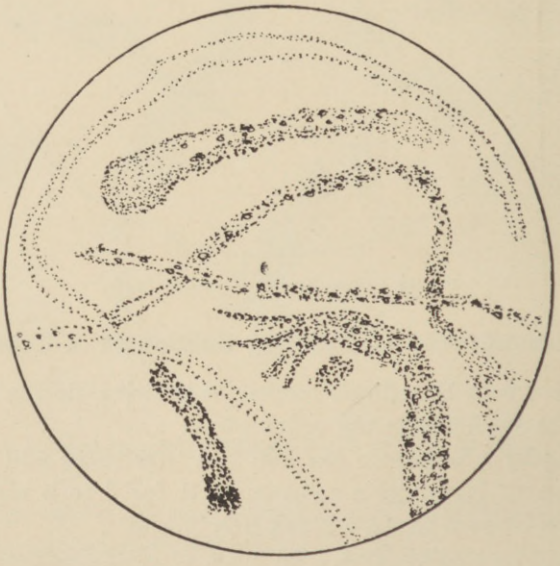


Fig. 14.—Cylindroids. (*After Peyer.*)

Fig. 1.—Osozones. (*After Hawk.*) Upper form, dextrosozone; lower form, maltosozone.

Fig. 2.—Uric acid crystals. Normal color. (*From Hawk, after Peyer.*)

PLATE II.

gently for 1 minute; and add 4-5 drops of 20 per cent NaOH. Heat for few minutes; let cool at room temperature. Characteristic yellow osozone crystals (Plate II) are obtained within 20 minutes if sugar be present. If the melting point of the crystals is to be determined, dissolve crystals in hot 50 per cent alcohol, pour into distilled water and evaporate the alcohol. The osozones crystallize out.

3. **Fermentation Test.**—Rub up a small piece of fresh yeast with 50 c.c. of boiled urine; fill fermentation tube (Fig. 15) and place in incubator. As control, use (a) normal boiled urine and yeast and (b) normal urine and yeast plus glucose to prove the activity of the yeast. If a fermentable sugar is present, gas will form in the fermentation tube.

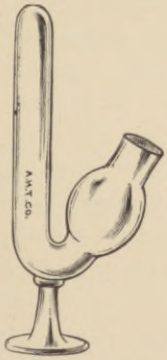


Fig. 15.—Fermentation tube.

4. **Polariscopic Test.**—Fill tube of polariscope and determine whether the substance rotates the plane of light to right or left or gives no rotation. (See page 39.)

Table I shows the reaction of the reducing substances commonly occurring in the urine to the tests given above.

If necessary for the identification, the following special tests for reducing substances may be used.

(1) **Barfoed's Test.**—Place about 5 c.c. of Barfoed's reagent in a test tube and heat to boiling. Add the urine slowly a few drops at a time, heating after each addition. Reduction is indicated by the formation of a red precipitate of cuprous oxide. If the precipitate does not form after boiliing ½ minute, allow the

tube to stand a few minutes and examine. Monosaccharides give a positive reaction; disaccharides do not react.

(2) **Levulose.**—Seliwanoff's Test.—Mix 5 to 10 c.c. of urine and an equal volume of 25 per cent hydrochloric acid (two parts of concentrated hydrochloric acid and one part of water); add a few grains of resorcin. Boil gently for a few seconds. If levulose is present a red color appears, usually followed by a brownish precipitate. Cool the fluid, pour into an evaporating dish or beaker, and treat with sodium carbonate in substance until the reaction is alkaline. Pour the fluid into a test tube and shake with ethyl acetate. If levulose is present, the ethyl acetate is colored yellow.

In performing the test prolonged boiling should be avoided.

(3) **Pentose.**—Phloroglucin Test.—To about 5 c.c. of urine in a test tube add an equal volume of concentrated hydrochloric acid and a liberal knife point of phloroglucin. Heat the mixture preferably on a water-bath. A red color appears and soon afterward a dark precipitate. Cool the contents of the tube and extract with amyl alcohol. Examine the amyl alcohol extract in the spectroscope. If pentose is present a band appears midway between D and E, a little to the right of the sodium line.

Glycuronic acid compounds give a positive phloroglucin test as well as pentose.

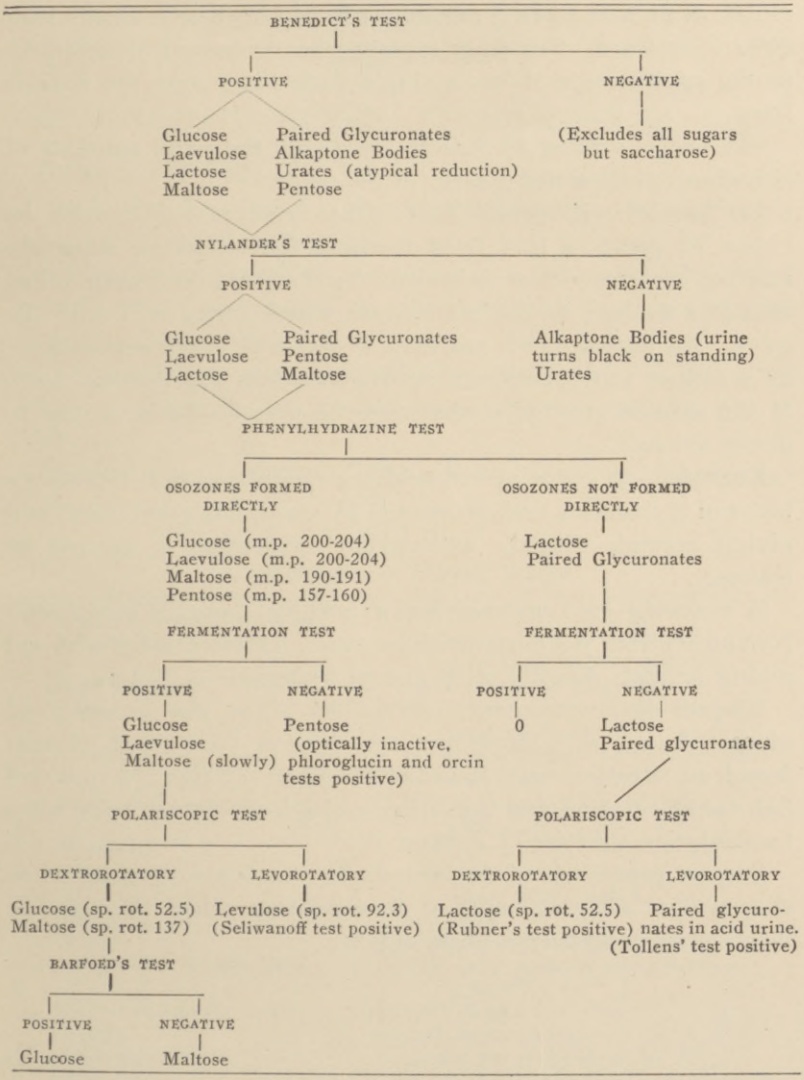
Orcin Test.—Mix equal parts of urine and hydrochloric acid; add a small knife point of orcin and boil gently. If pentose is present, a dark greenish color soon develops, and finally a turbidity, due to a dark green or blue precipitate. Cool the contents of the test tube until they are lukewarm, and then extract with amyl alcohol. The extract is a dark olive green color, the depth of which is proportional to the concentration of pentose in the urine. Spectroscopic examination shows a band at D, the sodium line.

The orcin test also is given by the paired glycuronic acid compounds, but the reaction is much less readily obtained than with the phloroglucin. Urine should not be filtered through paper before the test.

(4) **Paired Glycuronates.**—Tollens' Test.—To 5 c.c. of urine in a test tube add a bit of naphthoresorcin about the size of a

TABLE I

THE IDENTIFICATION OF REDUCING SUBSTANCES OCCURRING IN URINE



millet seed and 5 c.c. of concentrated hydrochloric acid. Boil gently about one minute and set the tube aside for about four minutes. Cool the contents of the tube under running water.

Extract with an equal quantity of ether. If glycuronates are present in the urine in excess, the ether extract is dark blue to violet, while with smaller amounts a faint bluish or reddish violet color is obtained. The ether extract when examined immediately in the spectroscope shows a single dark band near the sodium line.

The test is sufficiently delicate to detect the small amounts of glycuronates normally present in the urine.

(5) **Lactose.**—RUBNER'S TEST.—The urine is boiled with an excess of sugar of lead from three to four minutes, when the solution becomes yellow or brown. To the hot fluid is then added ammonia as long as the precipitate which forms will still dissolve. An intense brick red fluid is obtained which settles later as a copper red precipitate with a colorless supernatant fluid. If the specific gravity of the urine be over 1.020, it is best to dilute one-half.

Remarks.—A reducing substance giving a positive Benedict's test and Nylander's test, fermented by yeast, dextrorotatory, and giving crystals directly with phenyl hydrazine is glucose or maltose.

A reducing substance giving a positive Benedict's test and Nylander's test, *not* fermented by yeast, dextrorotatory and *not* giving crystals directly with phenyl hydrazine is lactose.

Suspect levulosuria with glycosuria when the quantity of glucose found on polariscopic examination falls short of that found on titration with Benedict's copper solution. A positive Seliwanoff reaction and the absence of a levorotatory body after fermentation practically confirm it.

Suspect pentosuria when the reduction tests are atypical, when they persist after attempts at fermentation, when the urine is inactive on polariscopic examination.

CHAPTER II

QUANTITATIVE CHEMICAL EXAMINATION OF URINE

Collection of Urine.—It is very important that all specimens of urine for quantitative chemical examination be accurately collected on the 24-hour period.

The container should be kept in a cool place and have added to it 10 c.c. of toluol or 5 c.c. of a saturated alcoholic solution of thymol for each liter of urine.

The calculation may ordinarily be simplified by diluting the entire specimen to the nearest round number as 1,000 c.c. or 1,500 c.c.

Composition of Normal Urine.—Table II shows the average

TABLE II.

COMPOSITION OF NORMAL URINE

Hydrogen-Ion concentration	4.80 -7.50 (mean 6.00).
Acidity by Titration	200-500 (c.c. N/10 alkali to neutralize 24-hour output).
Total nitrogen	12-18 grams in 24 hours.
Urea	30-35 grams (equals 80-90% of total nitrogen).
Ammonia	0.7 gram (equals 2.5-4.5% of total nitrogen).
Amino acid nitrogen	0.4-1.0 (equals 2-6% of total nitrogen).
Creatinine	1.25 gram.
Creatine	Few milligrams (children may show 10-50 mgm).
Uric acid	0.7 gram.
Glucose	0 (common qualitative tests).
Acetone and diacetic acid	3-15 mgm. (about one-fourth is acetone).
B-Hydroxybutyric acid	20-30 mgm.
Indican	4-20 mgm.
Total sulphates	1.5-3.0 gm. (expressed as SO_3).
Chlorides	10-15 gm. (expressed as sodium chloride).
Ethereal sulphates	0.1025 gm. (expressed as SO_3).
Total phosphates	2.5 gm. (expressed as P_2O_5).
Calcium	0.1-0.4 gram (expressed as CaO).
Magnesium	0.1-0.3 gram (expressed as MgO).

acidity and the daily excretion of the substances found in normal urine.

Acidity by Titration (Folin)

Principle.—Potassium oxalate is added to the urine to precipitate the calcium present. The urine is then titrated with tenth-normal sodium hydroxide solution.

Procedure.—Place 25 c.c. of urine in a 200 c.c. flask and add 15 to 20 grams finely powdered potassium oxalate and 1 to 2 drops 1 per cent phenolphthalein solution. Shake vigorously 1 to 2 minutes and titrate immediately with N/10 NaOH until a faint but unmistakable pink remains permanent on further shaking. Express the result in c.c. N/10 NaOH required to neutralize the 24-hour specimen.

Calculation.—The total acidity of the 24-hour specimen expressed in cubic centimeters of N/10 sodium hydroxide equals—

$$\frac{\text{Total vol. of urine}}{25} \times \text{c.c. N/10 sodium hydroxide used.}$$

Remarks.—The normal titration acidity is 200-500 expressed in terms of N/10 alkali required to neutralize the 24-hour output.

Determination of Hydrogen-Ion Concentration (True Acidity)

Principle.—The urine is treated with a few drops of the proper indicator and the color compared with that produced with the same amount of indicator and a solution of known hydrogen-ion concentration.

Reagents.—1. Standard Buffer Solutions.—A set covering the range P_H 5.0 to 7.0 will allow for the extreme variations encountered. For preparation see "Colorimetric Determination of the Hydrogen-Ion Concentration of Biological Fluids," page 234.

2. Indicators.—Methyl red, brom cresol purple, and phenol red are the most useful indicators. See page 239 for preparation of the indicator solutions.

Procedure.—Find the approximate P_H of the urine by adding a few drops of indicator beginning with phenol red and comparing with the standard buffer solutions containing indicator.

Measure 10 c.c. of urine into a test tube of the same bore as those containing the standard. Add 10 drops of the indicator

showing the sharpest color changes over the range within which the solution falls as determined by the preliminary test.

Compare the urine in a comparator (Fig. 60) with buffer solution containing the same indicator. The tubes are arranged as illustrated in Fig. 61. The hydrogen-ion concentration of the urine is equal to that of the standard buffer solution whose color it matches.

Remarks.—The normal values lie between 4.8 and 7.5 with a mean value almost exactly 6.0. For vegetarians the mean value is about 6.6.

Quantitative Estimation of Glucose

A. By Titration with Copper Solution (Benedict's Solution)

Principle.—The urine is heated with an alkaline copper sulphate solution containing potassium thiocyanate. A white precipitate of copper thiocyanate is formed on reduction instead of the usual red precipitate of cuprous oxide.

Reagents.—

1. Benedict's sugar reagent: (quantitative)—

Copper sulphate (crystallized)	18.0 grams
Sodium carbonate (crystallized, ½ the weight of the anhydrous salt may be used)	200.0 grams
Sodium or potassium citrate	200.0 grams
Potassium thiocyanate	125.0 grams
Potassium ferrocyanide (5% sol.)	5.0 c.c.
Distilled water to make a total volume of	1000.0 c.c.

With the aid of heat dissolve the carbonate, citrate and thiocyanate 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 are reduced by 50 mg. of glucose.

2. Sodium carbonate.
3. Powdered pumice stone.

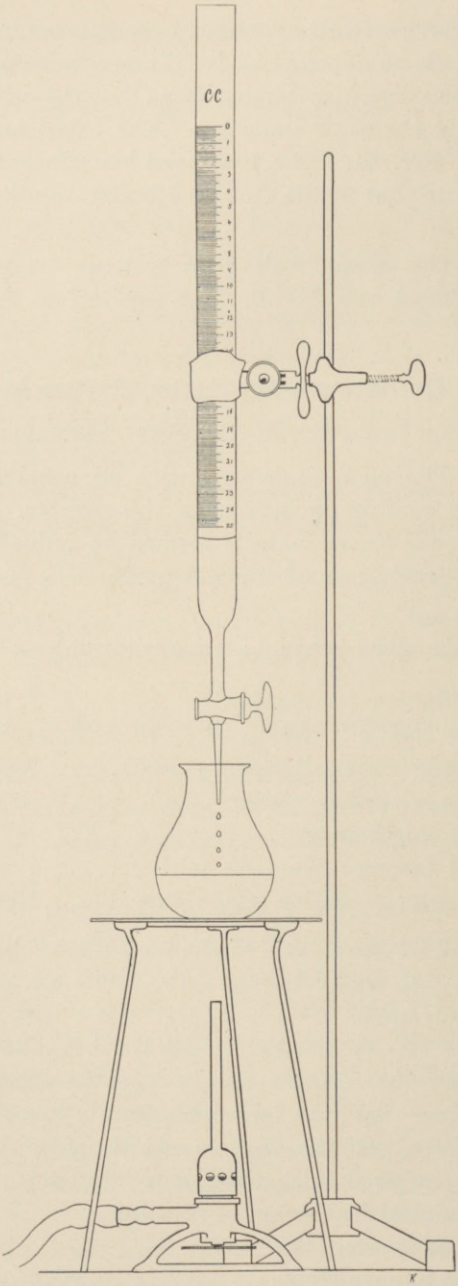


Fig. 16.—Showing Benedict's method for the quantitative estimation of sugar. (*From Gradwohl and Blaivas.*)

Procedure.—The urine, 10 c.c. of which should be diluted with water to 100 c.c. (unless the sugar content is believed to be low, when it may be used undiluted), is poured into a 50 c.c. burette up to the zero mark. Twenty-five c.c. of the reagent are measured with a pipette into a porcelain evaporating dish (25-30 cm. in diameter), 10 to 20 grams of crystallized sodium carbonate (or one-half the weight of the anhydrous salt), are added, together with a small quantity of powdered pumice stone or talcum, and the mixture heated to boiling over a free flame (Fig. 16) until the carbonate has entirely dissolved. The diluted urine is now run in from the burette, rather rapidly, until a 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. The solution must be kept vigorously boiling throughout the entire titration. If the mixture becomes too concentrated during the process, water may be added from time to time to replace the volume lost by evaporation.

Calculation.—The 25 c.c. of copper solution are reduced by exactly 50 mg. of glucose. Therefore, the volume run out of the burette to effect the reduction contained 50 mg. of the sugar. When the urine is diluted 1:10, as in the usual titration of diabetic urines, the formula for calculating the percentage of the sugar is the following:

$$\frac{0.050}{x} \times 1000 = \text{percentage in original sample}$$

wherein x is the number of cubic centimeters of the diluted urine required to reduce 25 c.c. of the copper solution.

Remarks.—In the use of this method, chloroform must not be present during the titration. If used as a preservative in the urine, it may be removed by boiling a sample for a few minutes, and then diluting to its original volume. Table III shows the percentage of glucose corresponding to the different amounts of urine required.

B. By Polariscopic Method

The urine must be acid, albumin-free, and clear. If cloudy, add 2 grams lead acetate, (sugar of lead), to a portion, shake and

TABLE III

DETERMINATION OF GLUCOSE IN URINE

Take 25 c.c. of Benedict's reagent and decolorize with urine diluted 1:10, if the sugar content is high, or undiluted if low.

Table shows the percentage of glucose corresponding to the different amounts of diluted or undiluted urine required.

URINE DILUTED 1:10						UNDILUTED URINE					
C.C. URINE	PER CENT GLUCOSE	C.C. URINE	PER CENT GLUCOSE	C.C. URINE	PER CENT GLUCOSE	C.C. URINE	PER CENT GLUCOSE	C.C. URINE	PER CENT GLUCOSE	C.C. URINE	PER CENT GLUCOSE
5.0	10.0	10.0	5.0	30	1.7	5.0	1.0	10.0	0.50	30	0.17
5.2	9.6	10.5	4.8	31	1.7	5.2	0.96	10.5	0.48	31	0.17
5.4	9.3	11.0	4.6	32	1.6	5.4	0.93	11.0	0.46	32	0.16
5.6	8.9	11.5	4.4	33	1.6	5.6	0.89	11.5	0.44	33	0.16
5.8	8.6	12.0	4.2	34	1.5	5.8	0.86	12.0	0.42	34	0.15
6.0	8.3	12.5	4.0	35	1.4	6.0	0.83	12.5	0.40	35	0.14
6.2	8.1	13.0	3.9	36	1.4	6.2	0.81	13.0	0.39	36	0.14
6.4	7.8	13.5	3.7	37	1.4	6.4	0.78	13.5	0.37	37	0.14
6.6	7.6	14.0	3.6	38	1.3	6.6	0.76	14.0	0.36	38	0.13
6.8	7.4	14.5	3.5	39	1.3	6.8	0.74	14.5	0.35	39	0.13
7.0	7.2	15.0	3.3	40	1.3	7.0	0.72	15	0.33	40	0.13
7.2	7.0	16	3.1	41	1.2	7.2	0.70	16	0.31	41	0.12
7.4	6.8	17	3.0	42	1.2	7.4	0.68	17	0.30	42	0.12
7.6	6.6	18	2.8	43	1.2	7.6	0.66	18	0.28	43	0.12
7.8	6.4	19	2.6	44	1.1	7.8	0.64	19	0.26	44	0.11
8.0	6.3	20	2.5	45	1.1	8.0	0.63	20	0.25	45	0.11
8.2	6.1	21	2.4	46	1.1	8.2	0.61	21	0.24	46	0.11
8.4	6.0	22	2.3	47	1.1	8.4	0.60	22	0.22	47	0.11
8.6	5.8	23	2.2	48	1.0	8.6	0.58	23	0.22	48	0.10
8.8	5.6	24	2.1	49	1.0	8.8	0.56	24	0.21	49	0.10
9.0	5.6	25	2.0	50	1.0	9.0	0.56	25	0.20	50	0.10
9.2	5.4	26	1.9			9.2	0.54	26	0.19		
9.4	5.3	27	1.9			9.4	0.53	27	0.19		
9.6	5.2	28	1.8			9.6	0.52	28	0.18		
9.8	5.1	29	1.7			9.8	0.51	29	0.17		

filter several times through the same filter until perfectly clear. If albumin is present, it must be removed by heat and acid and filtration.

Fill polariscope tube marked 189.4 mm. Place glass disc over end of tube and screw down cap. Avoid air bubbles. After focusing, readings are made, first without the urine to determine whether the zero point is accurate; next, after refocusing, with the tube of urine in place. Starting at zero, the handle is rotated until the entire field is equally illuminated. The reading gives the percentage of sugar directly. If the tube marked 94.7 mm. is used, multiply result by 2.

Remarks.—The polariscope may be used to determine the percentage (P) of any optically active substance in solution from the following equation:

$$P \text{ equals } \frac{A \times 100}{(\alpha)\ D \times L}$$

where A equals the angle of rotation
(α) D equals specific rotation of the substance in question
L equals length of polariscope tube in decimeters.

Quantitative Estimation of Albumin

Preparation of Tsuchiya Reagent.—

Phosphotungstic acid	15 gm.
Concentrated hydrochloric acid	50 c.c.
Ethyl alcohol (95 per cent) ad.q.s.	1000 c.c.

Procedure.—If the urine is alkaline, acidify with acetic acid. Fill an Esbach tube (Fig. 17) with urine to the mark "U" and then add Tsuchiya's reagent to mark "R." The tube is corked and inverted twelve times. Do not shake. Place in vertical position for twenty-four hours at room temperature and read the height of the precipitate on the scale marked on the tube. The figure obtained gives the albumin in grams per liter.

Quantitative Estimation of Chlorides

Principle.—Volumetric silver nitrate solution is added in excess to the urine precipitating the chlorides as silver chloride.

The excess of silver nitrate is titrated with a standard ammonium thiocyanate solution using iron alum as an indicator.

Reagents.—1. Silver Nitrate Solution.—Dissolve 29.075 grams of silver nitrate in 900 c.c. of 25 per cent nitric acid; add 50 c.c. of a 10 per cent solution of ferric ammonium sulphate; make up to one liter with distilled water. One c.c. of this solution is equivalent to 0.01 gram sodium chloride.

Standardize against tenth normal hydrochloric acid. Ten c.c. of the tenth normal acid should be completely precipitated by 5.85 c.c. of the silver nitrate solution.

2. Ammonium Thiocyanate Solution.—This solution is made of such a strength that 2 c.c. of it is equal to 1 c.c. of the standard

Fig. 17.—Esbach albuminometer.

silver nitrate solution. To prepare the solution dissolve 12.9 grams of ammonium thiocyanate, NH_4SCN, in a little less than two liters of water. In a small flask place 20 c.c. of the standard silver nitrate solution, add water to make the total volume 100 c.c. and thoroughly mix the contents of the flask. Now run in the ammonium thiocyanate solution from a burette until a permanent red-brown tinge is produced. This is the end-reaction and indicates that the last trace of silver nitrate has been precipitated. Take the burette reading and calculate the amount of water necessary to use in diluting the ammonium thiocyanate in order that 20 c.c. of this solution may be exactly equal to 10 c.c. of the silver nitrate solution. Make the dilution and titrate again to be certain that the solution is of the proper strength.

Procedure.—Mix 5 c.c. of urine and 10 c.c. of volumetric silver nitrate solution in a small flask. Add about 35 c.c. of distilled water. Run in the standard ammonium thiocyanate solution from a burette until a permanent red-brown tinge is produced.

Calculation.—If x be the number of c.c. of ammonium thiocyanate used in titrating the excess silver nitrate: $20 - x =$ grams per liter of chloride (expressed as sodium chloride).

Remarks.—When ammonium thiocyanate is added to silver chloride, a small part of the chloride is converted into silver thiocyanate. This may be avoided by filtering the solution after the silver chloride has been precipitated. The error due to this side reaction is so small, however, that the method as outlined is sufficiently accurate for routine clinical use. Table IV shows the number of grams of NaCl corresponding to the amount of NH_4CNS used to neutralize the excess of $AgNO_3$.

TABLE IV

DETERMINATION OF CHLORIDES IN URINE

Take 5 c.c. of urine and 10 c.c. of the standard $AgNO_3$ solution.
Table shows the number of grams of NaCl per liter corresponding to the number of cubic centimeters of NH_4CNS used to neutralize the excess of $AgNO_3$.

C.C. OF NH_4CNS USED	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
0	20.0	19.9	19.8	19.7	19.6	19.5	19.4	19.3	19.2	19.1
1	19.0	18.9	18.8	18.7	18.6	18.5	18.4	18.3	18.2	18.1
2	18.0	17.9	17.8	17.7	17.6	17.5	17.4	17.3	17.2	17.1
3	17.0	16.9	16.8	16.7	16.6	16.5	16.4	16.3	16.2	16.1
4	16.0	15.9	15.8	15.7	15.6	15.5	15.4	15.3	15.2	15.1
5	15.0	14.9	14.8	14.7	14.6	14.5	14.4	14.3	14.2	14.1
6	14.0	13.9	13.8	13.7	13.6	13.5	13.4	13.3	13.2	13.1
7	13.0	12.9	12.8	12.7	12.6	12.5	12.4	12.3	12.2	12.1
8	12.0	11.9	11.8	11.7	11.6	11.5	11.4	11.3	11.2	11.1
9	11.0	10.9	10.8	10.7	10.6	10.5	10.4	10.3	10.2	10.1
10	10.0	9.9	9.8	9.7	9.6	9.5	9.4	9.3	9.2	9.1
11	9.0	8.9	8.8	8.7	8.6	8.5	8.4	8.3	8.2	8.1
12	8.0	7.9	7.8	7.7	7.6	7.5	7.4	7.3	7.2	7.1
13	7.0	6.9	6.8	6.7	6.6	6.5	6.4	6.3	6.2	6.1
14	6.0	5.9	5.8	5.7	5.6	5.5	5.4	5.3	5.2	5.1
15	5.0	4.9	4.8	4.7	4.6	4.5	4.4	4.3	4.2	4.1
16	4.0	3.9	3.8	3.7	3.6	3.5	3.4	3.3	3.2	3.1
17	3.0	2.9	2.8	2.7	2.6	2.5	2.4	2.3	2.2	2.1
18	2.0	1.9	1.8	1.7	1.6	1.5	1.4	1.3	1.2	1.1
19	1.0	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1

Determination of Total Nitrogen

(Folin: Jour. Biol. Chem., 26, 473, 1916)

Principle.—The diluted urine is first heated with an acid mixture. By this procedure the nitrogen is converted into ammonium salts. The solution is then Nesslerized and read against a standard ammonium sulphate solution similarly treated.

Reagents.—1. Nessler's reagent. (See page 266.)

2. Standard ammonium sulphate solution:

Ammonium sulphate C.P.	0.4716	grams
Concentrated hydrochloric acid	1	c.c.
Water ad. q.s.	1000	c.c.

10 c.c. = 1 milligram nitrogen.

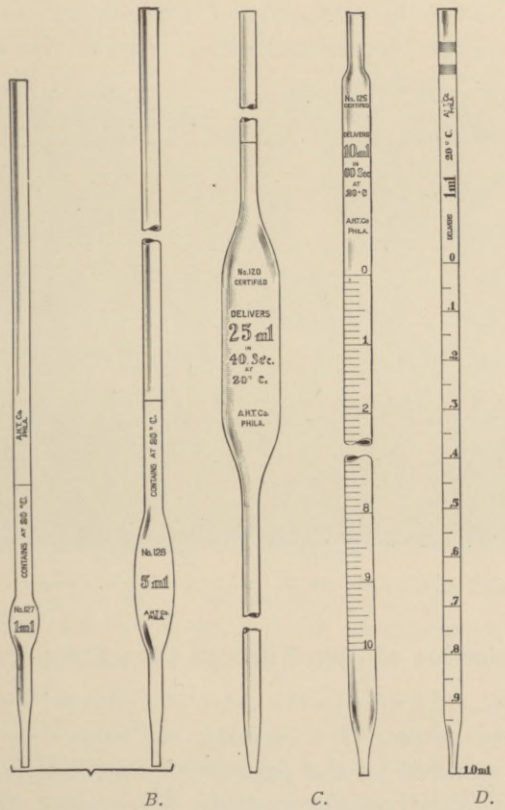
The ammonium sulphate should be dried in hot air for ½ hour at 110° centigrade.

3. Acid digestion mixture: Mix 300 c.c. of 85 per cent phosphoric acid with 100 c.c. of ammonia free sulphuric acid (concentrated). Transfer to a tall cylinder, cover well to exclude the absorption of ammonia and set aside for the sedimentation of calcium sulphate. To 100 c.c. of the clear acid add 10 c.c. of 6 per cent copper sulphate solution and 100 c.c. of water.

Procedure.—Dilute 5 c.c. of urine to 100 c.c., mix and with an Ostwald pipette (Fig. 18) transfer 1 c.c. of the diluted urine to a 200 × 20 mm. Pyrex tube graduated at 35 c.c. and 50 c.c. (The pipette must be drained for 15 seconds against the wall of test tube and then blown clean). With an ordinary pipette add 1 c.c. of the acid digestion mixture together with a pebble, to prevent bumping. Heat over a micro burner (no hood necessary) until the water is driven off and fumes become abundant within the tube (Fig. 19). This should take place in about two minutes. When filled with fumes, close the mouth of the test tube with a watch glass and continue the boiling at such a rate that the tube remains filled with fumes yet almost none escape. Within two minutes after the mouth of the test tube is closed the contents should become clear and bluish or light green. Continue the gentle boiling for 30 to 60 seconds longer, provided however, that the total boiling period with test tube

closed must not be less than two minutes. Remove the flame and let cool for a little less than two minutes, then add water to 35 c.c. mark.

Transfer 3 c.c. of standard ammonium sulphate solution containing 0.3 mg. of nitrogen into a 100 c.c. volumetric flask. Add 2 c.c. of the acid digestion mixture, to balance the acid in the un-



A. *B.* *C.* *D.*

Fig. 18.—Types of pipettes used in chemical and serological work.—*A*, Ostwald pipettes; *B*, volumetric pipette; *C*, Mohr pipette; *D*, serological pipette.

known, and dilute to a volume of 70 c.c. Whirl and add 30 c.c. of Nessler's reagent. To the test solution add 15 c.c. Nessler's reagent.

If the unknown Nesslerized digestion mixture is turbid, centrifuge a portion giving a crystal clear fluid above a white sed-

iment (silica). If the sediment is colored, the Nesslerization was not successful and the determination must be discarded.

Calculation.—Set standard at 20.

If R be the reading of unknown:

$$\frac{20}{R} = \text{grams nitrogen per 100 c.c. urine.}$$

Table V shows the percentage of nitrogen corresponding to the different colorimeter readings with a plunger type instrument.

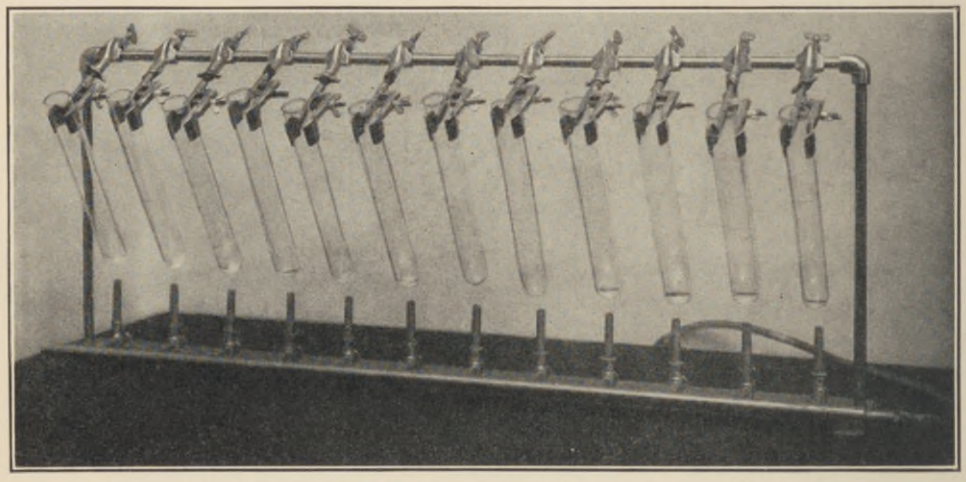


Fig. 19.—Kjeldahl apparatus for the determination of total nitrogen in blood and urine by the Folin micro-method. (*After Reyner.*)

Determination of Urea Nitrogen (Van Slyke and Cullen)

Principle.—The urea in the urine is converted into ammonium carbonate by urease. The ammonia is liberated by the addition of alkali, removed by the passage of a strong air current and collected in N/100 sulphuric acid. The excess of acid is then titrated with N/100 alkali.

Procedure.—Dilute 5 c.c. of urine to 50 c.c. with distilled water. Measure 5 c.c. of diluted urine into large test tube (Fig. 20) and add two pulverized urease tablets. Leave at room temperature for ½ hour. Add a layer of alcohol and kerosene, or a few drops of foam killer and 5 c.c. of saturated potassium carbonate. Connect at once with second tube "B" containing 50

TABLE V

DETERMINATION OF TOTAL NITROGEN IN URINE

Dilute 5 c.c. or 2 c.c. of urine to 100 c.c. Use 1 c.c. of the diluted urine in a final volume of 50 c.c. Compare with standard containing 0.3 mgm. nitrogen in 100 c.c.

Set standard at 20, or fill 20 mm. Bock-Benedict cell.

Table shows the total nitrogen in per cent corresponding to the different colorimeter readings with the two dilutions of urine, using a plunger type instrument.

COLORIMETER READING	5 C.C. URINE DILUTED TO 100 C.C. (Total nitrogen in per cent)					2 C.C. URINE DILUTED TO 100 C.C. (Total nitrogen in per cent)				
	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8
10	0.60	0.59	0.58	0.56	0.55	1.50	1.47	1.45	1.41	1.39
11	0.55	0.54	0.53	0.52	0.51	1.37	1.34	1.32	1.30	1.27
12	0.50	0.49	0.48	0.48	0.48	1.25	1.23	1.21	1.19	1.17
13	0.46	0.46	0.45	0.44	0.44	1.15	1.14	1.13	1.11	1.09
14	0.43	0.42	0.41	0.41	0.41	1.07	1.06	1.04	1.03	1.02
15	0.40	0.40	0.39	0.39	0.38	1.00	0.99	0.98	0.97	0.95
16	0.38	0.37	0.37	0.36	0.36	0.94	0.93	0.92	0.90	0.90
17	0.35	0.35	0.35	0.34	0.34	0.89	0.88	0.87	0.86	0.85
18	0.33	0.33	0.33	0.32	0.32	0.84	0.83	0.82	0.81	0.80
19	0.32	0.31	0.31	0.31	0.30	0.79	0.78	0.78	0.77	0.76
20	0.30	0.30	0.29	0.29	0.29	0.75	0.75	0.74	0.73	0.73
21	0.29	0.28	0.28	0.28	0.28	0.72	0.70	0.70	0.70	0.69
22	0.27	0.27	0.27	0.27	0.26	0.69	0.68	0.67	0.67	0.66
23	0.26	0.26	0.26	0.26	0.25	0.66	0.65	0.65	0.64	0.63
24	0.25	0.25	0.25	0.24	0.24	0.63	0.62	0.62	0.61	0.61
25	0.24	0.24	0.24	0.23	0.23	0.60	0.60	0.59	0.59	0.59
26	0.23	0.23	0.23	0.23	0.23	0.58	0.58	0.57	0.57	0.57
27	0.22	0.22	0.22	0.22	0.22	0.56	0.56	0.55	0.55	0.54
28	0.21	0.21	0.21	0.21	0.21	0.54	0.54	0.53	0.53	0.52
29	0.21	0.21	0.20	0.20	0.20	0.52	0.52	0.51	0.51	0.51

c.c. N/100 H_2SO_4 and two drops alizarin. Aspirate for one hour and titrate excess H_2SO_4 with N/100 NaOH.

Calculation.—The number of c.c. of N/100 H_2SO_4 neutralized multiplied by the factor 0.028 gives the number of grams of urea nitrogen plus ammonia nitrogen in 100 c.c. urine.

Determine the ammonia at the same time by using 5 c.c. of the undiluted urine. Layer with kerosene and alcohol or add a few drops of foam killer. Add 5 c.c. saturated potassium carbonate and aspirate into 50 c.c. N/100 H_2SO_4. The number of cubic centimeters of N/100 H_2SO_4 multiplied by the factor 0.0028 gives the number of grams of ammonia nitrogen per 100 c.c. urine. This subtracted from the result obtained in the first determination gives the number of grams of urea nitrogen per 100 c.c. urine.

Remarks.—A slow current of air should be used during the first two minutes of aspiration. The column of acid should be at least 50 mm. high in the receiving tube. The solution from which the ammonia is derived must contain at least one gram of potassium carbonate for each 2 c.c. of solution.

A blank determination should be run with each new lot of chemicals and the necessary deduction be made if any ammonia is found.

The air used for aerating should be run through a wash bottle containing sulphuric acid.

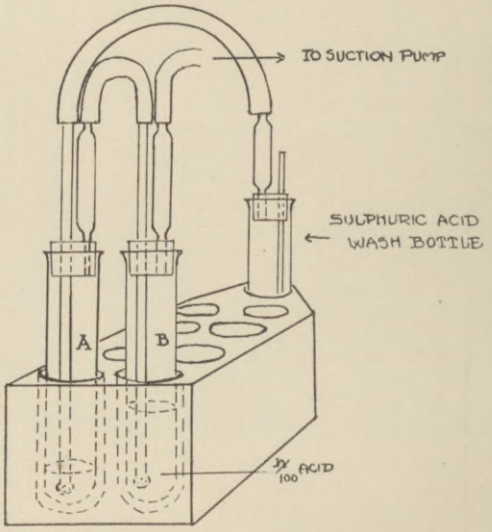


Fig. 20.—Van Slyke and Cullen apparatus for the determination of urea nitrogen.

The figure obtained for urea nitrogen may be converted into urea by dividing by the factor 0.467.

Determination of Ammonia

Principle.—The ammonia is liberated by the addition of an alkali, removed from the urine by the passage of a strong current of air, and is collected in N/100 sulphuric acid. The excess of acid is then titrated with N/100 alkali.

Procedure.—Use the same apparatus as that employed in the determination of urine by the urease method (Fig. 20). In the

first tube "A" place 5 cubic centimeters urine, 5 cubic centimeters of saturated potassium carbonate solution (90 grams to 100 c.c.) and a few drops of foam killer. In the second tube "B" place 50 c.c. of N/100 sulphuric acid and 2 drops of alizarin. Allow the air current to pass through for about an hour. Titrate excess of acid with N/100 NaOH.

Calculation.—Each cubic centimeter of N/100 sulphuric acid neutralized by the ammonia liberated corresponds to 0.0028 grams of ammonia N, or 0.0034 grams of ammonia per 100 c.c. urine.

Remarks.—A slow current of air should be used during the first two minutes of aspiration. The column of acid should be at least 50 mm. high in the receiving tube. The solution from which the ammonia is driven must contain at least 1 gram of potassium carbonate for each 2 c.c. of solution.

Determination of Uric Acid

(Benedict, S. R., and Franke, Elizabeth: Jour. Biol. Chem., 1922, lii, 387)

Principle.—The urine is diluted and a special reagent and sodium cyanide are added. A deep blue develops. This is compared with a standard solution of uric acid similarly treated.

Reagents.—1. Benedict's uric acid reagent. This is prepared as follows: 100 gm. of pure sodium tungstate are placed in a liter Pyrex flask and dissolved in about 600 c.c. of water; 50 gm. of pure arsenic acid (As_2O_5) are now added, followed by 25 c.c. of 85 per cent phosphoric acid and 20 c.c. of concentrated hydrochloric acid. The mixture is boiled for about 20 minutes, cooled and diluted to 1 liter. The reagent appears to keep indefinitely.

2. Sodium cyanide.—A 5 per cent solution of sodium cyanide, which should be prepared fresh once in about two months, is used.

3. Standard uric acid solution.—A standard solution of uric acid, acidified with hydrochloric acid, containing 0.2 mg. of uric acid in 10 c.c., is employed. A stock solution is prepared as follows: Dissolve 9 gm. of pure crystallized disodium hydrogen phosphate together with 1 gm. of monosodium dihydrogen phosphate in 200 to 300 c.c. of hot water. Filter if not perfectly clear and make up to about 500 c.c. with hot water. Pour this warm

solution upon exactly 200 mg. of uric acid suspended in a few c.c. of distilled water in a liter flask and shake gently until all the uric acid passes into solution. Cool the solution, add exactly 1.4 c.c. of glacial acetic acid, dilute to 1 liter, and mix. Five c.c. of chloroform are then added to prevent bacterial growth. Five c.c. of this standard solution contain 1 mg. of uric acid. Unless kept in an excessively warm place the solution may be relied on to keep for two months.

The standard solution for use in the test is prepared from the stock phosphate standard solution. Fifty c.c. (containing 10 mg. of uric acid) are measured into a 500 c.c. volumetric flask and diluted to about 400 c.c. with distilled water. Twenty-five c.c. of dilute hydrochloric acid (made by diluting 1 volume of the concentrated acid with 9 volumes of water) are added, and the solution is diluted to 500 c.c. and mixed. This dilute standard solution should be prepared fresh from the phosphate standard every ten days to two weeks.

Procedure.—The urine is so diluted that 10 c.c. will contain between 0.15 and 0.30 mg. of uric acid. (Usually a dilution of 1 to 20 is satisfactory.) Ten c.c. of the diluted urine are measured into a 50 c.c. volumetric flask, 5 c.c. of the 5 per cent sodium cyanide solution are added from a burette, followed by 1 c.c. of the arsenophosphotungstic acid reagent. The contents of the flask are mixed by gentle shaking, and at the end of 5 minutes diluted to the 50 c.c. mark with distilled water and mixed. This blue solution is then compared in a colorimeter with a simultaneously prepared solution obtained by treating 10 c.c. of the standard uric acid solution (0.2 mg. of uric acid) in a 50 c.c. flask with 5 c.c. of the sodium cyanide solution, 1 c.c. of the reagent, and diluting to the mark at the end of 5 minutes.

Calculation.—If R be the reading of the unknown:

$\frac{20}{R} \times 20$ = mg. uric acid per 100 c.c. of urine when 10 c.c. of urine are diluted to 100 c.c. for the test.

$\frac{20}{R} \times 40$ = mg. uric acid per 100 c.c. of urine when 5 c.c. of urine are diluted to 100 c.c.

$\frac{20}{R} \times 80$ = mg. uric acid per 100 c.c. of urine when 2.5 c.c. of urine are diluted to 100 c.c.

Remarks.—The proportional depth of color for uric acid concentrations varying between 0.15 and 0.30 mg. is almost absolutely exact under the conditions indicated, when 0.2 mg. of uric

TABLE VI

Dilute 2.5, 5 or 10 c.c. urine to 100 c.c. Take 10 c.c. of the diluted urine for the test and make up to a final volume of 50 c.c. Compare with standard containing 0.2 mg. uric acid made up to a similar volume.

Set standard at 20, or fill 20 mm. Bock-Benedict cell.

Table shows the uric acid in mgs. per 100 c.c. urine corresponding to the different colorimeter readings using a plunger type colorimeter.

	2.5 C.C. URINE DILUTED TO 100 C.C.					5 C.C. URINE DILUTED TO 100 C.C.					10 C.C. URINE DILUTED TO 100 C.C.				
COLORIMETER READING	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8
12	133	131	129	127	125	67	65	64	63	62	33	33	32	32	31
13	123	121	119	118	116	61	61	60	59	58	31	30	30	30	29
14	114	113	111	109	108	57	56	56	55	54	29	28	28	27	27
15	107	105	104	102	101	53	53	52	51	51	27	26	26	26	25
16	100	99	98	96	95	50	49	49	48	48	25	25	24	24	24
17	94	93	92	91	90	47	47	46	45	45	24	23	23	23	22
18	89	88	87	86	85	44	44	44	43	42	22	22	22	22	21
19	84	84	83	82	81	42	42	41	41	40	21	21	21	20	20
20	80	79	78	77	76	40	40	39	39	38	20	20	20	19	19
21	76	75	75	74	73	38	38	37	37	37	19	19	19	19	18
22	73	72	72	71	70	36	36	36	35	35	18	18	18	18	18
23	69	69	68	68	67	35	34	34	34	34	17	17	17	17	17
24	67	66	66	65	64	33	33	33	33	32	17	17	16	16	16
25	64	64	63	62	62	32	32	32	31	31	16	16	16	16	16
26	62	61	61	60	60	31	31	30	30	30	15	15	15	15	15
27	59	59	58	58	58	29	29	29	29	29	15	15	15	15	15

acid is used as standard. Outside of this range the results are not quite satisfactory, hence, if the colorimeter reading is below 14 or above 26, the test should be repeated using urine diluted 1 to 40, or 1 to 10.

It is essential that the volume of the unknown and of the standard be the same during the period of the reaction.

If albumin is present in appreciable amounts it is best to remove it by heat coagulation in the presence of a drop of acetic acid, and filtration.

Table VI shows the amount of uric acid corresponding to the colorimeter readings, using a plunger type instrument.

Determination of Creatinine (Folin)

Principle.—On adding picric acid and sodium hydroxide to a solution containing creatinine a deep red color is produced. The intensity of this color in a specimen of urine is compared with that of a standard solution of creatinine. Sugar and albumin do

not interfere but acetone and diacetic acid, if present, must be removed by heating.

Reagents.—(1) Standard creatinine solution: 1 gram of creatinine or 1.61 grams creatinine zinc chloride dissolved in 1000 c.c. of tenth normal hydrochloric acid (for method of preparation of creatinine and creatinine zinc chloride see page 267).

(2) Saturated picric acid solution (about 12 grams per liter). The picric acid should be tested for purity as follows: To 20 c.c. of a saturated solution of picric acid, add 1 c.c. of 10 per cent sodium hydroxide and let it stand for 15 minutes. The color of

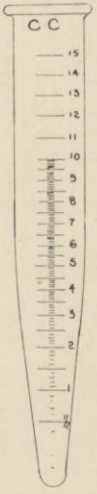


Fig. 21.—Fifteen c.c. graduated centrifuge tube.

the alkaline picrate solution must not be more than twice as deep as the color of the saturated acid solution. If the picric acid is unusually pure the color of the picrate solution will not be more than one and one-half times as deep as that of a saturated picric acid solution. If necessary the picric acid may be purified as described on page 269.

(3) Ten per cent NaOH.

Procedure.—By means of an Ostwald pipette (Fig. 18) transfer 1 c.c. or 2 c.c. of urine to a 100 c.c. volumetric flask. To another flask transfer 1 c.c. of the standard creatinine solution, 1 c.c. of which contains 1 mg. of creatinine. To each flask add 20

Table VII

Determination of Creatinine in Urine

Use 0.5, 1 or 2 c.c. of urine in a total volume of 100 c.c. and a standard containing 1 mg. of creatinine in 100 c.c.

Set standard at 20, or fill 20 mm. Bock-Benedict cell.

Table shows the creatinine in mg. per 100 c.c. of urine corresponding to the different colorimeter readings with the varying amounts of urine, using a plunger type instrument.

	USING 2 C.C. URINE (Creatinine in mg. per 100 c.c. urine)					USING 1 C.C. URINE (Creatinine in mg. per 100 c.c. urine)					USING 0.5 C.C. URINE (Creatinine in mg. per 100 c.c. urine)				
COLORIMETER READING	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8
15	67	66	65	64	64	133	132	130	128	127	267	263	260	256	254
16	63	62	61	61	60	125	124	122	121	119	250	247	244	241	238
17	59	58	58	57	56	118	116	115	114	112	236	233	230	227	224
18	56	55	55	54	53	111	110	109	108	106	222	220	218	215	213
19	53	52	52	51	51	105	104	103	102	101	211	209	206	204	202
20	50	50	49	49	48	100	99	98	97	96	200	198	196	194	192
21	48	47	47	47	46	95	94	93	93	91	190	189	187	185	183
22	46	45	45	44	44	91	90	89	88	87	182	180	179	177	175
23	44	43	43	43	42	87	86	86	85	84	173	172	171	169	168
24	42	42	41	41	41	83	83	82	81	81	167	165	164	163	161
25	40	40	40	39	39	80	79	79	78	78	160	159	158	156	155
26	38	38	38	38	38	77	76	76	75	75	154	153	152	150	149
27	37	37	37	37	36	74	74	73	73	72	147	147	146	145	144
28	36	36	36	35	35	71	71	71	70	69	142	142	140	140	139
29	35	35	34	34	34	69	69	68	68	67	137	137	136	135	134

c.c. of saturated picric acid solution, then add from a burette 1.5 c.c. of 10 per cent sodium hydroxide to each, and let stand for ten minutes. At the end of ten minutes dilute to mark with water and mix. Compare in colorimeter with standard set at 20.

Calculation.—If R be the reading of the unknown:

$$\frac{20}{R} = \text{Milligrams of creatinine in the quantity of urine taken.}$$

Remarks.—The normal excretion of creatinine is about 20 to 30 mg. per kilo of body weight, fat persons yielding less and thin persons more. On an average diet the creatinine nitrogen equals about 3 to 5 per cent of the total nitrogen.

Table VII shows the amount of creatinine corresponding to different readings with a colorimeter of the plunger type.

Determination of Creatine (Folin)

Principle.—On heating creatine with dilute mineral acid it is dehydrated and its anhydride creatinine 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.

Procedure.—Place 1 c.c. of the urine in a 100 c.c. volumetric flask and add 20 c.c. of saturated picric acid solution. Heat in the autoclave for twenty to thirty minutes at 15 pounds pressure, closing the mouth of flask with tin foil. Cool. Add 1.5 c.c. of sodium hydroxide. Determine the creatinine by the method described for creatinine. From the amount of creatinine so obtained deduct the amount of creatinine determined in the unheated urine. The difference will be the creatine content of the original urine in terms of creatinine. To obtain the amount of creatine 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.

Remarks.—Creatine occurs in the urine of normal adults in only very small amounts. In the urine of children as much as 10-15 mg. per day may be found.

Determination of Sulphates (Folin)

(A) Inorganic

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.—

(1) Dilute HCl (1 part concentrated HCl to 4 parts H_2O by volume).

(2) Barium chloride solution, 5 per cent.

Procedure.—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

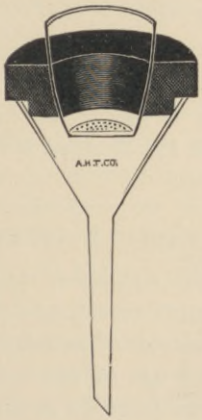


Fig. 22.—Gooch crucible and holder.

the urine is dilute take 50 c.c. instead of 25, and a correspondingly smaller amount of water. Ten c.c. 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, (Fig. 22), as described below. The precipitate is washed with at least 200 c.c. of water. The crucible is then dried, ignited, cooled and weighed. Report results in terms of SO_3.

Calculation.—One gram $BaSO_4$ = 0.3430 gm. SO_3.

Remarks.—The urine must be free of albumin.

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 degrees 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.

(B) Total Sulphates

Principle.—The ethereal sulphates are split by boiling with HCl, and the total sulphates resulting determined just as above.

Procedure.—Twenty-five c.c. 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 directed under determination of inorganic sulphates.

(C) Ethereal Sulphates (Folin)

The amount of these may be obtained by subtracting the amount of inorganic sulphates from that of the total sulphates.

(D) Neutral Sulphates

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 barium chloride solution.

Reagents.—

(1) Benedict's solution:

Crystallized copper nitrate..... 200 grams
Sodium or potassium chlorate..... 50 grams
Distilled water to.....1000 c.c.

(2) Barium chloride solution, 5 per cent.

(3) Dilute HCl (1 part concentrated HCl to 4 parts water by volume).

Procedure.—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.

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. Wash with at least 200 c.c. water; dry. Ignite, cool, and weigh.

Calculation.—1 gram $BaSO_4$ = 0.1374 gram sulphate.

Determination of Phosphates

(A) Total Phosphates

Principle.—The urine is first treated with a solution of sodium acetate to convert any monacid phosphate into diacid phosphate.

Standard uranium acetate is run into a measured quantity until all of the phosphate has been precipitated as insoluble uranium phosphate. An excess of uranium is indicated by a reddish coloration with potassium ferrocyanide. This method is accurate and gives practically the total phosphorus of urine inasmuch as the latter exists generally almost entirely as phosphates.

Reagents.—

1. Sodium acetate solution prepared by dissolving 100 grams of sodium acetate in 800 c.c. of distilled water, adding 100 c.c. of 30 per cent acetic acid to the solution, and making the volume of the mixture up to 1 liter with water.

2. Ten per cent potassium ferrocyanide.

3. Uranium acetate solution: Dissolve about 35.0 grams of uranium acetate in 1 liter of water with the aid of heat and 3 to 4 c.c. of glacial acetic acid. Let stand a few days and filter. Standardize against a phosphate solution containing 0.005 grams of P_2O_5 per cubic centimeter. For this purpose dissolve 14.721 grams of pure air-dry sodium ammonium phosphate ($NaNH_4HPO_4 + 4H_2O$) in water to make a liter. To 20 c.c. of this phosphate solution in a beaker add 30 c.c. of water and 5 c.c. of sodium acetate solution (see above) and titrate with the uranium solution to the correct end reaction as indicated in the method above. If exactly 20 c.c. of uranium solution are required, 1 c.c. of the solution is equivalent to 0.005 gram P_2O_5. If stronger than this, dilute accordingly and check again by titration.

Procedure.—To 50 c.c. of urine in a small beaker or Erlenmeyer flask add 5 c.c. of the sodium acetate solution and heat the mixture to the boiling point. From a burette, run into the hot mixture, drop by drop, the standard solution of uranium acetate 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 a drop of a 10 per cent solution of potassium ferrocyanide on a porcelain test-tablet (Fig. 23) produces instantaneously a brown-

ish-red coloration. Take the burette reading and calculate the P_2O_5 content of the urine under examination.

Calculation.—Multiply the number of cubic centimeters of uranium acetate solution used by 0.005 to determine the number of grams of P_2O_5 in the 50 c.c. of urine used. To express the result in percentage of P_2O_5 multiply the value just obtained by 2, e.g., if 50 c.c. of urine contained 0.074 gram of P_2O_5 it would be equivalent to 0.148 per cent.

Calculate, in terms of P_2O_5 the total phosphate content of the 24 hour specimen.

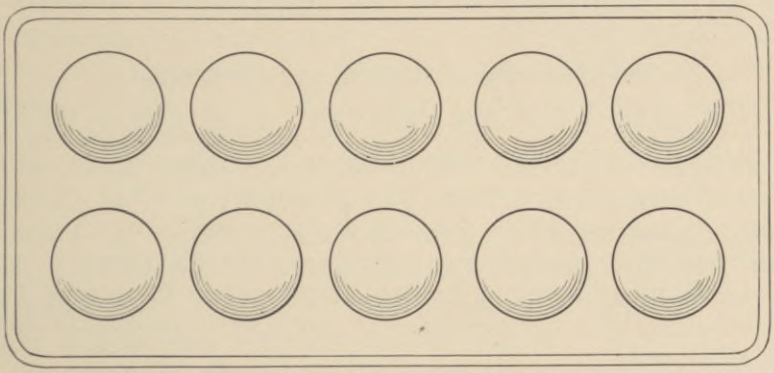


Fig. 23.—Porcelain mixing plate for use in the determination of phosphates and in blood grouping. (*From Gradwohl and Blaivas.*)

(B) Earthy Phosphates

Principle.—The earthy phosphates are precipitated by making the urine alkaline. The precipitate is filtered off, dissolved in acid, and titrated with uranium acetate.

Procedure.—To 100 c.c. of urine in a beaker add an excess of ammonium hydroxide and allow the mixture to stand 12 to 24 hours. Under these conditions the phosphoric acid in combination with the alkaline earths, calcium and magnesium, is precipitated as phosphates of these metals. Collect the precipitate on a filter paper and wash it with very dilute ammonium hydroxide. Pierce the paper, and remove the precipitate by means of hot water. Bring the phosphates into solution by adding a small amount of dilute acetic acid to the warm solution, and determine

the P_2O_5 content of the mixture according to the directions given under the Determination of Total Phosphates.

Calculation.—Multiply the number of cubic centimeters of uranium acetate solution used by 0.005 to determine the number of grams of P_2O_5 in the 100 c.c. of urine used. Since 100 c.c. of urine was taken this value also expresses the percentage of P_2O_5 present.

Calculate the quantity of earthy phosphates, in terms of P_2O_5, present in the 24 hour urine specimen.

The quantity of phosphoric acid present in combination with the alkali metals may be determined by subtracting the content of earthy phosphates from the total phosphates.

Determination of Calcium and Magnesium

(McCrudden: Jour. Biol. Chem., 1910, vii, 83; 1911, x, 187)

Principle.—Calcium is precipitated as the oxalate by the addition of ammonium oxalate. The precipitate is either ignited and weighed as CaO or determined volumetrically by titration with potassium permanganate. By the use of sodium acetate and hydrochloric acid such an acidity is attained as will prevent the interference with the procedure of magnesium, phosphates and iron.

Magnesium is determined in the filtrate from the calcium determination after the destruction of the organic matter. It is precipitated as ammonium magnesium phosphate, ignited and weighed as the pyrophosphate.

Reagents Required.—

(1) 2.5 per cent oxalic acid.

(2) 20 per cent sodium acetate.

(3) 0.5 per cent ammonium oxalate.

(4) N/10 potassium permanganate solution (for volumetric procedure).

(5) Conc. hydrochloric acid (sp.gr. 1.20).

(6) Alcoholic ammonia solution (1 part alcohol, 1 part dil. ammonia, 3 parts water).

(7) Dilute ammonia (sp.gr. 0.96).

Procedure for Calcium.—If the urine is alkaline make it neutral or slightly acid and filter. Take 200 c.c. of the filtered urine

for analysis. If it is only slightly acid to litmus add ten drops of concentrated hydrochloric acid (sp.gr. 1.20). If the urine is strongly acid it may be made just alkaline with ammonia and then just acid with hydrochloric acid, after which the ten drops of hydrochloric acid are added. Then add 10 c.c. of 2.5 per cent oxalic acid. Run in slowly with stirring 8 c.c. of 20 per cent sodium acetate. Allow to stand overnight at room temperature or shake vigorously for ten minutes. Filter off the precipitate of calcium oxalate on a small paper and wash free from chlorides with 0.5 per cent ammonium oxalate solution. The precipitate may then be dried, ignited to constant weight and weighed as calcium oxide or titrated volumetrically as described below.

Volumetric Titration.—If free from uric acid, the calcium oxalate precipitate may be washed three times with distilled water, filling the filter about two-thirds full and allowing it to drain completely before adding more. A hole is made in the paper and the calcium oxalate washed in the flask. The volume of the fluid is brought up to about 50 c.c. and 10 c.c. of concentrated sulphuric acid added. Titrate with N/10 potassium permanganate solution to a pink color which persists for at least a minute.

Calculation.—One c.c. of N/10 potassium permanganate solution is equivalent to 2.8 mg. of CaO.

Procedure for Magnesium.—Transfer the filtrate from the calcium determination to a porcelain dish, add about 20 c.c. concentrated nitric acid and evaporate to dryness. Heat the residue over a free flame until the ammonium salts are destroyed and the residue fuses. After cooling take the residue up with water and a little hydrochloric acid and filter if nceessary. Dilute to about 80 c.c., nearly neutralize with ammonia and cool. Add a slight excess of sodium acid phosphate and then ammonia drop by drop with constant stirring until the solution is alkaline and then add enough more slowly with constant stirring to make the solution contain one-fourth its bulk of dilute ammonia (sp.gr. 0.96). Allow to stand overnight. Filter and wash free from chlorides with alcoholic ammonia solution. The precipitate with filter paper is incinerated slowly and carefully with a good supply of air to prevent reduction, in the usual manner, and ignited and weighed as the pyrophosphate.

Calculation.—One gram magnesium pyrophosphate is equivalent to 0.3624 grams MgO.

Remarks.—The average excretion of calcium (as CaO) is 0.1 to 0.4 gm. per day. The kidneys excrete about the same amount of magnesium (as MgO) daily.

Determination of Acetone Bodies

(Van Slyke, D.D.: Jour. Biol. Chem., 1917, xxxii, 455)

Principle.—The method for the quantitative determination of acetone bodies in the urine is based on a combination of Shaffer's oxidation of beta-hydroxybutyric acid to acetone and Deniges' precipitation of acetone as a basic mercuric sulphate compound. Oxidation and precipitation 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 acetoacetic may be determined either with the beta-hydroxybutyric acid or separately. Neither the size of sample nor mode of procedure requires variation for different times. 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 quick drying and accurate weighing, but when facilities for weighing are absent, the precipitate can be dissolved in dilute hydrochloric acid and the mercury titrated with potassium iodide by the method of Personue.

Preservatives other than toluene or copper sulphate should not be used.

Reagents.—(1) Twenty per cent copper sulphate—200 grams of $CuSO_4.5H_2O$ dissolved in water and made up to 1 liter.

(2) Ten per cent mercuric sulphate—73 grams of pure red mercuric oxide dissolved in 1 liter of H_2SO_4 of 4N concentration.

(3) 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 must be readjusted if necessary to make it 17.0 N by titration.

(4) Ten per cent calcium hydroxide suspension—mix 100

grams of Merck's fine light "reagent" $Ca(OH)_2$ with 1 liter of water.

(5) Five per cent potassium dichromate—50 grams $K_2Cr_2O_7$ dissolved in water and made up to 1 liter.

(6) Combined reagents for total acetone body determination—1 liter of the above 50 per cent sulphuric acid, 3.5 liters of the mercuric sulphate, 10 liters of water.

Procedure.—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 water, 50 c.c. of copper sulphate solution and mix. Then add 50 c.c. of 10 per cent calcium hydroxide suspension, shake and test with litmus. If not alkaline, add more calcium hydroxide. Dilute to the 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 copper treatment is depended upon to remove interfering substances other than glucose, and should therefore never be omitted, even when glucose is absent. The filtrate may be tested for glucose by boiling a little in a test tube. A precipitate of yellow cuprous oxide 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.

Determination of Total Acetone Bodies (Acetone, Acetoacetic Acid, and B-hydroxybutyric Acid).—Place in a 500 c.c. Erlenmeyer flask 25 c.c. of urine filtrate. Add 100 c.c. of water, 10 c.c. of 50 per cent sulphuric acid, and 35 c.c. of the 10 per cent mercuric sulphate. Or in place of adding the water and reagents separately, add 145 c.c. of the "combined reagents." Connect the flask with a reflux condenser having a straight condensing tube of 8 or 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 1½ hours. The yellow precipitate which forms consists of the mercury sulphate-chromate compound of the preformed acetone, and the acetone which has been formed by decomposition of acetoacetic acid and by

oxidation of the B-hydroxybutyric acid. It is collected in a Gooch (Fig. 22) or "medium density" alundum crucible, washed with 200 c.c. of cold water, and dried for an hour at 110°. The crucible is allowed to cool in room air (a desiccator is unnecessary and undesirable) and weighed. Several precipitates may be collected, one above the other, without cleaning the crucible. As an alternative to weighing, the precipitate may be dissolved and titrated as described below.

Determination of Acetone and Acetoacetic Acid.—The acetone plus the acetoacetic acid, which completely decomposes into acetone and CO_2 on heating, is determined without the B-hydroxybutyric acid exactly as the total acetone bodies, except that (1) no dichromate is added to oxidize the B-hydroxybutyric acid and (2) the boiling must continue for not less than 30 nor more than 45 minutes. Boiling for more than 45 minutes splits off a little acetone from B-hydroxybutyric acid even in the absence of chromic acid.

Determination of B-hydroxybutyric Acid.—The B-hydroxybutyric acid alone is determined exactly as total acetone bodies except that the preformed acetone and that from the acetoacetic acid are first boiled off. To do this, the 25 c.c. of urine filtrate plus 100 c.c. of water are treated with 2 c.c. of the 50 per cent sulphuric acid and boiled in the open flask for 10 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 sulphuric acid and 35 c.c. of mercuric sulphate are added. The flask is connected under the condenser and the determination is continued as described for total acetone bodies.

Titration of the Precipitate in the Above Methods.—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. In place of using either a

Gooch or alundum crucible, one may, when titration is employed, wash the precipitate without suction on a small quantitative filter paper, which is transferred with the precipitate to the beaker and broken up with a rod in 15 c.c. of normal HCl.

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 it has been found that the addition of excess sodium acetate is the most satisfactory means. Six to 7 c.c. of 3 m. acetate are added to the cooled solution of redissolved precipitate. Then the 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 at once forms, 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 mg. 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 way 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 $HgCl_2 + 4KI = K_2HgI_4 + KCl$, 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 iodine is standardized by titration against it. A slight error appears to be introduced if the iodine solution is gravimetrically standardized and used for checking the mercury solution, instead of vice versa.

In standardizing the mercuric chloride the following procedure has been found convenient: 25 c.c. of 0.05 m. $HgCl_2$ are measured with a calibrated pipette, diluted to about 100 c.c. and H_2S is run in until the black precipitate flocculates and leaves a clear solution. The HgS, collected in a Gooch crucible and dried at 110°, should weigh 0.2908 gram if the solution is accurate.

Both by gravimetric analyses of the basic mercuric sulphate-acetone precipitate and by titration, the mercury content of the precipitate has been found to average 76.9 per cent. On this

basis, each c.c. of 0.2 m. KI solution being equivalent to 10.0 mg. of Hg. is equivalent to 13.0 mg. of the mercury acetone precipitate.

Titration is not quite so accurate as weighing, but except when the amounts determined are very small, the titration is satisfactory.

Calculation.—One mg. of B-hydroxybutyric acid yields 8.45 mg. of precipitate. One mg. of acetone yields 20.0 mg. of precipitate. One c.c. of 0.2 m. KI solution is equivalent to 13 mg. of precipitate in titration of the latter.

TABLE VIII

SPECIAL FACTORS FOR CALCULATION OF RESULTS WHEN 25 C.C. OF URINE FILTRATE EQUIVALENT TO 2.5 C.C. OF URINE, ARE USED FOR THE DETERMINATION

Determination performed.	Acetone bodies, calculated as gm. acetone per liter of urine, indicated by	
	1 gm. of prec.	1 c.c. of 0.2 m. KI sol.
Total acetone bodies	24.8	0.322
B-hydroxybutyric acid	26.4	0.344
Acetone—acetoacetic acid	20.0	0.260

In order to calculate the acetone bodies as B-hydroxybutyric acid rather than acetone, use the above factors multiplied by the ratio of the molecular weights $\frac{\text{B-acid}}{\text{acetone}} = \frac{104}{58} = 1.793$. In order to calculate the acetone bodies in terms of molecular concentration, divide the factors in Table VIII by 58. To calculate c.c. of 0.1 m. acetone bodies per liter of urine use the above factors multiplied by $\frac{10{,}000}{58} = 172.4$.

Remarks.—The maximum daily excretion of acetone bodies found by Van Slyke in normal men, under usual conditions, was 0.42 grams calculated as acetone, or 0.75 grams calculated as beta-hydroxybutyric acid. Normal adults on a mixed diet excrete on an average of 3-15 mg. of acetone and diacetic acid bodies per day. Usually about one-fourth is in the form of acetone. Beta-hydroxybutyric acid may occur in normal urine to the extent of 20 to 30 mg. per day.

Mosenthal Nephritic Test Meal

(Mosenthal, H. O.: Arch. Int. Med. 1915, lxvi, 733)

Principle.—The nephritic test meal is a method for measuring kidney function by the elimination of fluid, salt and nitrogen, and by the specific gravity of the urine. A fixed, weighed, and salt-free diet is given for one day. With each meal 2.3 grams of salt are given. Urine specimens are collected at 2 hour intervals from 8:00 A.M. to 8:00 P.M. and saved separately. The total night urine is collected at 8:00 A.M. on the morning following the test. Any food refused is weighed and noted on the diet chart. The evening meal must be taken at least three hours before the collection of the night urine is begun.

Procedure.—Measure and take the specific gravity of each specimen. Mix the seven day specimens and determine the total nitrogen and total chlorides. Make similar determinations of the total night urine.

Table IX shows the diet given, the fluid intake, and the nitrogen percentage of each article of food. Calculate the amount of nitrogen in any food refused and subtract from the total nitrogen of the test meal. Similarly any fluid or salt not taken is to be subtracted from the total offered. Calculate the fluid, salt and nitrogen balance.

Record results as follows:

MOSENTHAL TEST

TIME	VOL.	SP.GR.	SODIUM CHLORIDE		NITROGEN	
			%	GMS.	%	GMS.
8-10						
10-12						
12-2						
2-4						
4-6						
6-8						
Total Day						
Total Night 8-8						
Total Excretion (24 hours)						
Total Intake						
Balance						

TABLE IX

	FLUID C.C.	NITROGEN		FAT GRAMS	PROTEIN GRAMS	CARBOHYDRATE GRAMS	CALORIES
		%	GRAMS				
Breakfast—8 A. M.							
Boiled oatmeal—100 gm.	...	0.448	0.448	0.5	2.8	11.5	63
Sugar, 1/2 teaspoonfuls	...	...	.....	....	....	5	20
Milk, 30 c.c.	30	0.528	0.158	1.2	1.0	1.5	21.6
Two slices bread (30 gm. each)	...	1.47	0.880	0.8	5.5	31.9	160.8
Butter, 20 gm.	...	0.16	0.032	17.0	0.2	....	159
Coffee, 160 c.c.	160	...	.....	....	....	....	.....
Sugar, 1 teaspoonful 200 c.c.	...	...	.....	....	....	5	20
Milk, 40 c.c.	40	0.528	0.211	1.6	1.3	2	28.8
Milk, 200 c.c.	200	0.528	1.056	8.0	6.6	10	144
Water, 200 c.c.	200	...	.....	....	....	....	.....
Dinner—12 noon.							
Meat Soup, 180 c.c.	180	0.70	1.264	0.7	7.9	1.9	46.8
Beefsteak, 100 gm.	...	4.416	4.416	7.7	27.6	....	185
Potato (baked, mashed or boiled) 130 gm.	...	0.406	0.528	0.1	3.3	27.2	126.1
Green vegetables, as desired	...	...	.....	....	....	....	.....
Two slices bread (30 gm. each)	...	1.47	0.880	0.8	5.5	31.9	160.8
Butter, 20 gm.	...	0.16	0.032	17.0	0.2	....	159
Tea, 180 c.c.	180	...	.....	....	....	....	....
Sugar, 1 teaspoonful 200 c.c.	...	...	.....	....	....	5	20
Milk, 20 c.c.	20	0.528	0.105	0.8	0.6	1.0	14.4
Water, 250 c.c.	250	...	.....	....	....	....	.....
Pudding (tapioca or rice) 110 gm.	...	0.116	0.128	0.3	0.8	79	359
Supper—5 P. M.							
Two eggs, cooked in any style	...	2.11	2.53	14.4	15.8	....	202.8
Two slices bread (30 gm. each)	...	1.47	0.880	0.8	5.5	31.9	160.8
Butter, 20 gm.	...	0.16	0.032	17.0	0.2	....	159
Tea, 180 c.c.	180	...	....	....	....	....	.....
Sugar, 1 teaspoonful 200 c.c.	...	...	.....	....	....	5	20
Milk, 20 c.c.	20	0.528	0.105	0.8	0.6	1	14.4
Fruit stewed or fresh—1 portion	...	...	.....	....	....	10	40
Water, 300 c.c.	300	...	.....	....	....	....	.....
TOTAL	1760	...	13.736	89.5	85.4	260.8	2295.3

Remarks.—The following outline shows the normal standard for the Mosenthal Nephritic Test Meal.

Maximum specific gravity	1.020 or plus
Degrees variation of specific gravity, usually 9 or plus	
Specific gravity of night urine	Of no significance
Volume of night urine	750 c.c. or less
N and NaCl per cent in night urine, or highest per cent in any specimen.	Normal if 1 per cent or higher, not necessarily abnormal if less.

Mosenthal has recently suggested (Medical Clinics of North America, 4,209, 1920), a modification of this test, in which the special diet is eliminated.

McLean Index of Kidney Function

(McLean: Jour. Am. Med. Assn., 1916, lxvi, 415)

Principle.—The McLean Index expresses the relationship between the concentration of urea in the blood and the rate of urea excretion by the kidney.

It is based upon the two laws of Ambard that in normal cases: (1) when the concentration of the urea in the urine is constant the quantity of urea excreted varies proportionately as the square of the concentration of the urea in the blood; i.e., if the quantity of urea in the blood is doubled, the amount excreted in a given time is quadrupled, and (2) when the concentration of the urea in the blood remains constant, the quantity excreted in the urine varies inversely as the square root of the concentration in the urine, i.e., a quadrupling of the concentration results in a halving of the rate of the output.

The ideal normal rate of excretion is taken as 100. The index expresses in direct percentage the rate of urea excretion found as compared with the rate of excretion of a normal individual under the same condition as to concentration in the blood, concentration in the urine and body weight.

Procedure.—The patient is given a glass of water to insure a free flow of urine. The bladder is emptied (by catheter if necessary) and the time is noted to within one minute. One hour later 5 to 10 c.c. of blood is withdrawn and prevented from clotting by the addition of 5 drops of potassium oxalate solution. At the end of two hours from the time of voiding, the bladder is

again emptied and the entire second voiding, 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.

Make a quantitative estimation of the urea in the blood and the urea in the urine.

Calculation.—The formula for the index of urea excretion follows:

$$\text{Index of urea excretion (1)} = \frac{D \sqrt{C} \times 8.96}{\text{Wt.} \times \text{Ur}_2}$$

D = Grams of urea excreted per twenty-four hours (calculated from the above two hour period).

C = Grams of urea per liter of urine.

Ur = Grams of urea per liter of blood.

Wt = Body weight of individual in kilograms.

Remarks.—Usually the urea index (1) is 100 to 200. Variations between 80 and 300 are not infrequently observed in normal individuals. An index below 50 indicates a considerable degree of impairment of renal function.

Phenolsulphonephthalein Test for Kidney Function

(Rowntree and Geraghty)

Principle.—A dyestuff, phenolsulphonephthalein, which is eliminated rapidly by the kidneys, is injected. The urine is collected over a fixed period of time and the amount of dyestuff it contains determined colorimetrically.

Procedure.—A few minutes before the test is begun the patient is given a glass of water to insure a free flow of urine. The patient then empties the bladder and is given intravenously, or intramuscularly in the deltoid or lumbar muscles, 1 c.c. of sterile phenosulphonephthalein solution, containing 6 mg. of the drug. The patient voids exactly one hour and ten minutes later and again two hours and ten minutes from the time of injection. The two specimens are kept separate.

Determination of Amount of Phthalein Excreted.—Each specimen is measured, rendered alkaline with 40 per cent sodium

hydroxide solution and then diluted to 1,000 c.c. with water. The amount of dye in the solution so diluted is determined in a Dunning colorimeter (Fig. 24) by comparison with tubes of known concentration, or in a Hellige colorimeter (Fig. 34) using a standard solution containing 6 mg. of phthalein per liter.

If a Hellige colorimeter is used it should be calibrated as described on page 98.

Remarks.—The normal excretion of phthalein after intramus-

Fig. 24.—Dunning colorimeter used in phenolsulphonephthalein test of kidney function.

Fig. 25.—Record syringe for injecting phenolsulphonephthalein solution.

cular injection is 50 to 70 per cent in two hours; after intravenous injection 60 to 80 per cent is excreted in two hours.

The results after intramuscular injection are unreliable if there be present any condition which will interfere with absorption, such as edema.

It is very necessary that the phthalein injected be accurately measured. The injection is best made with a calibrated 2 c.c. Record syringe (Fig. 25).

The following form is convenient in keeping the record of the test:

Phenolsulphonephthalein Test

Be sure the patient empties the bladder.
Be sure to save the entire amount of urine voided.
Patient's Name ..... Case No. .....
Date.....
Bladder emptied at .....
Amount of urine obtained ..... c.c.
1 c.c. phenolsulphonephthalein injected (intravenously)
(into buttocks), at .....
Note: Amount injected must be accurately measured)
1 glass of water taken at .....
(This should follow the injection immediately)
Injected by .....
1st collection of urine at ..... empty bladder.
Amount of urine collected ..... c.c.
(This collection should be made 1 hour and 10 minutes after injection of phenolsulphonephthalein.)
2nd glass of water taken at .....
(This should follow first collection)
2nd collection of urine at ..... empty bladder
Amount of urine collected.....c.c.

KEEP SPECIMENS SEPARATE

Urine lost .....
(Note amount, when, how lost, etc.)
Collected by .....

Alkali Tolerance Test

(Peabody: Arch. Int. Med., 1915, xvi, 958. Palmer and Van Slyke: Jour. Biol. Chem., 1917, xxxii, 499)

Principle.—Sodium bicarbonate is administered by mouth in small amounts until the reaction of the urine reaches that of the blood ($P_H = 7.4$).

Procedure.—Give two grams of sodium bicarbonate in 100 c.c. water every half hour, and at the same time collect a specimen of urine. Record the number of grams of sodium bicarbonate taken before the urine (examined fresh) shows a H-ion concentration of 7.4 (page 36).

Remarks.—The high normal limit of sodium bicarbonate is 10 grams. If no acidosis is indicated by the test, its absence can be accepted; but if acidosis is indicated, the finding must be confirmed by blood analysis as there may be an alkali retention due to the inability of the kidney to excrete alkali.

Instead of determining the hydrogen-ion concentration, litmus paper may be used to roughly indicate the reaction of the urine.

Determination of Urobilin and Urobilinogen

(Wilbur, R. L., and Addis, Thos.: Arch. Int. Med. 1913, xiii, 235)

Collection of Specimen.—Collect total urine for 24 hours in dark brown bottle and keep in darkness. Add a few thymol crystals as a preservative.

Procedure.—Measure urine. Mix 10 c.c. of urine with 10 c.c. of absolute alcohol and one gram of zinc acetate and filter. Ten c.c. of the filtrate are taken and 1 c.c. of Ehrlich's solution, (paradi-methyl-amido-benzaldehyd, 20 grams; concentrated hydrochloric acid, 150 c.c.; water 150 c.c.), is added. Keep in dark for one hour. Transfer filtrate to a spectroscope cell 1 cm. in

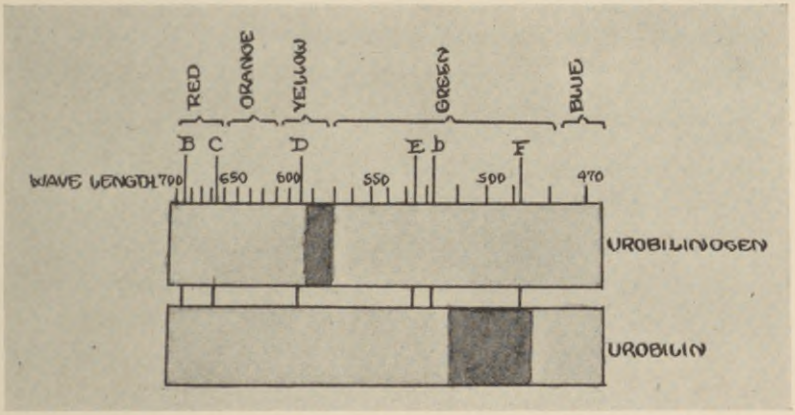


Fig. 26-*A*.—Spectrum of urobilin and urobilinogen.

thickness. Examine the solution with the spectroscope for the characteristic absorption bands of urobilin and urobilinogen.

The presence of urobilin is marked by a broad band in the blue end of the spectrum. The violet rays are completely absorbed, and if there is much urobilin present nearly all of the green may be obliterated. Urobilinogen absorbs a narrow portion of the spectrum in the yellow at the edge of the green (Fig. 26-A), and if present in large amount the band may be broad enough to obliterate the entire yellow portion of the spectrum. It is located by its proximity to the "D" line while urobilin extends from between "b" and "F" lines to the violet end of the visible spectrum.

Dilute the solution with tap water until the absorption disappears with full light but can be made out faintly when the spectroscope slit is narrowed to just half of its former opening. The urobilin and urobilinogen bands differ in their intensity, consequently the disappearance of the absorption bands will occur with different dilutions.

Calculation.—The number of dilutions required to obliterate the urobilinogen band plus the number required to obliterate that of urobilin gives the dilution value for 5 c.c. of urine. Multiply the sum of these two by the number of 5 c.c. quantities in the 24 hour time. This gives the number of dilutions which would have been necessary if all the urobilin and urobilinogen had been concentrated in a volume of 5 c.c. of urine.

Remarks.—With this method a positive result in the 24 hour specimens of urine indicates an abnormal increase over the amount usually present.

Instead of diluting the specimen until the bands disappear, a Hellige colorimeter with spectroscope attachment, may be employed. (Boyd, J. D., Jour. Lab. and Clin. Med., 1919, iv. 495.)

CHAPTER III

ANALYSIS OF GASTRIC JUICE

The Test Meal

The patient is given a glass of water, and two arrowroot biscuits or a slice of bread. A small stomach tube is passed and specimens of the gastric contents removed by aspiration at thirty minute intervals over a period of two hours.

Routine Examination

1. Note the number of minutes which elapse between the time of ingestion of the test meal and the removal of the specimen.
2. Measure each specimen.
3. Note the gross appearance. Look for blood, mucus, bile, and retained food such as raisins.
4. Determine the amount of free and total acid, or acid deficit on each specimen, and do guaiac test on pooled specimens.
5. Examine microscopically the sediment of the pooled specimens.

Chemical Examination of the Gastric Contents

A. **Reagents.**—

1. Gunzburg's Reagent.—

Phloroglucin	2.0 gm.
Vanillin	1.0 gm.
Alcohol, absolute	30.0 c.c.

Dissolve and keep in a brown bottle. The reagent deteriorates in a few months.

2. Topfer's Reagent.—Dissolve 0.5 gm. of dimethylaminoazobenzol in 100 c.c. of 95 per cent alcohol.

B. **Qualitative Test for Free Hydrochloric Acid.**—A few drops of Gunzburg's reagent are evaporated to dryness by warming gently over a Bunsen burner. A drop of gastric contents is

brought in contact with the yellowish-brown stain left and is evaporated. If free hydrochloric acid is present, an intense red color develops where the reagent and gastric juice have mixed. Organic acids do not give the test.

C. **Titration of Free Hydrochloric Acid.**—Add one drop of Topfer's reagent to 10 c.c. of clear gastric juice placed in a porcelain dish. In the presence of free hydrochloric acid an intense red color develops. Run in N/10 sodium hydroxide until the red color changes to a pure yellow.

Calculation.—The number of cubic centimeters of tenth normal alkali required to neutralize the acid multiplied by 10 gives the "acidity per cent" of free hydrochloric acid.

D. **Titration of Total Acidity.**—Add one drop of phenolphthalein to 10 c.c. of clear gastric contents. Run in N/10 sodium hydroxide until the whole mixture takes on a permanent faint pink color.

Calculation.—The number of cubic centimeters of alkali used, multiplied by 10, gives the total "acidity per cent."

If desired the phenolphthalein may be added to the specimen on which the free hydrochloric acid determination was made, and N/10 alkali be added until the pink color develops. The amount of sodium hydroxide used plus that required for the neutralization of the free hydrochloric acid multiplied by 10 gives the total "acidity per cent."

E. **Determination of Hydrochloric Acid Deficit.**—If the free hydrochloric acid is absent as shown by the test with Gunzburg's reagent, determine the amount of hydrochloric acid it is necessary to add until a reaction for free acid is obtained.

Add N/10 hydrochloric acid to 10 c.c. of clear gastric contents until free hydrochloric acid is present as shown by testing with Congo-red paper. In the presence of free hydrochloric acid the paper turns blue when a small drop of the gastric juice is placed on it.

Calculation.—The number of cubic centimeters of N/10 HCl used multiplied by 10 gives the "deficit per cent."

F. **Qualitative Test for Lactic Acid.**—To 5 to 10 c.c. of gastric juice in a test tube, add a few drops of dilute hydrochloric acid, and about 10 c.c. of ether. Shake carefully and decant ether into

another test tube. Add to the ether a small amount of 0.2 per cent ferric chloride solution. A yellow color develops in the presence of lactic acid.

G. **Guaiac Test for Occult Blood.**—Make 5 c.c. of stomach juice strongly acid with acetic acid. Layer with a mixture of saturated alcoholic solution of gum guaiac and 3 per cent hydrogen peroxide. A blue color develops at the line of contact in the presence of blood.

In a fractional gastric analysis the guaiac test is done on a sample from the pooled specimen.

Microscopic Examination of the Gastric Contents

Place a drop of the sediment on a slide, cover with a cover glass and examine for:

(a) Starch granules. These are normally present in moderate numbers. They turn blue on the addition of an iodine solution.

(b) Red blood cells.

(c) Pus cells.

(d) Yeast cells, which are oval and may be distinguished by the budding which some cells show.

(e) Sarcinae, which may be recognized by the typical bundle arrangement of the cocci.

(f) Opper-Boas bacilli. These are large, long, gram-positive rods occurring in chains. They are found only in the absence of free hydrochloric acid.

In a fractional gastric analysis the microscopic examination is made on the sediment from the pooled specimens.

Determination of Urobilin and Urobilinogen in Duodenal Contents

(Giffen, Sandford and Szlapka: Am. Jour. Med. Sc., 1918, clv, 562)

Collection of Specimen.—Duodenal contents are collected by means of an Einhorn duodenal tube. The fluid should be faintly alkaline, clear and light yellow to chocolate brown in color.

Reagents.—

(1) Schlesinger's Solution.—A saturated solution of zinc acetate in absolute alcohol.

(2) Ehrlich's Reagent.—

Paradimethylaminobenzaldehyde,	4 gms.
Hydrochloric acid	30 c.c.
Distilled water	30 c.c.

Procedure.—To 10 c.c. of duodenal contents add an equal amount of Schlesinger's solution. Shake thoroughly. Filter through a single layer of coarse filter paper. To 10 c.c. of the filtrate add 1 c.c. of Ehrlich's reagent. Set in dark for fifteen minutes.

Fill the solution into a spectrum cell with parallel sides of such dimensions that the path of the rays of light in passing through the fluid are exactly 1 cm. Examine in spectroscope.

The presence of urobilin is marked by a broad band in the blue end of the spectrum (Fig. 26-*A*). The violet rays are completely absorbed, and if there is much urobilin present the entire blue portion and nearly all the green may be obliterated. Urobilinogen absorbs a narrow portion of the spectrum in the yellow at the edge of the green, and if present in large amounts the band may be broad enough to obliterate the entire yellow portion of the spectrum. It is located by its proximity to the "D" Fraunhofer line while urobilin extends from between the "b" and "F" lines to the violet end of the visible spectrum.

Dilute the solution with 60 per cent alcohol until the absorption bands disappear but can be made to reappear faintly when the slit is narrowed to just half its former opening. The urobilin and urobilinogen bands will disappear with different dilutions.

The amount of urobilin and urobilinogen is estimated according to the Wilbur and Addis method for 1,000 c.c. by multiplying the number of dilutions by 200. The number of units of urobilin and urobilinogen are added together and the total number of units reported.

Remarks.—The duodenal contents normally contain 500 to 1000 units of urobilin and urobilinogen.

CHAPTER IV

EXAMINATION OF SPUTUM

Collection of Specimen.—The patient should be directed to cough up sputum from the deeper bronchi, and expectorate this into a sterile container. Specimens can usually be best obtained on awakening in the morning. Saliva and pharyngeal material are worthless for examination.

Routine Examination

1. Note gross appearance: (a) mucoid; (b) mucopurulent; (c) purulent; (d) serous; (e) bloody.
2. Examine preparation of fresh sputum.
3. Wash sputum and make culture on blood agar plate. (See page 203.)
4. Make a smear from a bit of the washed sputum and stain by gram.
5. Make film preparation and stain for tubercle bacilli.

Examination of Fresh Sputum

Transfer a portion of the fresh specimen of sputum to glass plate about the size of a microscope stage, and cover with a second smaller glass plate. Examine under microscope with the low power objective.

Note in the preparation:

(a) Elastic fibers, which are usually in the small greyish yellow particles. They are very refractive and have curling ends.

(b) Moulds. These may be recognized by the branching mycelium and the spores.

(c) Pus cells.

(d) Eosinophilic leucocytes. These are distinguished by the large granules in the protoplasm which stain red with eosin.

(e) Alveolar epithelial cells, which are 4 to 5 times the size of a leucocyte, are oval, their protoplasm coarsely granular, and

with one or several large oval, vesicular nuclei. They may contain large brown granules of blood pigment.

(f) Charcot-Leyden crystals. These are long, narrow diamond shaped crystals which resemble two very sharp pyramids with their bases together. They have a slight yellowish refractivity.

(g) Curschman's spirals, which are spirally twisted strands of mucus enclosing many pus cells and Charcot-Leyden crystals.

Examination for Tubercle Bacilli

1. Ziehl-Neelsen Method.—Pour the sputum into a Petri dish and select for examination the yellowish particles of pus. Spread a small particle in a thin film on a clean glass slide. Dry in air, and fix by passing through flame. Cover with carbolfuchsin (see page 196) and steam on hot plate for two minutes. Wash under tap. Decolorize in acid alcohol (2 per cent hydrochloric acid in 80 per cent alcohol) until the thicker parts show only a faint pink color. Counterstain with Loeffler's methylene blue. (See page 196.)

2. Antiformin Method.—Put total specimen of sputum in 50 c.c. centrifuge tube, dilute with distilled water, and add antiformin until the preparation contains 20 per cent. Stir well, warm over flame, and leave in incubator for one hour. Dilute well with distilled water. Centrifuge for thirty minutes. Mix sediment with one drop of blood serum, make slide preparation and stain as directed above.

Examination for Elastic Tissue

Boil the sputum with 20 per cent NaOH solution until it is homogeneous, centrifugalize, and examine the sediment pressed out on a glass plate as directed in the examination of fresh sputum.

The fibers of elastic tissue are characterized by their intense refractivity, wavy outline, sharp edges, uniform diameter and curling ends.

CHAPTER V

EXAMINATION OF FECES

Routine Examination

1. Note gross appearance; color, presence of blood, or mucus, or remnants of food.

2. Test for occult blood with benzidine.

3. Make a microscopic examination of a portion of stool emulsified in water.

Qualitative Tests

1. Occult Blood

(a) **Benzidine Test.**—Take up a bit of powdered benzidine on the tip of a toothpick and dissolve in 2 c.c. of glacial acetic acid. Add 20 drops of 3 per cent hydrogen peroxide.

Smear a small portion of the stool on a white card with a toothpick; add a drop of the benzidine suspension and mix thoroughly. If blood is present the mixture turns blue.

An atypical or doubtful reaction should be checked up with the guaiac test.

(b) **Guaiac Test.**—Emulsify a portion of the specimen of stool in a test tube with glacial acetic acid. Extract with a few cubic centimeters of ether. Layer the ether extract in a test tube on a mixture of equal parts alcoholic solution of gum guaiac (1 gram in 60 c.c. 95 per cent alcohol) and 3 per cent hydrogen peroxide. A blue color develops in the ether if blood is present.

2. Bilirubin and Hydrobilirubin

Schmidt Test.—Emulsify a bit of stool in a porcelain dish with a saturated solution of mercuric chloride. Allow to stand for 24 hours. If hydrobilirubin is present the specimen becomes pink. Bilirubin causes a green color.

Examination for Large Parasites, Gallstones, and Other Foreign Bodies

Mix the specimen of stool with a large amount of water, stir well, and filter through a fine mesh wire sieve.

Microscopic Examination

Place a drop of water on a slide and mix with it bits of the stool from different portions of the specimen. Cover with a cover

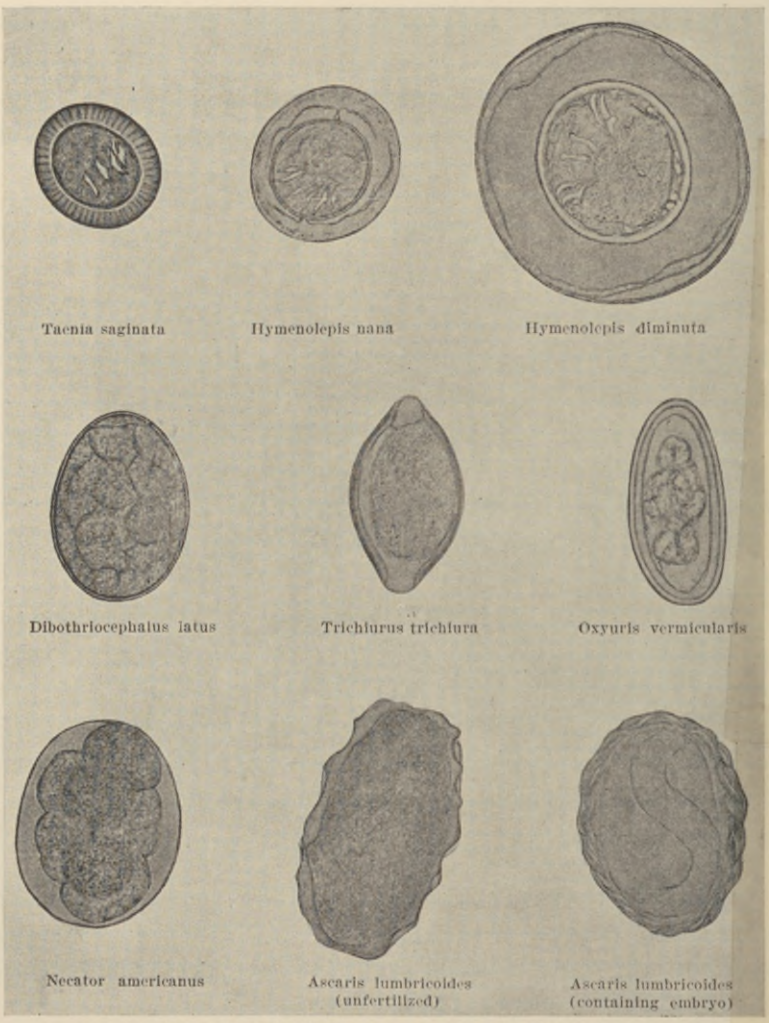


Fig. 26-*B*.—Ova in human feces. (*After Barker.*)

Table X

Classification of Ova of the Commoner Intestinal Parasites

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	35x25	Tenia saginata
		Thick and transparent		30–40	Dipylidium caninum
	Three transparent membranes			68	Hymenolepis nana
An operculum: Ova brown				75x45	Dibothricephalus latus

II. Classification of Ova of Nemathelminthes

Ova with a single envelope	Wall smooth	Thick	Bulging on one side, flat on the other	50x23	Oxyuris vermicularis
			Clear plug at each pole	55x25	Trichocephalus trichiuris
		Transparent	2 to 4 blastomeres	60x40	Ankylostomum duodenale
			2 to 4 blastomeres	70x40	Necator americanus
			Thick embryo, folded in two	54x32	Strongyloides intestinalis
	Wall ornamented		Mamillated, brown	60x44	Ascaris lumbricoides
			Regularly cribbed with depressions, yellow	75x65	Ascaris canis

(Abbreviated from Medical War Manual No. 6.)

glass and examine under the lower power of the microscope for (1) Pus cells; (2) Red blood cells; (3) An excess of undigested muscle fibers; (4) Parasites; (5) Ova.

The ova in the stool, if present, may be concentrated by the following procedure (Kofoid and Barber): Emulsify the specimens with a saturated salt solution. Make a filter ⅛ to ¼ inch in thickness of No. 0 or No. 1 long fiber steel wool and pour the emulsion through it.

Allow the preparation to stand one hour. Loop off the surface, film to an ordinary gloss, slide and examine under the low power of the microscope. If amebae are suspected, examine as indicated under "Examination of Stool for Amebae."

Table X summarizes the characteristics of the ova of the commoner intestinal parasites. The ova commonly found in the stool are illustrated by Fig. 26B.

Determination of Reaction

(Bruce, W. J.: Jour. Lab. and Clin. Med., 1920, v, 61)

Prepare a 1 per cent aqueous solution of alizarin. Place two small drops of the indicator on a white index card 1½ inches apart. Dip a toothpick into the liquid part of the specimen (or if the feces are formed, merely puncture the mass). Mix thoroughly in one of the drops, using the other drop as a control. An alkaline reaction is indicated by a reddish violet color, neutral, no change, an acid a light yellow color. The density of the colors will depend upon the amount of acid or alkali present.

Examination of Stool for Amebae

(From "Parasitic Amebae of Man," Craig)

A very small portion of a freshly passed stool should be placed upon a microscopic slide and covered with a cover glass, gentle pressure being used to spread the specimen. The material selected for examination should preferably be a drop of the liquid portion of the stool rather than solid particles. It is always well to give a saline cathartic before making an examination as this tends to wash the amebae from the intestinal walls. If present, a particle of mucus or any blood-stained material should be ex-

amined as well. Most stools from amebic dysentery cases contain gelatinous material which frequently contains numerous amebae, and such material should always be fully examined.

The feces should be examined as quickly as possible after they have been passed as the amebae are much more easily recognized when they are motile. No disinfectant should be used in stools which are to be examined for amebae, neither should urine be mixed with the stools. In temperate regions, especially in the winter, the receptacle used in collecting the specimen should be warmed, but care should be taken if water is used for this purpose, that it be boiled, as otherwise water amebae might be mistaken for parasitic amebae, having reached the feces in this manner.

In making fresh preparations it is always well to dilute a loopful of the stool with normal salt solution or distilled water. One of the most frequent mistakes made in examining such preparations is the use of too thick a preparation. One should not be satisfied with the examination of a single slide, but should thoroughly examine at least six or eight preparations before a negative result is reported.

The hanging drop method is often valuable in the examination of these organisms, a small drop of the stool being placed in the center of a cover glass which is then inverted upon a hollow ground slide and ringed with vaseline. If a film preparation is used it should always be ringed with vaseline, for unless this is done evaporation occurs and the preparation becomes useless.

The preparation should be examined with a one-sixth inch lens and a one- or two-inch eyepiece. For the finer details regarding the structure of the cytoplasm and the nucleus, as well as the reproductive changes, it is necessary to use the one-twelfth inch oil immersion objective. Table XI summarizes the important differential features of Entameba Coli and Entameba Histolytica.

Neutral Red.—The use of a solution of $\frac{1}{10,000}$ of neutral red is often of great service in those cases in which the amebae are few in number and for the study of structural details. This solution is very quickly absorbed by the amebae, coloring them pink or red, and does not interfere with their movements if it is not used in too strong a dilution. It is a most useful method in

TABLE XI

DIFFERENTIAL FEATURES OF ENTAMEBA COLI AND ENTAMEBA HISTOLYTICA

	ENTAMEBA COLI-SCHAUDINN, 1903	ENTAMEBA HISTOLYTICA-SCHAUDINN, 1903
SIZE:	15 to 20 microns. Average diam. 25-35 microns. Generally smaller than entameba histolytica.	10 to 70 microns. Generally from 15 to 40 microns.
PSEUDOPODIA:	Small, blunt and not clearly differentiated from rest of parasite.	Blunt or slender and finger-shaped. Very refractive and clearly differentiated from rest of the parasite.
MOTILITY:	Sluggish, less progressive and more indefinite in direction as compared with entameba histolytica.	Active.
CYTOPLASM:	Ectoplasm not distinct except when moving and then only because it is free from granules. Is grayish in color and not very refractive. Endoplasm is gray, finely granular, few non-contractile vacuoles. Is not generally phagocytic for red blood corpuscles but may contain bacteria and crystals.	Ectoplasm is very distinct and refractive, in some instances even when motionless. Glassy appearing. Endoplasm is granular, contains numerous non-contractile vacuoles and red blood corpuscles when latter are present in the feces.
NUCLEUS:	Distinct, having a well defined nuclear membrane, much chromatin and large karyosome.	In acute dysentery the nucleus is usually more or less indistinct; has no well defined nuclear membrane; contains but little chromatin and has a minute karyosome. In chronic dysentery the nucleus is distinct, has thick nuclear membrane formed by chromatin, large karyosome and clear area surrounding the ventricle.
CYST FORMATION:	Present. Cysts have eight nuclei, are 16-25 microns in diam., have thick wall, no chromidial bodies and are more refractive than those of entameba histolytica.	Present. Cysts contain four nuclei. Measure from 11-14 microns, are covered with a thin membrane, are less refractive than those of entameba coli and contain chromidial bodies. Four amebae develop within cyst.

TABLE XI (Continued)

DIFFERENTIAL FEATURES OF ENTAMEBA COLI AND ENTAMEBA HISTOLYTICA

	ENTAMEBA COLI-SCHAUDINN, 1903	ENTAMEBA HISTOLYTICA-SCHAUDINN, 1903
CULTIVATION:	Doubtful.	Negative.
METHODS OF REPRODUCTION:	By simple division, autogamous sexual reproduction in cyst and by schizogony with the production of eight daughter amebae. Eight amebae are produced within the cyst.	By simple division and by autogamous sexual reproduction within cyst, four amebae being formed.
PATHOGENESIS:	Is not pathogenic, occurring in a large percentage of healthy individuals and in patients suffering from diseases other than dysentery.	Is the cause of a form of amebic dysentery.

distinguishing between parasitic amebae and leucocytes, as the latter do not stain with this substance. In specimens in which the amebae are in scant numbers they are easily distinguished by the reddish color given them by the neutral red, as other cells occurring in feces are not colored distinctly by this dye. The dilution should be made with normal salt solution.

Quantitative Estimation of Urobilin and Urobilinogen in Stools

(Wilbur, R. L., and Addis, Thos.: Arch. Int. Med., 1914, xiii, 235)

Collection of Specimen.—All the feces passed in 24 hours are collected in the same receiver and kept in the dark.

Procedure.—The total stool is then washed into a large graduate and thoroughly ground up with water into a homogeneous paste and water added to 0.5, 1 or 2 liters, depending upon the size of the stool.

After thoroughly mixing, 25 c.c. are taken and 75 c.c. of acid alcohol (95 per cent alcohol, 1600 c.c., concentrated hydrochloric acid, 25 c.c. and water 800 c.c.) are added. Put mixture in a shaker for about one half an hour. To 10 c.c. add an equal quantity of absolute alcohol and 1 gram of zinc acetate. Filter. Add 1 c.c. of Ehrlich's reagent (page 19) to 10 c.c. of the filtrate and put aside in a dark place until the next day. Transfer to a

spectroscope cell 1 cm. in thickness and examine with spectroscope for the characteristic absorption bands of urobilin and urobilinogen.

The presence of urobilin is marked by a broad band (Fig. 26-*A*) in the blue end of the spectrum. The violet rays are completely absorbed, and if there is much urobilin present the entire blue portion and nearly all of the green may be obliterated. Urobilinogen absorbs a narrow portion of the spectrum in the yellow at the edge of the green, and if present in large amounts the band may be broad enough to obliterate the entire yellow portion of the spectrum. It is located by its proximity to the "D" line while urobilin extends from between "b" and "F" lines to the violet end of the visible spectrum.

Dilute with 60 per cent alcohol until the absorption bands disappear with dull light but reappear when the spectroscope slit is narrowed to one just half of its former opening.

The urobilin and urobilinogen bands differ in their intensity, consequently the disappearance of the absorption bands will occur with different dilutions.

Calculation.—The total amount of urobilin and urobilinogen in terms of dilutions equals:

$$\frac{V}{5} \times 8 \times (R \text{ plus } S)$$

where

V equals the volume to which the stool was diluted;

R equals dilutions required to obliterate urobilin band in the 10 c.c. of filtrate, and

S equals dilutions required to obliterate urobilinogen band.

Remarks.—Normally the total 24-hour specimen of stool does not contain more than 6500 dilutions of urobilin and urobilinogen.

Preservation of Intestinal Parasites

Wash carefully in salt solution and place in 2 per cent formalin.

If it is desired to clear the specimen after fixation in 2 per cent formalin for 14 to 16 hours, it is then placed in the following solution until clear:

Glucose	480 grams
Water	520 c.c.
Methyl alcohol	200 c.c.
Glycerine	100 c.c.
Camphor (q.s. to keep)	

After the specimen is cleared sufficient quantity of glycerine jelly is dropped on a slide and the specimen transferred to it. Cover with a cover glass and allow the jelly to harden.

The glycerine jelly is made by melting 14 grams of the best Gold Mark gelatine in 120 c.c. of hot water and adding 120 c.c. glycerine. This is then cooled to 50° C.

The carefully separated whites of two eggs are then added and the fluid heated gently without stirring. This is filtered, the volume made up by adding water to 240 c.c. and 1 c.c. of pure carbolic acid is added. This jelly is solid at ordinary temperature but is easily melted under the hot water tap.

Preservation of Stools Containing Ova

The stools are diluted to a soup-like consistency, and one-tenth volume of formalin is added.

CHAPTER VI

QUALITATIVE EXAMINATION OF BLOOD

Counting the Blood Cells

I. **Reagents.**—(1) Hayem's Solution.—

Mercuric chloride	0.25 gram
Sodium chloride	0.5 gram
Sodium sulphate	2.5 grams
Distilled water	100 c.c.

(2) Turck's Solution.—One and one-half per cent acetic acid. Color with gentian violet.

(3) Blood Platelet Solutions:

Solution I:

"Brilliant cresyl blue"	1.0 gram
Distilled water	300.0 c.c.

Dissolve. Keep on ice to prevent the growth of yeast.

Solution II:

Potassium cyanide	1.0 gram
Distilled water	1400.0 c.c.

This solution should be made up every ten days.

II. **The Diluting Pipettes.**—Each pipette (Fig. 27) consists of a capillary tube which opens into a bulb containing a glass pearl.

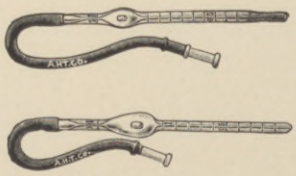


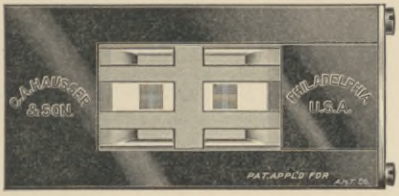
Fig. 27.—Blood counting pipettes for red and white corpuscles.

The capillary tube is divided into ten equal parts. In the red pipette the bulb when filled to the line on its upper outlet (marked

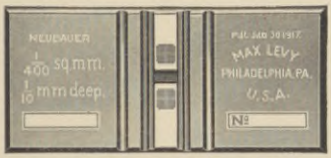
101) holds one hundred times the contents of the ten divisions of the capillary tube.

The bulb of the white pipette contains ten times that of the capillary tube.

III. **The Counting Chamber.**—A counting chamber (Fig. 28) of the Burker type with double Neubauer ruling is used. On this instrument there are two ruled areas three millimeters on a side or nine millimeters square. Each area (Fig. 29) is divided into nine large squares, each of which is one millimeter on a side or one square millimeter. The central square millimeter which is used for counting the red blood cells is subdivided into 400



A.



B.

Fig. 28.—*A.*—Hausser counting chamber of Burker type with double Neubauer ruling. The glass parts are in one piece which is mounted in a Bakelite holder. This is by far the most desirable type of instrument to buy.

B.—Levy counting chamber of Burker type with Neubauer ruling. The ruled area is cemented on to the slide which is made of glass.

small squares, each of which is $\frac{1}{20}$ millimeter on a side, and has therefore an area of $\frac{1}{400}$ square millimeter. By means of double lines these smallest squares are grouped into blocks of twenty-five. Each of the remaining eight large squares (1 square millimeter each) is subdivided into sixteen small squares. The depth of the chamber is 0.1 millimeter.

Accurately calibrated pipettes and counting chamber are absolutely necessary if satisfactory blood counting is to be done.

It is best to use only apparatus certified as correct by the Bureau of Standards.

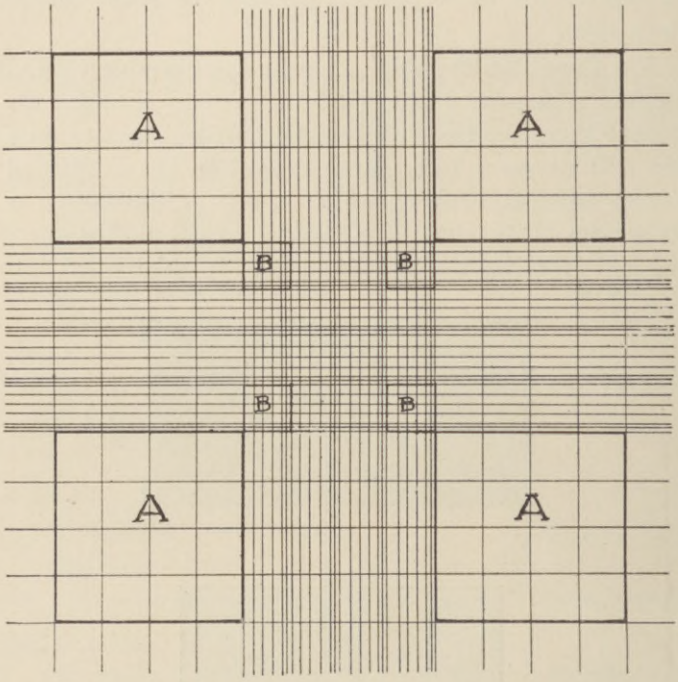


Fig. 29.—Showing the Neubauer ruling of counting chamber as it appears under the microscope. *A*, unit used in counting white corpuscles; *B*, unit used in counting red corpuscles and platelets.

Procedure in Counting the Red Blood Cells

The finger or ear is punctured with a blood lancet or with a Hagedorn needle. The blood should flow freely. The first drop is wiped away and the second used. The blood is sucked cautiously into the capillary tube of the pipette to the line marked 0.5. If the blood is accidentally sucked above the line, it may be lowered by drawing the finger across the tip of the pipette, provided the column of blood has not passed more than 1 millimeter above the line; if it has extended farther, the blood adhering to the wall of the tube will be sufficient to introduce a serious error in dilution. The end of the pipette is wiped free

of blood. Before drawing in the diluting fluid care should be taken not to expel any of the blood in the capillary tube.

The pipette is now filled to the line marked 101 with Hayem's fluid. While the dilution fluid is being drawn up, the pipette, held between the thumb and fingers, is revolved to keep the glass pearl within the bulb in motion. This mixes the blood and diluting fluid and also prevents bubbles adhering to the pearl.

Place pipette in shaking machine (Fig. 30) and shake for three minutes, or shake by hand for three minutes.

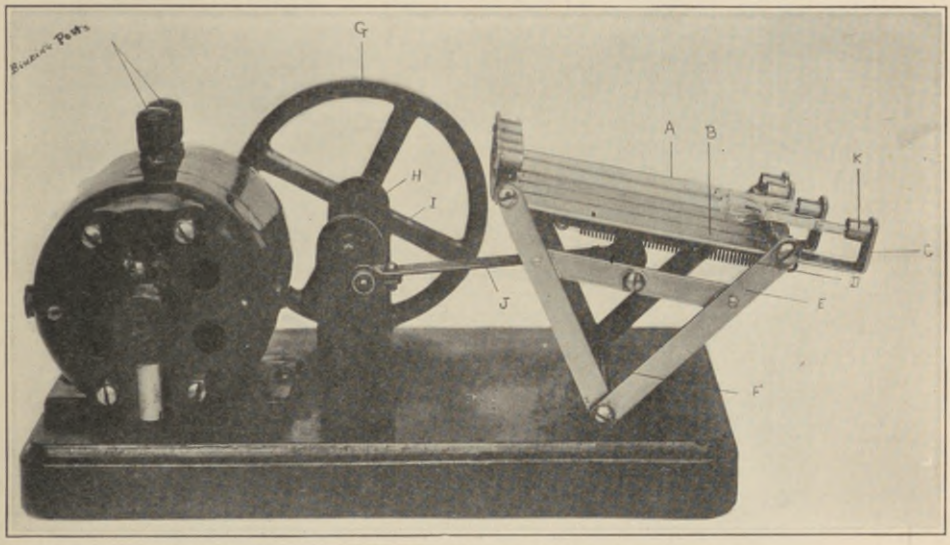


Fig. 30.—Machine for shaking blood counting pipettes.

Clean cover glass and counting chamber carefully. Discard the first drop of fluid in the pipette.

With the cover glass in position, the space between cover glass and ruled area is filled by capillary attraction, when the tip of the pipette touches the rectangular ruled strip at the edge of the cover glass.

Allow the cells to settle in the chamber. Count the cells in the four squares marked "B" on the diagram of the ruled area. (Fig. 29.)

Cells touching the upper and right boundary line are consid-

ered in the square, those touching the left and lower boundary as out of the square. The number of cells in each square should not vary more than 25 per cent of the average number per square.

Calculation.—This area counted equals 100 small squares, equals $100 \times \frac{1}{400} \times 0.1$ equals $\frac{1}{40}$ c.mm.

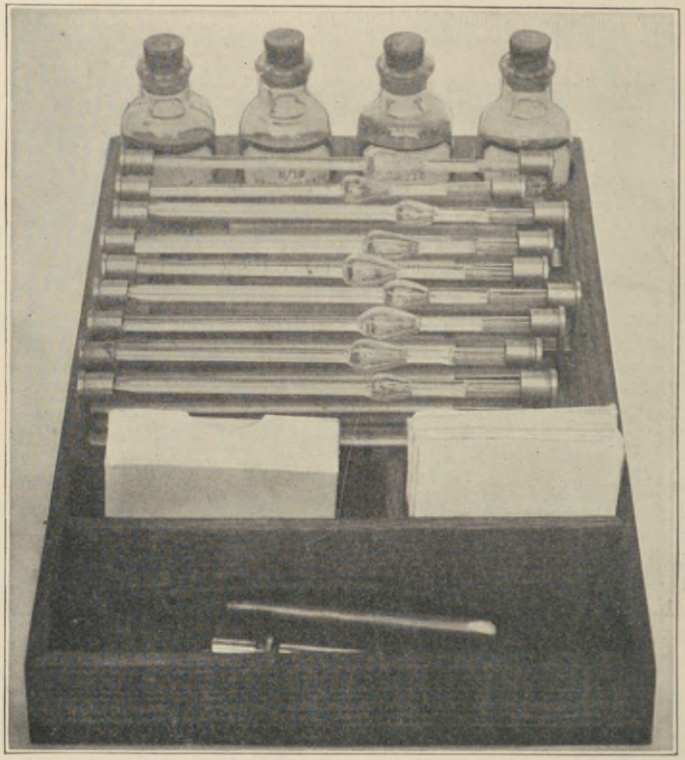


Fig. 31.—Tray for holding pipettes and materials for blood counting.

The blood was diluted 200 times. Hence the number of cells in the 100 squares counted × 40 x 200 equals number of red blood cells in 1 c.c. of undiluted blood.

Remarks.—The red blood cell count in normal men varies from 4,500,000 to 5,500,000; in women from 4,000,000 to 5,000,000.

Procedure in Counting the Leukocytes

Draw blood up to 0.5 line on white blood cell counting pipette and fill to the eleven mark with Turck's solution.

Make preparation in counting chamber as directed for red blood cells. Allow cells to settle.

Count all white cells in four squares marked "A" on the diagram of ruled area. (Fig. 29.)

Calculation.—Each square has a cubic content of 0.1 c.mm., since the side of the square is 1 mm. and the chamber is 0.1 mm. deep.

The sum of leukocytes in the four large squares divided by 4 gives the average number of cells in 0.1 c.mm. of diluted blood. To obtain the number of cells in 1 c.mm. of undiluted blood, this number is multiplied by 10 and by 20, or to put it simply, the total number of cells in the four squares counted, multiplied by 50 gives the number of white blood cells per cubic millimeter of undiluted blood.

Remarks.—The white blood cell count varies normally from 6,000 to 8,000. A count over 10,000 is a leucocytosis, a count under 5,000 is a leucopenia.

Procedure in Counting the Blood Platelets (Wright and Kinnicutt)

Draw blood up to the 1 mark on a red blood cell pipette and fill to the 101 mark with diluting fluid. The diluting fluid is made by mixing two parts of blood platelet Solution I with three parts of Solution II and filtering.

Shake the pipette well by hand or in the pipette shaking machine. Fill the counting chamber as for a red blood cell count and leave at rest for 10-15 minutes in order that the platelets may settle to the bottom.

The platelets appear as sharply outlined, round or oval or elongated, lilac-colored bodies, some of which form a part of small spheres or globules of hyalin, unstained substance. The red cells are decolorized and appear only as shadows. The nuclei of the white cells are stained a dark blue, the protoplasm light blue.

Count the platelets in 16 large squares marked "B" on diagram of ruled area, (Fig. 29) using the high power dry objective.

Calculation.—The total number of platelets in the sixteen squares multiplied by 1000 gives the number per cubic millimeter of blood.

Remarks.—The platelet count of normal adults varies between 225,000 and 350,000 per cubic millimeter, the general average being about 300,000.

Cleaning Counting Chamber and Pipettes

The counting chamber is cleaned with soap and water, using a soft cloth.

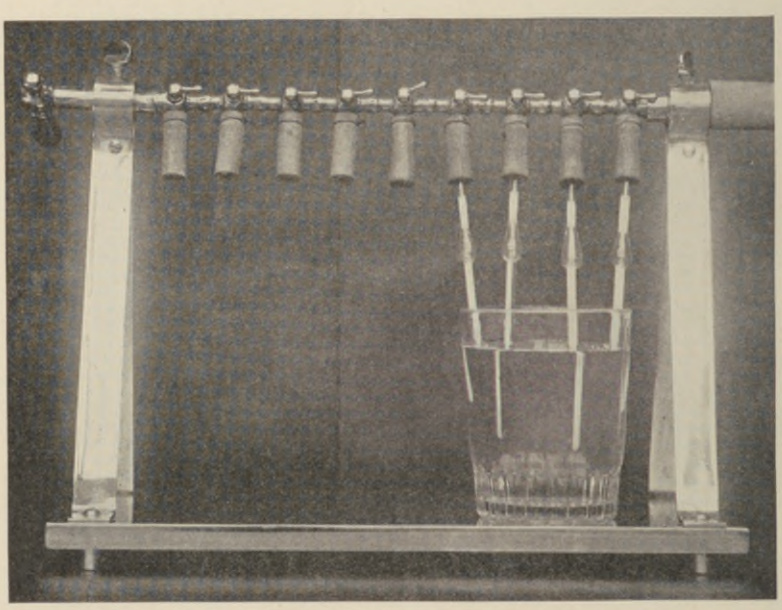


Fig. 32.—Apparatus used in cleaning blood counting pipettes. This is made from 3-way stopcocks soldered together.

The pipette is first emptied and then water, alcohol, ether and air are aspirated through in the order named (Figs. 32 and 33). If any albuminous material remains in the bulb, it may be removed by filling the pipette with the following alkaline pancreatin solution and leaving in the incubator overnight.

Sodium carbonate .....5 grams
Pancreatin .....0.5 gram
Water .....1000 c.c.
Chloroform .....a few drops

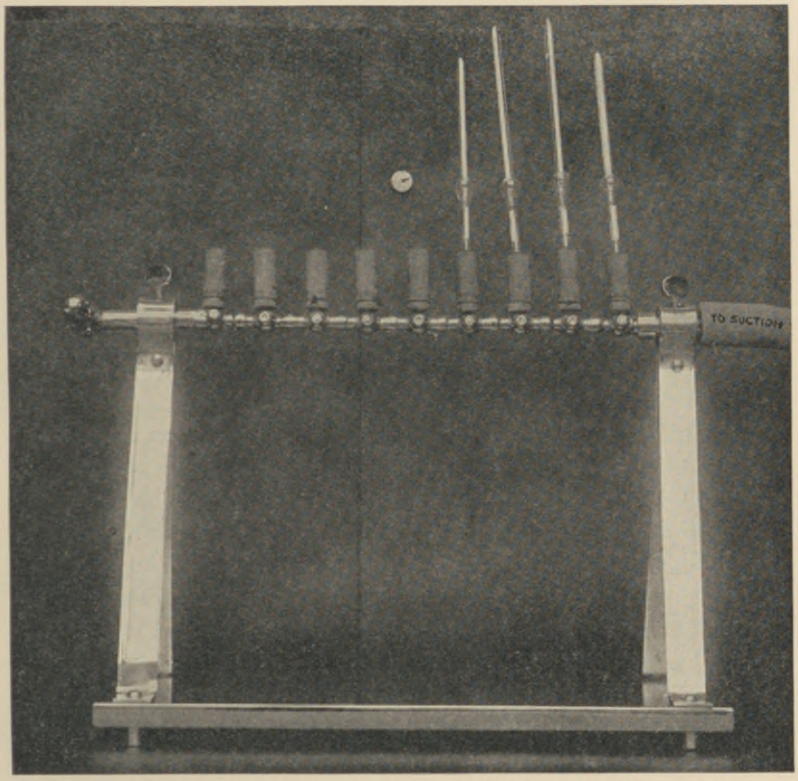


Fig. 33.—Pipette cleaning apparatus in position for emptying pipettes.

Determination of Hemoglobin

Choice of Method.—The oxygen capacity method with the Van Slyke apparatus affords the most accurate means for the estimation of hemoglobin. This method should be used in all cases in which a very exact hemoglobin determination is of value.

The acid hematin method of Sahli is probably the most valuable for routine clinical use. Good results will be obtained if the pipette is correctly graduated, the standard and comparison

tubes are of equal bore, and the standard solution is accurately made.

The acid hematin method as adapted to the Hellige colorimeter affords a procedure which is especially valuable in hospitals or wherever it is desirable to collect the specimen for the hemoglobin determination and make the reading in the laboratory. The blood is diluted and transported in an ordinary red blood counting pipette.

In all the methods given the hemoglobin reading of a normal blood containing 5 million red cells per c.mm. is taken as 100 per cent. Such a blood contains 15.6 grams of hemoglobin per 100 c.c.

Determination by the Oxygen Capacity Method

The procedure for the determination of hemoglobin by this method is given in detail on page 171.

Determination by a Method of Sahli

Standard Solution.—A stock acid hematin solution is made as described below in the method adapted to the Hellige colorimeter. A 1 per cent solution is made from the stock 10 per cent solution using equal parts of glycerine and N/10 hydrochloric acid as the diluent, and filled into the standard tube. The standard tube as supplied with the instrument is seldom accurate.

Procedure.—With a dropper run N/10 hydrochloric acid into the graduated tube to the mark 10. With the Sahli pipette draw up blood to the 20 c.mm. mark. Blow the blood into the acid in the graduated tube. The pipette is thoroughly cleaned of blood by sucking up and blowing out the acid several times. The hydrochloric acid will in a few minutes change the hemoglobin to acid hematin. It is then diluted with distilled water until its tint corresponds to that of the standard tube. The tubes should be compared with the light transmitted through the milk glass background. The hemoglobin in per cent is read off directly from the graduation on the tube.

Determination with the Hellige Colorimeter

Calibration of the Hellige Colorimeter.—The accuracy of the readings with the Hellige colorimeter (Fig. 34) will depend upon

the care with which the instrument is calibrated. The calibration is easily done as follows. With the same solution in both cup and wedge read the colorimeter scale when the color of the solution in the cup matches that of the wedge. This determines the 100 per cent mark when the unknown and standard are equally diluted. We may call this point "Y." Now raise the wedge until the bottom is just above the lower level of the aperture through which the readings are made. This determines the point on the scale equivalent to 0 per cent. This scale reading may be designated "X." Now at any point "R" on the scale

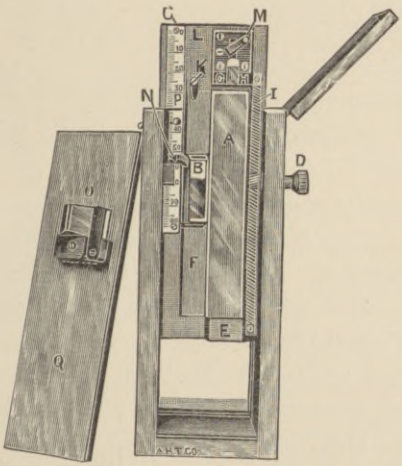


Fig. 34.—Hellige colorimeter. *A*, Wedge for standard. *B*, Specimen cup. *C*, scale. *F*, Ground glass plate. *I*, *D*, Rack and pinion. *K*, Catch for holding ground glass plate. *L*, Back slide. *M*, Setscrew for holding wedge in position. *N*, Cup holder. *O*, Helmholtz double plate. *P*, Pointer. *Q*, Front slide.

at which the colors match, the percentage of a substance in solution in the cup is equal to:

$$(R\text{-}X) \times \frac{100}{(Y\text{-}X)} \times \frac{A}{B}$$

Where R is the reading of the scale.

A is the concentration of the standard in the wedge.

B is the concentration of the substance determined.

In making the hemoglobin estimation the blood is diluted

1:100 to make a 1 per cent solution. It is convenient to so dilute the standard as to make the factor $\frac{100}{Y\text{-}X} \times \frac{A}{B}$ equal a whole number, preferably two. If X equals 5 and Y equals 85, and the blood in the standard be in the dilution of 1:62.5, or 1.6 per cent then the above factor equals $\frac{100}{80} \times \frac{1.6}{1.0}$ or 2.

A flat piece of wood about the thickness of a tongue depressor should be fitted in the bottom of the cup holder. This raises the cup on a level with the bottom of the aperture, thus enabling one to make readings with the fluid in even the smallest red cell pipette.

Reagents.—

1. Tenth normal hydrochloric acid. This may be made sufficiently accurate by diluting 11.7 c.c. of conc. HCl with distilled water to a volume of 1000 c.c.

2. Acid hematin standard. This is prepared as follows: Withdraw by venipuncture 50 c.c. of blood, carefully defibrinate by whipping and strain through gauze. Determine the hemoglobin of the defibrinated blood in the Van Slyke apparatus by the oxygen capacity method. Dilute the blood with N/10 HCl to make a 20 per cent solution of a blood containing 15.6 grams of hemoglobin, or an oxygen capacity of 20.9 volumes per cent per 100 c.c. of blood. Mix well and let stand for 24 hours. Add an equal volume of glycerine. Store in a glass stoppered bottle preferably in cool spot away from the light. This solution will keep for months. The solution represents a 10 per cent solution of a blood containing 15.6 grams of hemoglobin per 100 c.c. From this standard any desired dilution, usually 1.6 per cent, may be made, using equal parts of glycerine and N/10 HCl as the diluting fluid.

Procedure.—Draw blood accurately up to the 1 mark in a red cell counting pipette and fill to 101 with N/10 HCl. This makes a 1:100 dilution of the blood.

Allow the preparation to stand for 10 minutes or more. At least 10 minutes is required for approximate complete conversion of the hemoglobin into acid hematin. There is very little change

after this time so the reading may be made 24, or even 48 hours after dilution.

Blow the contents of the pipette into the cup of the colorimeter after discarding the first drop.

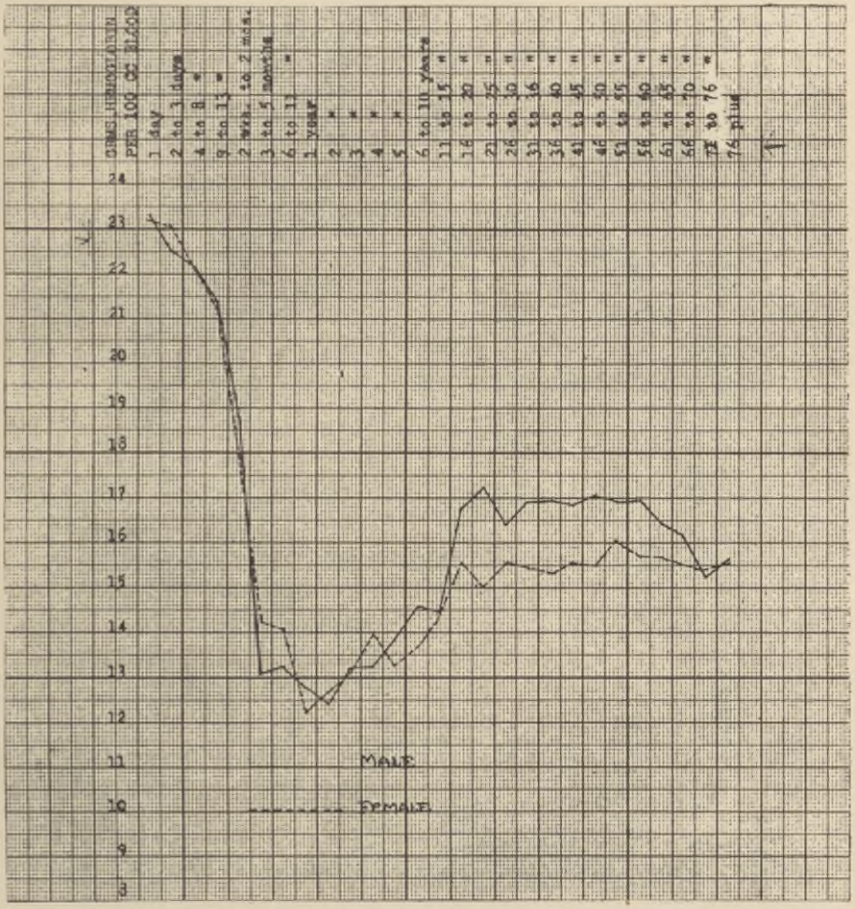


Fig. 35.—Scale showing the average amount of hemoglobin in grams per 100 c.c. blood at different ages. (*After Williamson.*)

Read the scale at the point at which the color of the unknown in the cup matches that of the wedge.

Calculation.—If the blood in the standard is so diluted as to make the factor $\frac{100}{X\text{-}Y} \times \frac{A}{B}$ equal 2, the hemoglobin corresponding

to any reading "R" on the scale is 2(R-X) per cent or $2(R\text{-}X) \times \frac{15.6}{100}$ grams per 100 c.c. blood.

Remarks.—The colorimeter cup must be kept clean by rubbing the inside surfaces with a small moist cotton swab. A small amount of acid hematin adhering to the sides will in time make a large error in the reading.

The wedge holding the standard must be shaken daily to keep the acid hematin in suspension.

It is convenient to figure a table, such as Table XII, for each colorimeter. The hemoglobin can then be read off directly in per cent or in grams per 100 c.c. blood from the scale reading.

Figure 35 shows the hemoglobin values for different ages as given by Williamson. These results are obtained with the spectrophotometer and do not take into account the red cell count. They are higher than those obtained with the Van Slyke apparatus.

TABLE XII

The Table shows the hemoglobin in per cent and in grams per 100 c.c. of blood with Hellige colorimeter No. 2312. On this instrument a scale reading of 87 equals 100, and a reading of 7 equals 0.

The blood is diluted 1:100, the standard is a 1.6 per cent solution or a dilution of 1:62.5 of a blood having a hemoglobin content of 15.6 grams per 100 c.c. Hence the hemoglobin in per cent corresponding to any point "R" on the scale is:

$$\frac{100}{87\text{-}7} \times (R\text{-}7) \times \frac{1.6}{1.0} \text{ or } 2(R\text{-}7) \text{ per cent}$$

$$\text{or } 2(R\text{-}7) \times \frac{15.6}{100} \text{ grams per 100 c.c. blood.}$$

COLORIMETER READING	PER CENT HEMOGLOBIN	GRAMS HEMOGLOBIN PER 100 C.C.
67	120	18.7
66	118	18.4
65	116	18.1
64	114	17.8
63	112	17.5
62	110	17.2
61	108	16.9
60	106	16.5
59	104	16.2
58	102	15.9
57	100	15.6
56	98	15.3

TABLE XII

COLORIMETER READING	PER CENT HEMOGLOBIN	GRAMS HEMOGLOBIN PER 100 C.C.
55	96	15.0
54	94	14.7
53	92	14.4
52	90	14.1
51	88	13.8
50	86	13.4
49	84	13.1
48	82	12.8
47	80	12.5
46	78	12.2
45	76	11.9
44	74	11.6
43	72	11.3
42	70	11.0
41	68	10.6
40	66	10.3
39	64	10.0
38	62	9.7
37	60	9.4
36	58	9.1
35	56	8.8
34	54	8.4
33	52	8.1
32	50	7.8
31	48	7.5
30	46	7.2
29	44	6.9
28	42	6.6
27	40	6.3
26	38	5.9
25	36	5.6
24	34	5.3
23	32	5.0
22	30	4.7
20	28	4.4
18	26	4.1

Color Index

The color index is the quotient obtained by dividing the percentage of hemoglobin by the percentage of red corpuscles, 5,000,000 cells per cubic millimeter being considered as 100 per cent of corpuscles. It is calculated as follows:

$$\text{Color Index (C I)} = \frac{\text{per cent hemoglobin}}{100} \text{ divided by } \frac{\text{number of red cells}}{5{,}000{,}000} = \frac{\text{per cent hemoglobin} \times 50{,}000}{\text{number of red cells}}$$

In normal blood the color index is about 1. A color index above 1 means that the cells are larger than normal. A color index below 1 means that the cells contain a smaller amount of hemoglobin or are smaller than normal.

Volume Index

The term "Volume Index" is an expression used to designate the volume of red cells relative to the normal. It is the quotient of the percentage volume of the erythrocytes divided by the percentage number of cells. It is a measure of the relative size of the red cells.

To determine the volume index 10 c.c. of blood are withdrawn by venipuncture into a dry well vaselined syringe. The blood is run immediately into a 15 c.c. graduated hematocrit tube (Fig. 21) containing 2 c.c. of 1.6 per cent sodium oxalate and mixed by inversion.

The tube is then centrifuged for 30 minutes at 2500 revolutions per minute.

With normal blood and a red cell count of 5,000,000, the red cells comprise about 46 per cent. This value for a normal blood should be determined for each centrifuge however. The value so determined represents 100 per cent volume. The erythrocytes are counted at the same time that the volume determination is made.

The volume index is equal the volume per cent divided by the number per cent of red cells with 5,000,000 corpuscles being considered 100 per cent.

In normal blood the volume index is 1. An index below 1 shows that the average size of the cells is less than 1 and index above 1 indicates that the average size of the cells is greater than normal.

Saturation Index

The color index expresses the relationship between the number of red blood cells and the amount of hemoglobin but does not give the true concentration of hemoglobin in the cells. The relationship is a function of both the size of the red cell and the amount of hemoglobin contained therein. The actual percentage

of hemoglobin in the red cells is given by the "saturation index." This is obtained by dividing the hemoglobin in per cent by the percentage volume of red cells.

To calculate the saturation index blood is obtained as for a volume index determination, centrifuged, and the volume of red cells read off. The per cent of normal volume is determined. A hemoglobin estimation on the same specimen of blood is made. The hemoglobin in per cent divided by the percentage volume of red cells gives the saturation index. For example, 10 c.c. of blood are centrifuged and 2.3 c.c. or 23 per cent of packed cells is obtained. Normal blood with a red count of 5 million per c.mm. would give with the same centrifuge 4.6 c.c., or 46 per cent packed cells. The hemoglobin is 50 per cent. The saturation index is 50 divided by $\frac{2.3}{4.6}$ or 1.00.

Examination of Fresh Blood

The exact size and shape of red corpuscles are best determined in preparations of fresh blood.

The fresh preparations are made by taking a very small drop of blood on the center of a cover slip cleaned carefully as for a blood smear and laying it on a slide similarly cleaned. The blood will spread out leaving the red cells separate. Seal the edges of the cover slip to the slide with a small amount of vaseline if the preparation is to be kept under observation for some time.

Note in the fresh preparation the size, shape and color of the red blood cells; the relative number of white blood cells; and the presence of parasites.

Preparation and Staining of Blood Films

Cleaning the Cover Glasses.—

(1) Immerse the cover glasses in concentrated sulphuric acid for about 24 hours.

(2) Pour off the acid and wash in running water.

(3) Drain off the water and cover the glassware with 95 per cent alcohol for an hour or longer.

(4) Wipe dry with towel and place in hot air sterilizer for several hours.

Preparation of Blood Films.—The film is made by placing a small drop of blood in the center of one cover slip (Fig. 36) and quickly placing a second cover over it until there is a suitable spread of the blood. The cover slips are then drawn apart with a sliding motion. The films are allowed to dry in the air.

Staining the Film.—

1. **Wright's Stain.**—(a) *Preparation of Stain*: To a 0.5 per cent aqueous solution of sodium bicarbonate, add methylene blue (B.X. or "medicinally pure") in the proportion of 1 gram of the dye to each 100 c.c. of the solution. Heat the mixture in a steam

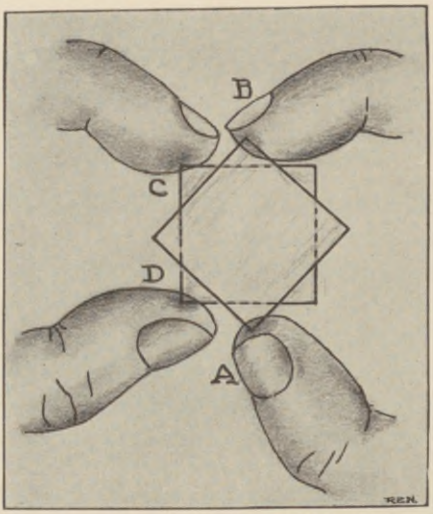


Fig. 36.—Blood film by the cover glass method. With thumb and forefinger of the right hand firmly grasp the upper cover glass (⅞ inch square No. 1) at the diagonal corners, *a* and *b*, and the lower one at the adjacent corners, *c* and *d*, and quickly pull apart, keeping the two parallel. (*After McJunkin.*)

sterilizer at 100° C. for one full hour, counting the time after the sterilizer has become thoroughly heated. The mixture is to be contained in a flask, or flasks, of such size and shape that it forms a layer not more than 6 centimeters deep. After heating, the mixture is allowed to cool, placing the flask in cold water if desired, and is then filtered to remove the precipitate which has formed in it. It should, when cold, have a deep purple red color when viewed in a thin layer by transmitted yellowish artificial light. It does not show this color while it is warm.

To each 100 c.c. of the filtered mixture add 500 c.c. of a 0.1 per cent aqueous solution of "yellowish, water-soluble" eosin and mix thoroughly. Collect on a filter the abundant precipitate which immediately appears. When the precipitate is dry, dissolve it in methyl alcohol (Merck's "reagent") in the proportion of 0.1 gram to 60 c.c. of the alcohol. In order to facilitate solution the precipitate is to be rubbed with alcohol in a porcelain dish or mortar with a spatula or pestle.

(b) *Method of Staining.*—Cover the blood film (Fig. 37) with a noted quantity of the stain (about 7 drops for a ¾ inch cover glass.) After one minute add an equal number of drops of a phosphate buffer solution with P_H equal to 6.4, and allow to remain for 5 to 6 minutes (McJunkin). Wash quickly, blot, dry, and mount in balsam. If the buffer solution is not available, distilled water may be used.

The phosphate buffer solution with P_H equal to 6.4 is made as follows:

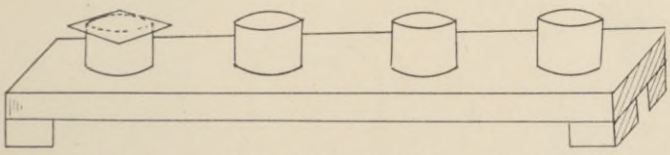


Fig. 37.—Convenient stand for staining blood films made on cover glasses.

Recrystallized primary potassium phosphate	6.63	grams.
Recrystallized secondary sodium phosphate (exposed to air for two weeks to lose its water of crystallization)	3.20	grams.
Distilled water, add quantity sufficient	1000	c.c.

2. **Ehrlich's Stain.**—

(a) *Preparation of Stain.*—

Saturated aqueous solution of orange G	13.0	c.c.
Saturated aqueous solution of acid fuchsin	7.0	c.c.
Distilled water	15.0	c.c.
Absolute alcohol	15.0	c.c.
Saturated aqueous solution of methyl green	17.5	c.c.
Absolute alcohol	10.0	c.c.
Glycerine	10.0	c.c.

The fluids are mixed with the same graduated cylinder which should not be rinsed. The receiving flask should be shaken vigorously after the addition of each constituent which is added in the order given in the formula. It is essential to add the methyl green, second portion of alcohol and glycerine slowly, shaking well after each addition. The mixture is ready for use immediately and does not deteriorate with age.

(b) *Method of staining.*—Fix the blood film on a copper bar at the spheroidal point for 30 to 45 seconds. Cover with Ehrlich's stain, and allow to remain 5 to 10 minutes. Wash quickly in water, blot dry, and mount in balsam.

Differential Leucocyte Counts

A blood film stained with Wright's stain is used for a routine count.

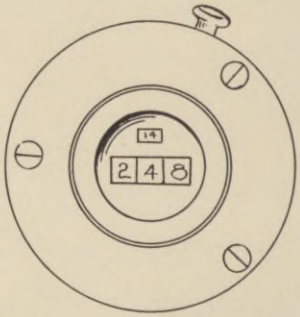


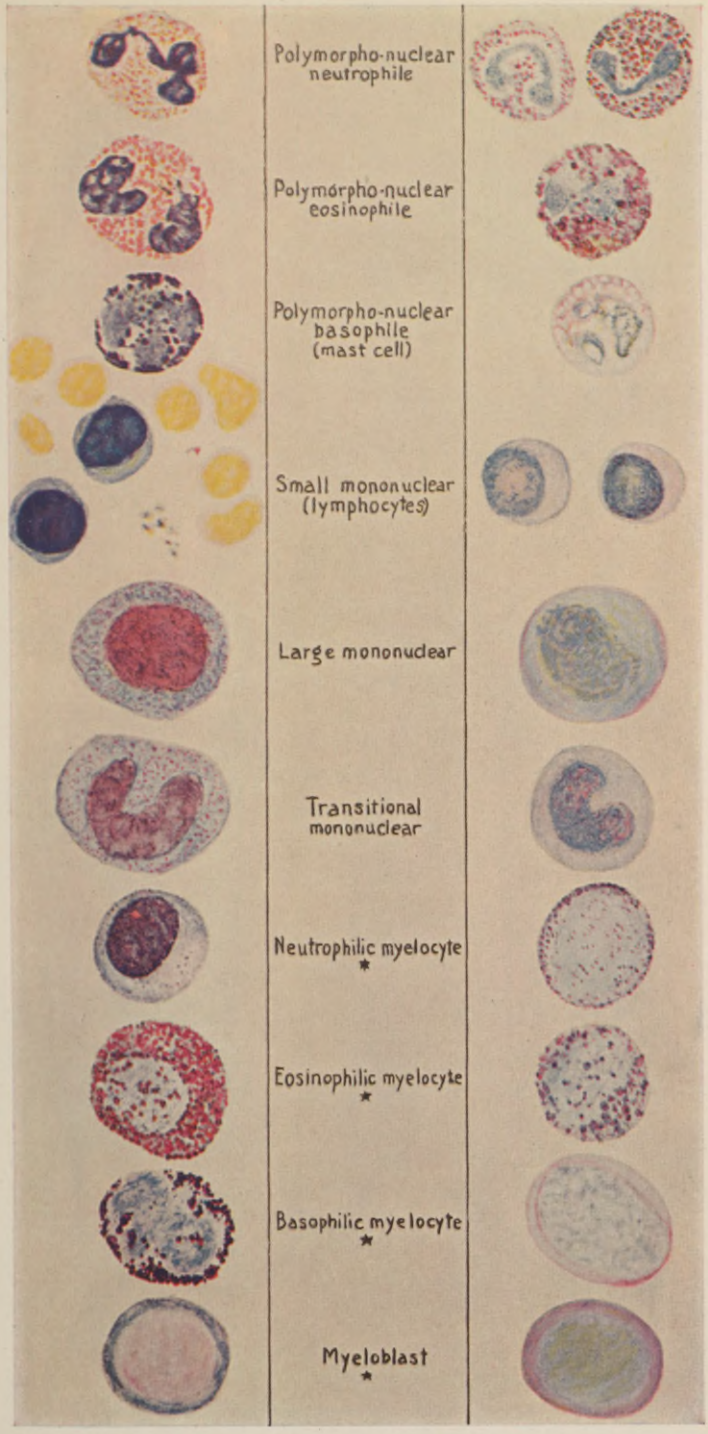
Fig. 38.—Tallying register used in making differential leucocyte counts.

Count 250 leucocytes, employing the 4 millimeter objective and 10 X eyepiece. The counting is facilitated by the use of a tallying register (Fig. 38).

The normal leucocytes are classified as follows: (Plate III)

1. *Polymorphonuclear neutrophilic leucocytes* (P.M.N.) are cells with polymorphous nuclei, in whose cytoplasm are numerous fine neutrophilic granules. These cells are 9 to 12 micromillimeters in diameter.

2. *Polymorphonuclear eosinophilic leucocytes* (P.M.E.) are similar to the above, except for the presence of coarse eosinophilic or acidophilic granules in the protoplasm. They are usually larger than the neutrophiles.



*Cells not present in normal blood.

PLATE III.

White blood corpuscles with Wright and Ehrlich stains. *(After Barker.)*

TABLE XIII*

CELLS OF NORMAL BLOOD	STAIN	NUCLEUS	PROTOPLASM	GRANULES	SIZE IN MICRA	%	ABS. NO.	ORIGIN	REMARKS
# 1	Wright	None	Light buff or pink. Rarely any structure seen.	None	7½ Micra	100 %	4½ to 5 millions	Bone Marrow	Round or slightly oval. Slaty blue color usually denotes insufficient washing.
R.B.C.	Ehrlich	None	Buff	None					Lemon yellow—over fixed; red brown—under fixed.
# 2	Wright	More polymorphous than polynuclear, chromatin deep purple, slightly reticular.	Relatively abundant		(Average 10 micra) 8 -12 micra	63.5 %	4900	Bone Marrow	Generally round, become very large from crushing. Protoplasm in young forms often blue.
P.M.N.	Ehrlich	Blue green or robin's egg color. Little evidence of structure.	Faint pink	Lilac if fixed O.K. True red color with quick fixation. Stain is specific.					Black nuclear mass is due to stain precipitate.

*The absolute numbers and percentages are taken from Miller, S. R.: Bull. Johns Hopkins Hosp., 1914, xxv, 284.

TABLE XIII—CONTINUED.

CELLS OF NORMAL BLOOD	STAIN	NUCLEUS	PROTOPLASM	GRANULES	SIZE IN MICRA	%	ABS. NO.	ORIGIN	REMARKS
# 3	Wright	Larger than #2, less pyknotic, stains lighter, shows fewer lobulations, as rule reticulated.	Scanty, faint pink if seen at all	Red	Average is slightly larger than #2	2.8 %	218	Bone Marrow	Size and brilliancy of the granules stained with Wright distinguishes these cells from #2 similarly stained.
P.M.E.	Ehrlich	Light green color	Generally not seen	Dark red or crimson					
# 4	Wright	Chromatin scanty, stains light purple, not very polymorphous.	Faint pink						Cells are rarely ever increased in number.
P.M.B.	Ehrlich	Light green reticular.	None	Do not stain, appear as colorless vacuoles	Generally smaller than Nos. 2 or 3	0.5 %	42	Bone Marrow	

TABLE XIII—CONTINUED.

CELLS OF NORMAL BLOOD	STAIN	NUCLEUS	PROTOPLASM	GRANULES	SIZE IN MICRA	%	ABS. NO.	ORIGIN	REMARKS
# 5	Wright	Large chromatin, round oval or slightly notched, deep purple clear outer zone due to nuclear contraction.			4-10 micra	22 %	1725	Lymphoid tissue, spleen, and glands	Nucleus central in large forms. Easily overlooked when stained with Ehrlich.
S.M.	Ehrlich	Light blue green.	Light pink or violet often not seen	None					
# 6	Wright	Large oval indented and vesicular, chromatin poor, light purple or blue, and generally eccentric.	"Azure" granules occur.		12-20 micra	8 %	610	Endothelium	Group includes cell of type #5 larger than an average size P.M.N. Extremely fine lilac granules occasionally seen with Ehrlich stain.
L.M.	Ehrlich	Very pale blue or green, hard to see.		None					
# 7	Wright	Horseshoe, kidney shape, or irregular plump nucleus, deeper purple than #6.	Less relatively than in #6.	May be a few like those of a P. M. N.	Largest cells seen	2.8 %	218	Are probably senile forms of #6	Cells are not transition form of #2.
Trans.	Ehrlich	Light blue easily seen.		Few occasionally.					Are probably senile forms of #6.

3. *Polymorphonuclear basophilic leucocytes* (P.M.B.) show in the protoplasm, basophilic granules which are variable in size, the majority being about as coarse as the eosinophilic granules. The nucleus is usually simply indented or lobulated.

4. *Small mononuclear leucocytes* (S.M.) are cells having a single round or oval nucleus and a scanty rim of protoplasm. The protoplasm is nongranular, although about 30 per cent of the leucocytes of normal blood possess azurophile granules which are demonstrable after staining with methylene azure, but not with other stains. The granules vary greatly in number and size. Classify as small mononuclears all mononuclear cells smaller than an average sized polymorphonuclear neutrophiles. The small mononuclears are lymphocytes.

5. *Large mononuclear leucocytes* (L.M.) are mononuclear cells as large or larger than an average sized polymorphonuclear neutrophile. The nucleus is poorer in chromatin and therefore stains less intensely. These cells have relatively more protoplasm than the small mononuclears. The protoplasm may contain azurophilic granules.

6. *Transitional leucocytes* (Trans.) differ from the large mononuclears in the shape of the nucleus, which is horseshoe-shaped, lobulated, or deeply indented, and in the constant presence in the protoplasm of numerous dust-like, azurophile granules.

Table XIII summarizes the important features of the normal red blood cells and leucocytes with Wright and Ehrlich stains.

Pathological Red Blood Cells and Leucocytes

In anemias the following abnormalities of the red blood cells should be looked for in fresh preparations and in stained films. (Plates III and IV.)

1. Irregularity in size—anisocytosis (note especially the presence of very large cells), and irregularity in shape—poikilocytosis. These features can be best observed in fresh blood. Cells less than 6 micra in diameter are classed as microcytes; those with a diameter of 9 to 12 micra are classed as macrocytes.

2. Nucleated cells: Normoblasts have the diameter of the average red cell, the nucleus is round and pyknotic; microblasts are abnormally small nucleated red cells; megaloblasts are large

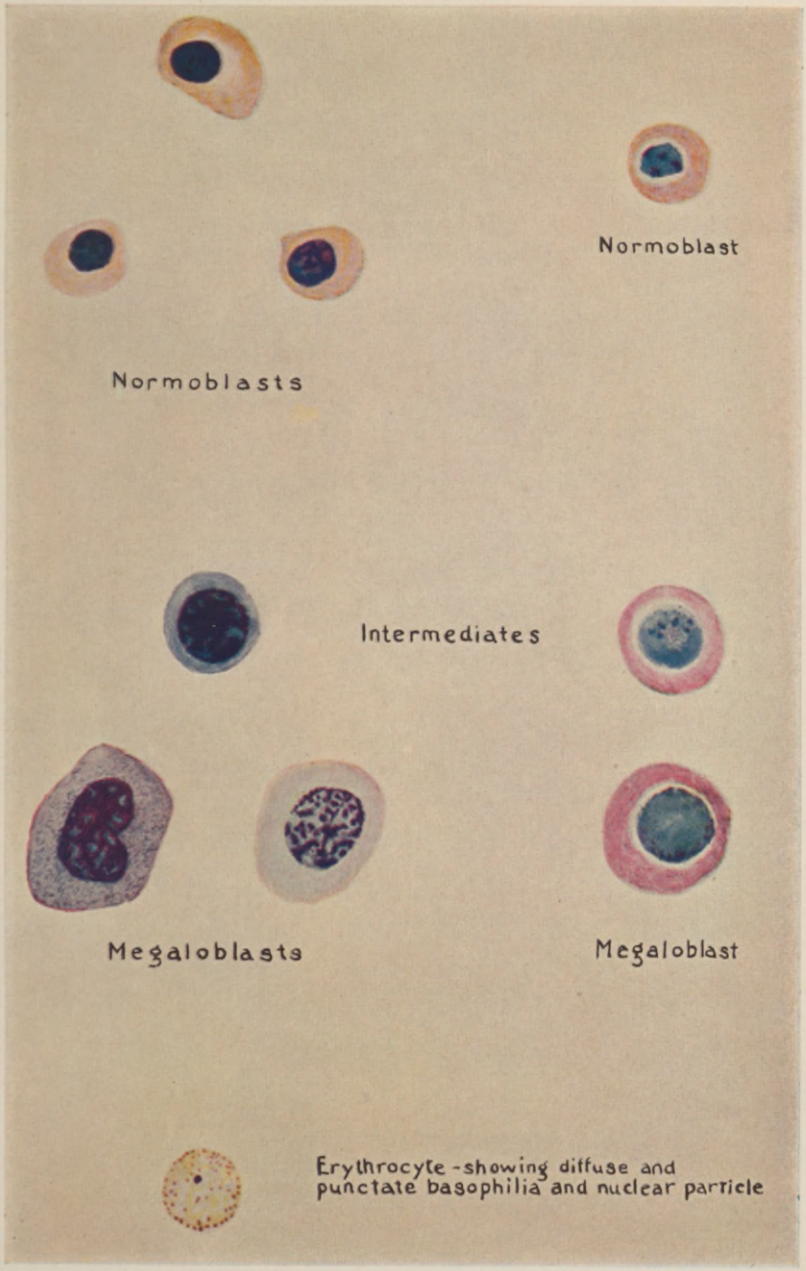


PLATE IV.

Nucleated red blood corpuscles with Wright and Ehrlich stains. *(After Barker.)*

and have a large oval or round nucleus, which may show beautiful chromatin network.

3. Basophilia in which the cells take the basic dye and with Wright's stain appear blue. The basophilia may be diffuse in which a uniform basic staining occurs or punctate in which the cells contain basic staining granules.

Pathological leucocytes are seen chiefly in leukemia. The most important forms are given below:

1. Myelocytes—neutrophilic, which have a round or oval nucleus and show neutrophilic granules in the protoplasm; eosinophilic in which the granules are eosinophilic; basophilic in which the granules are basophilic.

2. Myeloblasts which differ from the myelocytes in having no granules in the protoplasm. Both the myelocytes and the myeloblasts show a positive oxydase reaction.

3. Pathological lymphocytes may differ very much from the normal. They vary much in size. The nucleus may be indented or convoluted forming the so-called Rieder cells.

Platelets

Any relative variation from the normal number of blood platelets in the smear should be noted.

Determination of the Fragility of Erythrocytes

(Griffin and Sandford: Jour. Lab. and Clin. Med., 1919, iv, 465)

Principle.—Fresh blood is mixed with sodium chloride solutions of varying concentration. The strength of the solutions in which hemolysis begins and in which hemolysis is complete is noted.

Reagent.—Five-tenths per cent solution of chemically pure sodium chloride. The sodium chloride should be dried in hot air oven at 170° C. for two hours, cooled in a desiccator and weighed accurately on the chemical balance. The water should be measured in a volumetric flask.

Procedure.—Set up twelve 4 × ⅜ inch test tubes in each of two racks (Fig. 39). Number the tubes in each rack from left to right 25, 24, 23, 22, 21, 20, 19, 18, 17, 16, 15, 14.

With a capillary pipette put into each tube the number of drops of 0.5 per cent sodium chloride indicated by the figure on the tube. Add distilled water with the same pipette so that the total number of drops in each tube is 25.

Withdraw blood from a vein with a dry sterile syringe and run one drop of blood (Fig. 40) into each tube of one rack. In a

Fig. 39.—Dilutions are made of 0.5 per cent sodium chloride solution by adding distilled water by the drop method so that each tube contains twenty-five drops of hypisotonic solution. (*After Giffin and Sanford.*)

similar manner obtain blood from a normal control and put one drop into each tube of the second rack.

Allow the racks to stand at room temperature for one to two hours and read. The dilution in which there is just a slight tingeing of the supernatant fluid due to laking of a few of the least resistant corpuscles is noted as the point of initial hemolysis. Reading from left to right the first tube, in which there is no

corpuscular residue evident by shaking the tube, indicates complete hemolysis.

The percentage of salt in any tube can be immediately determined by multiplying the number on the tube by 0.02.

Remarks.—Normal blood shows initial hemolysis in 0.42 to 0.38 per cent sodium chloride solution and complete hemolysis in 0.36 to 0.32 per cent.

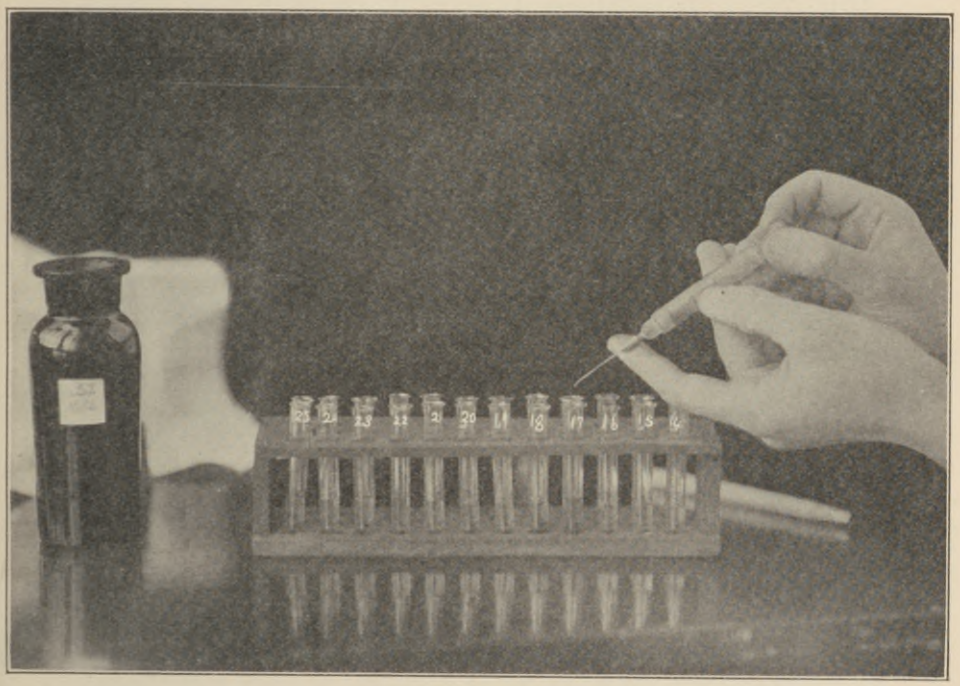


Fig. 40.—One drop of whole blood is added to each tube of hypisotonic solution. (*After Giffin and Sanford.*)

Technic for Vital Blood Stain

Reagent.—Mix 5 c.c. of a saturated aqueous solution of brilliant cresyl blue and one cubic centimeter of two per cent sodium oxalate solution and filter. Draw up 1 to 2 drops of this mixture into each of the several capillary pipettes and place in incubator until the stain is evaporated to dryness. These preparations will keep indefinitely.

Procedure.—Puncture the finger so as to get a free flow of

blood. With a rubber bulb draw up a good sized drop of blood into one of the capillary pipettes containing the stain. Mix thoroughly. After standing for several minutes make films on cover slips. Dry in air and stain with Wright's stain in the usual way.

In the cells taking the vital stain a granulo-reticulo-filamentous substance is seen. This appears in the form of granular particles which are sometimes discrete but are more often threads which are woven into a network, or wreaths which fill a great part of the cell.

Determine the percentage of reticulated cells using the oil immersion objective. The counting is facilitated by using a small circle cut from cardboard on the diaphragm of the eye piece.

Remarks.—In the normal blood of adults less than one per cent of the red blood cells show the granulo-reticulo-filamentous substance.

If brilliant cresyl blue is not available, Unna's methylene blue may be used.

Determination of Coagulation Time of Blood

(Howell, W. H., Arch. Int. Med., 1914, xiii, 80)

A clean syringe is dried, rinsed with albolene and normal salt solution. The space in the syringe between the end of the plunger and the needle is filled with salt solution so that the blood does not come in contact with air. A vial (Fig. 41) about 21 millimeters in diameter is thoroughly cleaned with bichromate cleaning solution, rinsed in distilled water and dried with alcohol and ether.

Blood is withdrawn by venapuncture and 2 c.c. run into the vial. The time of withdrawal of the blood is noted accurately. The specimen is examined at short intervals for coagulation. The test of coagulation is ability to invert the tube slowly without dislodging the blood.

By this technic normal blood at room temperature (20° C.) clots in 20 to 40 minutes. For each degree below this temperature, about 1 to 2 minutes will probably be added to the coagulation time.

Decreasing the diameter of the tube shortens the coagulation time. Lee and White suggest that a test-tube of 8 mm. bore be used. One c.c. of blood is withdrawn with a small syringe and run at once into the tube. The same precautions as to preparation of the syringe and tube noted above must be observed. The normal coagulation time by this method is 5 to 8 minutes.

A rough idea of the coagulation time may be obtained by filling a capillary tube with blood from a puncture of the ear or finger. The tube is broken off at intervals of one-half minute. Coagulation is easily recognized by the string of fibrin. The coagulation time by this method varies from 2 to 6 minutes.

Fig. 41.—Tube used in the determination of the coagulation time of blood.

Determination of the Bleeding Time

The bleeding time is determined by the method suggested by Duke. A small incision is made in the finger or in the lobe of the ear and at half-minute intervals the blood is blotted up with smooth filter paper. The cut should be such that the diameter of the first blot is about 2 cm. without any squeezing. Each blot represents a half minute's flow of blood. The bleeding time is the total duration of the hemorrhage. It varies from 1 to 3 minutes in normal individuals.

Schultze's Oxydase Reaction

(Evans, F. A.: Arch. Int. Med., 1916, xvii, 1)

Principle.—Many white blood cells possess an oxidizing ferment which they disclose by forming synthetically naphthol blue

TABLE XIV*

BLOOD REPORT

Name: No:

Case No: Date:

Service: Stain used:

I. RED BLOOD CELLS:
1. Total number per c.mm.
2. Size in fresh preparation
3. Shape in fresh preparation
4. Color in fresh preparation
5. Regeneration forms:
   (a) Nucleated R.B.C. –
   (b) Basophilia: punctate or diffuse –
   (c) Nuclear particles –
   (d) Cabot Rings –
6. Resistance to hypotonic salt solution: maximal %; minimal %
7. Percentage of reticulated cells in vital stain:

II. WHITE BLOOD CELLS:
1. Total number per c.mm.
2. Differential count: Cells counted:
   PMN = % PME = % PMB = %
   SM = % LM = % TRANS = %
3. Presence of abnormal forms:
   (a) Myelocytes: type?
   (b) Myeloblasts –
   (c) Irritation forms –

III. HEMOGLOBIN: % (Hemoglobinometer)

IV. COLOR INDEX:

V. VOLUME INDEX:

VI. PLATELETS:

VII. COAGULATION TIME: (Method)

VIII. PARASITES:

IX. REMARKS:

X. IMPRESSIONS:
1. Summary of Important Evidence:
2. Probable Diagnois:

NAME OF EXAMINER.

*This form is modeled after one in use at the Johns Hopkins Hospital.

when they are treated first with a-naphthol and then with dimethyl-phenylendiamin. The method is particularly useful for differentiating myeloblasts and myelocytes from cells of the lymphocyte series. The myelocytes and myeloblasts give a positive reaction, while the lymphocytes are negative.

The granules which exhibit the oxydase reaction are stained deep blue. The preparations are not permanent.

Procedure.—Make smears on cover slips as for differential leucocyte count.

(a) *Staining.*—

1. Fix smears eight hours (under bell jar) in formaldehyde vapor.
2. Stain in saturated aqueous safranin for eight minutes.
3. Wash quickly in water and dry immediately by careful blotting.

(b) *Oxydase Reaction:* Put on slide.

1. One drop of 1 per cent aqueous solution of dimethylparaphenylenediamin, and
2. One drop of freshly prepared 1 per cent solution of alpha naphthol in 1 per cent KOH dissolved by heating.
3. Mount previously stained smear in this and examine at once.

Examination of Blood for Malarial Organisms

Make smears as for differential leucocyte count. Stain with Wright's stain. The staining should be done with distilled water instead of buffer solution. A preparation of fresh blood should also be made by placing a small drop of blood on a clean slide and covering with a cover glass.

Examine both preparations with the oil immersion lens.

Tables XV and XVI summarize the characteristics of the different malarial organisms in fresh and stained preparations.

TABLE XV

THE APPEARANCE OF MALARIAL PLASMODIA IN FRESH BLOOD

	PL. VIVAX (tertian)	PL. FALCIPARUM (estivoautumnal)	PL. MALARIA (quartan)
I. HYALINES			
(a) Shape	Often irregular, occasionally ring forms.	Usually ring forms, occasionally irregular.	Irregular or ring forms.
(b) Refractivity	Difficult to see; much like the red cell. Ring forms more refractive.	Easily seen; refractive.	Rather easily seen.
(c) Motility	Actively ameboid.	Occasionally active. Rings sluggish.	Sluggish usually.
(d) Multiple infections	Infrequent.	Frequent. May be six or more hyalins in a single cell.	Infrequent.
II. PIGMENTED FORMS			
(a) Shape	Ameboid, very irregular. Three-quarters and full grown parasites round.	Usually no pigmented forms in circulating blood. Round or oval, when seen.	Irregular, soon becoming round or oval. "Band" forms not infrequent.
(b) Refractivity	Young forms difficult to see.	Easily seen; refractive	Easily seen.
(c) Motility	Young forms actively ameboid.	Sluggish.	Sluggish.
(d) Pigment	Fine brown granules scattered throughout parasite.	Fine, dark brown granules, centrally placed.	Coarse brown granules peripherally placed.
(e) Motility of pigment	Very active in younger forms.	Sluggish.	Very sluggish.
(f) Merozoites or daughter parasites	12 to 24, usually about 16.	8 to 24, usually 12 to 16, small.	6 to 12, often 8.
III. SEXUAL FORMS Shape	Round.	Usually crescentic, at times round or oval.	Round.
IV. INFECTED RED CELLS	Swollen and pale.	Often brassy, shrunken or crenated.	Often brassy, no swelling.

TABLE XVI

THE APPEARANCE OF MALARIAL PLASMODIA IN STAINED BLOOD FILMS (WRIGHT STAIN)

	PL. VIVAX (tertian)	PL. FALCIPARUM (aestivoautumnal)	PL. MALARIA (quartan)
I. HYALINES	Ring of blue protoplasm. Round chromatin mass; red or violet with achromatic zone. Whole parasite encloses unstained area of R.B.C. Chromatin located at thin area of ring.	Protoplasm scanty; stains faint blue. Chromatin in 1/3 masses of filaments often projecting beyond the ring.	Generally the same but chromatin is not dense but rather an irregular clump of red violet granules. Chromatin is nearer the center than in tertian. Protoplasm stains a darker blue. Parasite as whole is smaller than tertian.
II. PIGMENTED FORMS (a) Pigment	Fine, irregularly scattered, takes a greenish brown stain. Collected in masses at the periphery of presegmenting forms. Single mass at center of segmenters.	Amount and distribution as in fresh blood. Takes deep brown stain.	Little influenced by stain. Size and distribution as in fresh blood. Dark greenish brown or blue black in color.
(b) Chromatin	Becomes more irregular with growth of parasite. In full grown parasite it breaks up into 15-20 clusters each in achromatic zone. Stains a deep red violet.	Often in several masses in ring forms.	Chromatin less dense in hyalines. In older forms is a cluster of fine granules with an achromatic zone. Stains a more intense red than with tertian.
(c) Protoplasm	Stains a definite blue, thickest opposite the chromatin in hyaline. Irregular in outline. With growth of parasite tends to stain more deeply.	Protoplasm throughout scantier than in other forms. Tends to stain a delicate blue in hyaline form and not very dark in mature form.	Protoplasm throughout stains more intensely and is generally regular in outline. Circular.

TABLE XVI—CONTINUED.

THE APPEARANCE OF MALARIAL PLASMODIA IN STAINED BLOOD FILMS (WRIGHT STAIN)

	PL. VIVAX (tertian)	PL. FALCIPARUM (aestivoautumnal)	PL. MALARIA (quartan)
III. SEXUAL FORMS (a) Female	Intense blue protoplasm. Chromatin is scanty and tends to be peripheral. Stains a brilliant red, and is compact. Pigment is blue; black rods situated peripherally or as a nidcentral areola.	Long and slender. Chromatin compact and central. Protoplasm deep blue with polar intensification. Pigment is near center in masses or in wreath about the chromatin. It is coarse and black.	In general as in tertian. These forms show more intense staining reaction, are apt to be smaller, and show coarser, darker pigments as in fresh blood.
(b) Male	Protoplasm stains a faint blue. Chromatin is abundant and loose. Stains intense red. Central chromatin has an achromatic zone. Pigment is greenish blue, fine and diffuse. Chromatin breaks up into 4-8 masses just prior to flagellation.	Kidney shaped. Protoplasm stains much lighter blue than female. Chromatin is not so brilliant and forms loose network often scattered throughout. Pigment finer, diffuse, and greenish brown in color.	
IV. INFECTED CELLS	Enlargement noticeable. Often distorted, stains poorly. Red or purple stippling may occur, especially common in tertian type and occurs in cells with early forms.	Small size; darker staining. Stippling does not occur.	Normal or smaller—takes a dark reddish stain corresponding to brassy look. Stippling very common.

Qualitative Tests for Bile Pigments, Bile Salts and Urobilin in Blood Plasma

(Blankenhorn, M. A.: Arch. Int. Med., 1917, xix, 344)

I. **Bile Pigments.**—Put nitric acid under the plasma in a test tube with a small pipette. A white coagulum is formed at the junction, which soon develops into a white layer of a certain thickness and remains fairly constant as the acid dissolves the coagulum at the lower border and forms it at the upper, thus

ascending through the plasma. The blue green color develops in a line at the midst of the white zone if bile pigment be present and remains in the same relative position. When the bilirubin is present in small amounts, the blue color may not appear for half an hour.

The bilirubin content of the blood is expressed by the dilution required to diminish the staining to a point where it is just perceptible in a column one centimeter deep. The plasma must be free from hemolysis. In diluting the plasma with distilled water, a faint precipitate usually appears in suspension, which can be put in solution by a drop of ammonium hydroxide.

The end point of the dilution, that is where the yellow color is just perceptible, is found by comparing the solution in a small test tube with a similar column of distilled water. To facilitate reading, the tubes are held parallel and observation made down the length of the tubes. A device which serves to bring the two columns to still more equal terms is to immerse the ends of the tubes in an inch or two of water in an evaporating dish.

II. **Bile Salts.**—Three c.c. of plasma are dialyzed into 9 c.c. of water. The collected dialysate is concentrated and the Pettenkofer test applied, and the spectroscopic examination made, if any color develops. For the test, two cubic centimeters of concentrated dialysate are placed in a small flask with two or three drops of a 1 to 1000 aqueous solution of furfurol and two cubic centimeters of concentrated sulphuric acid are added, drop by drop, from a pipette. The contents of the flask is kept at 60 degrees Centigrade by immersing the flask in a bath and keeping the mixture agitated. When the acid is added too rapidly a dark reddish brown color sometimes appears from the charring action of the acid on the small amounts of protein or sugar that dialyze. If the temperature remains much below 60° C., the color fails to develop. The spectrum of the Pettenkofer test for bile salts shows a wide absorption band in the blue when the test is first made and is of a cherry red color. Later as the cherry turns to purple, the band in the blue fades and a smaller band develops in the orange (near I, between D and C). Small amounts of bile salts which give the Pettenkofer test may not give a characteristic spectrum.

Test dialysate for bilirubin also.

III. **Urobilin.**—Precipitate the blood protein by adding one to two volumes of freshly prepared saturated alcoholic solution of zinc acetate and centrifugalize. Add 5 to 10 drops of Ehrlich's aldehyde reagent (page 19) and a color from pink to dark red develops, which gives the characteristic spectrum if urobilin or urobilinogen is present (Fig. 26-*A*).

CHAPTER VII

QUANTITATIVE CHEMICAL EXAMINATION OF BLOOD

Collection of Blood for Chemical Analysis

Blood for chemical analysis should be collected in the morning before the patient has had breakfast.

All specimens except those for calcium determination are to have oxalate added. The optimum amount is one drop of a saturated (about 30 per cent) solution of potassium oxalate to each 5 c.c. of blood. If too much oxalate be added the coagulation of the proteins and the uric acid precipitation will be interfered with.

For a calcium determination 10 c.c. of blood is run into a test tube containing 0.1 gram of solid sodium citrate and well shaken.

The following amounts of blood are necessary:

(1) Complete analysis including,

Non-protein nitrogen Urea nitrogen Creatinine Uric acid Sugar Chlorides Bicarbonate content	20 c.c.

(2) Single determination of any one of the above: 5 c.c.

The specimen for the determination of the bicarbonate content is to be collected in a centrifuge tube containing paraffin oil and potassium oxalate.

It is very important that specimens for sugar determination be sent to the laboratory immediately after withdrawal as the sugar is quite rapidly destroyed on standing.

The blood may be preserved if necessary by adding 1 drop of commercial formalin for each 5 c.c. of blood.

TABLE XVII

COMPOSITION OF NORMAL BLOOD AND CHEMICAL CHANGES IN CERTAIN PATHOLOGICAL CONDITIONS[1]

	NORMAL	CHRONIC HYPERTENSIVE VASCULAR DISEASE	CHRONIC NEPHRITIS	UREMIA	EARLY OR MILD DIABETES	SEVERE DIABETES	MODERATE ACIDOSIS	SEVERE ACIDOSIS	GOUT	ARTHRITIS	CHOLELITHIASIS
Hydrogen Ion Concentration (Plasma)	7.6-7.8						7.55-7.4	7.4-7.2			
Total Nonprotein Nitrogen	25-35	25-35	35-200	100-375		25-50			25-35	25-100	
Urea Nitrogen	10-18	10-20	25-175	75-325		15-30					
Uric Acid	1-4	1-4	1-6	5-25		4-10			4-10	1-4	
Creatinine	1-2	1-2	1-5	4-33							
Creatine	3-7	3-7	3-10	7-18							
Sugar	70-110		120-180	120-230	120-300	300-600					
Chlorides as NaCl (Plasma)	550-650	550-700	550-750	450-650							
Cholesterol	150-180	150-180	170-350		150-300						280-950
Alkali Reserve (c.c. CO_2 in 100 c.c. plasma)	50-75						30-40	Below 30			
Inorganic Phosphorus as H_2PO_4 (Plasma)	7-10		Up to 75								
Lipoid Phosphorus as H_2PO_4 (Plasma)	20-25										
Calcium as Ca (Plasma)	10										

[1]Compiled from results in the author's own experience and from numerous other observers. FIGURES IN MILLIGRAMS PER 100 C.C. BLOOD OR PLASMA.

Fig. 42 shows a convenient bottle to use in obtaining specimens of blood for chemical examination.

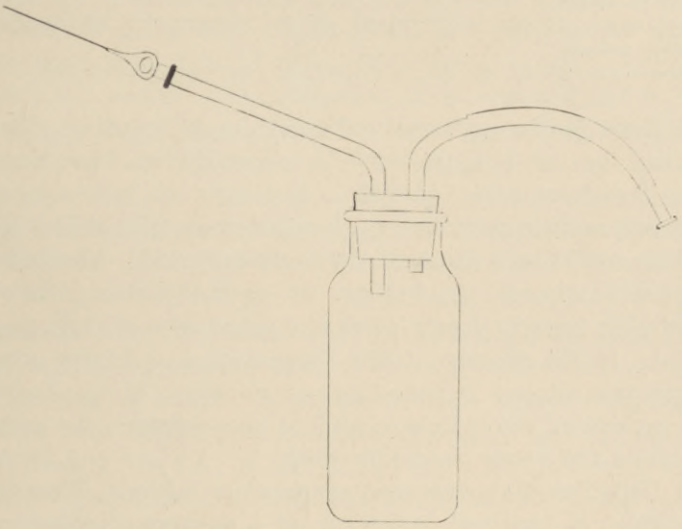


Fig. 42.—Blood chemical bottle used for collecting blood.

Determination of the Relative Hydrogen-Ion Concentration

(Levy, Rowntree and Marriott: Arch. Int. Med., 1915, xvi, 389)

Principle.—The blood is dialyzed against normal salt solution and the H-ion concentration of the protein-free dialysate is determined by the indicator method, using phenolsulphonephthalein.

Reagents.—(1) Standard phosphate mixtures prepared according to Sorensen's directions as follows:

1/15 mol. acid or primary potassium phosphate. 9.078 grams of the pure recrystallized salt (KH_2PO_4) is dissolved in freshly distilled water and made up to 1 liter.

1/15 mol. alkaline or secondary sodium phosphate. The pure recrystallized salt (NA_2HPO_4 $12H_2O$) is exposed to the air for from ten days to two weeks, protected from dust. Ten molecules of water of crystallization are given off and a salt of the formula Na_2HPO_4 $2H_2O$ is obtained. 11.876 grams of this is dissolved in freshly distilled water and made up to 1 liter. The solution should give a deep rose-red color with phenolphthalein. If only a faint pink color is obtained, the salt is not sufficiently pure.

The solutions are mixed in the proportions indicated below to obtain the desired P_H.

P_H	6.4	6.6	6.8	7.0	7.1	7.2	7.3	7.4	7.5	7.6	7.7	7.8	8.0	8.2	8.4
Primary potassium phosphate c.c.	73	63	51	37	32	27	23	19	15.8	13.2	11.0	8.8	5.6	3.2	2.0
Secondary sodium phospate c.c.	27	37	49	63	68	73	77	81	84.2	86.8	89.0	91.2	94.4	96.8	98.0

(2) Eight-tenths per cent sodium chloride solution. Before applying the test it is necessary to ascertain whether the solution is free from acids other than carbonic. To determine this, a few cubic centimeters of the salt solution are placed in a pyrex test tube and 1 or 2 drops of the indicator added, whereupon a yellow color appears. On boiling, carbon dioxide is expelled, and the solution loses its lemon color and takes on a slightly brownish tint. In the absence of this change other acids are present, and the salt solution is therefore not suitable. If, on the other hand, on adding the indicator, pink at once appears, the solution is alkaline and hence cannot be used.

(3) Collodion dialyzing sacs, prepared as follows: One ounce of celloidin is dissolved in 500 c.c. of a mixture of equal quantities of ether and ethyl alcohol. The solid swells up and dissolves with occasional gentle shakings, in 48 hours. As a small amount of brown sediment separates out at first, the solution should stand for at least three or four days, after which the clear supernatant solution is ready for use. A small test tube (120 by 9 mm., inside measurement) is filled with this mixture, inverted, and half the contents poured out. The tube is then righted, and the collodion allowed to fill the lower half again. A second time it is inverted and rotated on its axis, the collodion being drained off. Care must be taken to rotate the tube in order to secure a uniform thickness throughout. The tube is clamped in the inverted position and allowed to stand for ten minutes, until the odor of ether finally disappears. It is filled five or six times with cold water, or it is allowed to soak five minutes in cold water. A knife blade is run around the upper rim, so as to loosen the sac from the rim of the test tube, and a few cubic centimeters of water are run down between the sac and the glass tube. By gentle pulling the tube is extracted, after which it is preserved by complete immersion in water.

Procedure.—One to 3 c.c. of clear serum or of blood is run, by

means of a blunt-pointed pipette, into a dialyzing sac which has been washed outside and inside with salt solution. The sac is lowered into a small test tube (100 × 10 mm., inside measurement), containing 3 c.c. of salt solution, until the fluid on the outside of the sac is as high as on the inside. From 5 to 10 minutes are allowed for dialysis. The collodion sac is removed and 5 drops of the indicator (0.01 per cent solution of phenolsulphonephthalein) are thoroughly mixed with the dialysate. The tube is then compared with the standards until the corresponding color is found, which indicates the hydrogen-ion concentration present in the dialysate. Readings should be made immediately against a white background. Results are expressed in logarithmic notation.

Remarks.—Oxalated blood from normal individuals gives a dialysate with a P_H varying from 7.4 to 7.6, while that of serum ranges from 7.6 to 7.8. In clinical acidosis figures from 7.55 to 7.2 have been noted by this method for serum and for oxalated blood from 7.3 to 7.1. A rise in the H-ion concentration of the blood is significant because it indicates a failure on the part of the protective mechanism of the body to preserve the proper reaction.

Preparation of Protein Free Blood Filtrates

(Folin and Wu: Jour. Biol. Chem., 1919, xxxviii, 81)

Collection of Blood Sample.—The blood should be collected over finely powdered potassium oxalate, about 20 mg. for 10 c.c. of blood. Large amounts of oxalate interfere with the coagulation of the protein, and may precipitate some of the uric acid.

Reagents.—

1. Ten per cent solution of sodium tungstate.
2. Two-thirds normal sulphuric acid solution. This may be prepared by diluting 35 grams of concentrated C.P. sulphuric acid up to a volume of 1 liter. Check by titration. One c.c. of the acid is neutralized by 6⅔ c.c. of N/10 alkali.

Procedure.—Transfer a measured amount of oxalated blood, preferably 10 c.c., into a flask having a capacity of fifteen to twenty times that of the volume taken. Dilute with seven volumes of water and mix. Add one volume of 10 per cent solution

of sodium tungstate and mix. With another pipette add one volume of ⅔ normal sulphuric acid. Close the mouth of flask with a rubber stopper and give it a few vigorous shakes. Let stand for five minutes. The color of the coagulum changes from bright red to dark brown. If this change in color does not occur, the coagulation is incomplete, usually because too much oxalate has been added. The sample may be saved by adding 10 per cent sulphuric acid one drop at a time, shaking vigorously after each

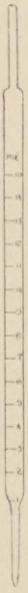


Fig. 43.—Folin and Wu pipette.

drop and continuing until there is no foaming and until the dark brown color has set in.

Pour the mixture on a filter large enough to hold the entire contents of the flask and cover with a watch glass. The filtration should be begun by pouring the first few cubic centimeters down the double portion of the filter and withholding the remainder until the whole filter is wet.

If the filtrate is to be kept longer than two days, a few drops of toluol should be added.

Remarks.—To test for excess of H_2SO_4 moisten congo red paper

with the filtrate. It should show negative, or at most, a bare trace of acid. An excess of acid will precipitate some of the uric acid.

Some examples of sodium tungstate contain too much sodium carbonate. This may be tested as follows: To 10 centimeters of a 10 per cent solution of the sodium tungstate add one drop of phenolphthalein and titrate with 0.1 N HCl. Each cubic centimeter of HCl is equivalent to 1.06 per cent of Na_2CO_3. The amount of acid used should not exceed 0.4 centimeters if the specimen is to be satisfactory for use.

Folin states that difficulty is sometimes encountered because the sodium tungstate is not alkaline enough. This may be corrected as follows: Prepare 100 c.c. of 10 per cent sodium tungstate using heat if necessary. Cool. Titrate 10 c.c. with normal NaOH to a permanent pink color using phenolphthalein as the indicator. The pink color should persist for 3 minutes after the last addition of alkali. Add to the remainder of the tungstate and to subsequent dilutions the amount of alkali indicated by the titration.

The preparation of the protein free filtrate is facilitated by the use of the special Folin pipette (Fig. 43).

Determination of Nonprotein Nitrogen

(Folin and Wu: Jour. Biol. Chem., 1919, xxxviii, 81)

Principle.—The protein-free blood filtrate is treated with an acid mixture, which converts the nitrogen into ammonia. The solution is Nesslerized and read against a standard ammonium sulphate solution similarly treated.

Reagents.—(1) Acid digestion mixture: Mix 300 c.c. of phosphoric acid syrup with 100 c.c. of ammonia-free sulphuric acid (concentrated). Transfer to a tall cylinder, cover well to exclude the absorption of ammonia, and set aside for sedimentation of calcium sulphate. To 100 c.c. of the clear acid add 10 c.c. of 6 per cent copper sulphate solution and 100 c.c. of water.

(2) Nessler's reagent. (See page 266.)

(3) Standard ammonium sulphate solution. (See page 44.)

Procedure.—Introduce 5 c.c. of the protein-free blood filtrate (page 129) into a dry 75 c.c. Pyrex test tube (200 × 25 milli-

TABLE XVIII

DETERMINATION OF NON-PROTEIN NITROGEN IN BLOOD

Use standard containing 0.3 mg. nitrogen made up to a total volume of 100 c.c.
Set standard at 20 or fill 20 mm. Bock-Benedict cell.
Make total volume of unknown blood up to 50 c.c.
Table shows the non-protein nitrogen in mg. per 100 c.c. blood corresponding to the different readings, with 5 c.c., 2 c.c., or 1 c.c. of blood filtrate, using a plunger type colorimeter.

	USING 5 C.C. OF BLOOD FILTRATE (Non-protein nitrogen in mg. per 100 c.c. blood)					USING 2 C.C. OF BLOOD FILTRATE (Non-protein nitrogen in mg. per 100 c.c. blood)					USING 1 C.C. OF BLOOD FILTRATE (Non-protein nitrogen in mg. per 100 c.c. blood)				
Colorimeter reading	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8
10	60.0	58.8	57.8	56.4	55.4	150.0	147.0	144.5	141.0	138.5	300	294	289	282	277
11	54.6	53.6	52.6	51.8	50.8	136.5	134.0	131.5	129.5	127.0	273	268	263	259	254
12	50.0	49.2	48.2	47.6	46.8	125.0	123.0	121.0	119.0	117.0	250	246	242	238	234
13	46.0	45.6	45.0	44.4	43.6	115.0	114.0	112.5	111.0	109.0	230	228	225	222	218
14	42.8	42.2	41.6	41.0	40.6	107.0	105.5	104.0	102.5	101.5	214	211	208	205	203
15	40.0	39.5	38.9	38.5	38.0	100.0	98.8	97.3	96.3	95.0	200	198	195	193	190
16	37.5	37.0	36.6	36.1	35.7	93.8	92.5	91.5	90.3	89.8	188	185	183	181	179
17	35.3	34.9	34.5	34.1	33.7	88.3	87.3	86.3	85.3	84.3	177	175	173	171	169
18	33.3	33.0	32.6	32.3	31.9	83.3	82.5	81.5	80.8	79.8	167	165	163	162	159
19	31.6	31.2	30.9	30.6	30.3	79.0	78.0	77.3	76.5	75.8	158	156	155	153	152
20	30.0	29.7	29.4	29.1	28.9	75.0	74.3	73.5	72.8	72.3	150	149	147	146	145
21	28.5	28.2	28.0	27.7	27.5	71.3	70.5	70.0	69.3	68.8	143	141	140	139	138
22	27.3	27.0	26.8	26.5	26.3	68.3	67.5	67.0	66.3	65.8	137	135	134	133	132
23	26.1	25.9	25.7	25.4	25.2	65.5	65.0	64.5	63.5	63.0	131	130	129	127	126
24	25.0	24.8	24.6	24.4	24.2	62.5	62.0	61.5	61.0	60.5	125	124	123	122	121
25	24.0	23.8	23.6	23.4	23.3	60.0	59.5	59.0	58.5	58.3	120	119	118	117	116
26	23.0	22.9	22.8	22.7	22.5	57.5	57.3	57.0	56.8	56.3	115	115	114	114	115
27	22.4	22.2	22.0	21.8	21.6	56.0	55.5	55.0	54.5	54.0	111	111	110	109	108
28	21.4	21.3	21.1	21.1	20.8	53.5	53.3	52.8	52.5	52.0	107	107	106	105	104
29	20.7	20.5	20.4	20.3	20.2	51.8	51.3	51.0	50.8	50.05	103	103	102	102	101

meters) graduated at 35 c.c. and 50 c.c. Add a quartz pebble and 1 c.c. of the sulphuric phosphoric acid mixture. Mix well. Boil vigorously over a micro burner (Fig. 19) until the characteristic dense acid fumes begin to fill up the test tube. Cut down the flame so that the contents just boil and close the mouth of the tube with a watch glass. Heat for two minutes counting from the time the test tube became filled with the fumes. Cool for seventy to ninety seconds and add 15 to 25 c.c. of water. Cool further to room temperature and add water to the 35 c.c. mark. Add 15 c.c. of Nessler solution. If turbid centrifuge. Compare in colorimeter with standard containing 0.3 milligrams nitrogen (as ammonium sulphate). Add to it 2 c.c. of the sulphuric phosphoric acid mixture, about 50 c.c. of water, and 30 c.c. of Nessler Solution. Make up to 100 c.c. and mix.

Nesslerize the unknown and the standard at approximately the same time.

Calculation.—

Set standard at 20

If X is the reading of the unknown.

$$\frac{20}{X} \times 30 = \text{mg. nonprotein nitrogen per 100 c.c. of blood.}$$

Remarks.—Normal blood gives by this method 25 to 35 mg. of nonprotein nitrogen per 100 c.c.

If the reading is high the determination should be repeated employing 2 or 1 c.c. of the filtrate.

A method for checking total nonprotein nitrogen determination is given on page 250.

Table XVIII gives the amount of nitrogen corresponding to the colorimeter readings, with a plunger type instrument.

Determination of Urea Nitrogen

(Van Slyke and Cullen: Modification of Marshall Method, Jour. Amer. Med. Assn., 1914, lxii, 1558)

Principle.—The urea in the blood is converted into ammonium carbonate by urease. The ammonia is liberated by a strong alkali, removed by the passage of a strong air current, and col-

lected in N/100 sulphuric acid. The excess of acid is then titrated with N/100 alkali.

Reagents.—

(1) Urease tablets (Hynson, Westcott and Dunning or Squibb).

(2) N/100 sulphuric acid.

(3) N/100 sodium hydroxide.

(4) Saturated solution of potassium carbonate (90 gm. per 100 c.c.).

(5) Alizarin (1 per cent sodium-alizarin sulphonate in 50 per cent alcohol).

Procedure.—Run 3 c.c. of oxalated blood into a large test tube (Fig. 20) containing about 10 c.c. of ammonia-free distilled water. Add 2 urease tablets (powdered). Leave at room temperature for 30 minutes. Add a teaspoonful of salt, a few drops of foam killer, (see page 272) and finally 5 c.c. of a saturated solution of potassium carbonate. Drive off the ammonia by aspiration into another tube "B" containing 15 c.c. of hundredth-normal sulphuric acid and 1 drop of alizarin for one hour. Titrate the excess of acid with hundredth-normal sodium hydroxide.

Calculation.—Each cubic centimeter of acid neutralized indicates 10 mg. of urea per 100 c.c. blood, or 4.67 mg. of urea nitrogen per 100 c.c. blood. In case the blood is one of the rare samples containing over 150 mg. urea per 100 c.c., all the acid will be neutralized and it will be necessary to repeat the determination, using only 1 c.c. blood. Fresh blood contains so little ammonia it may be disregarded.

Remarks.—The amount of urea nitrogen found in normal blood is 10 to 18 mg. per 100 c.c.

A slow current of air should be used during the first two minutes of aspiration.

The column of acid in the tube should be at least 50 mm. high.

The solution from which the ammonia is driven must contain at least 1 gram of potassium carbonate for each 2 c.c. of solution.

A blank determination should be run with each new lot of chemicals and the necessary deduction be made if any ammonia is found.

The air used for aerating should be run through a wash bottle containing sulphuric acid.

The figure obtained for urea nitrogen may be converted into urea by dividing by the factor 0.467.

Table XIX shows the blood urea nitrogen values corresponding to the amount of sodium hydroxide used to titrate the excess of sulphuric acid.

A method for checking urea nitrogen determinations is given on page 250.

TABLE XIX

DETERMINATION OF BLOOD UREA NITROGEN

Take 3 c.c. of blood and 15 c.c. of N/100 H_2SO_4.

Table shows the blood urea nitrogen in milligrams per 100 c.c. of blood corresponding to the number of c.c. of N/100 NaOH required to titrate the excess of N/100 H_2SO_4.

C.C. NaOH REQUIRED	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
0	70.05	69.58	69.12	68.65	68.18	67.71	67.25	66.78	66.31	65.85
1	65.38	64.91	64.45	63.98	63.51	63.05	62.58	62.11	61.64	61.18
2	60.71	60.24	59.78	59.31	58.84	58.38	57.91	57.44	56.97	56.51
3	56.04	55.57	55.11	54.64	54.17	53.71	53.24	52.77	52.30	51.83
4	51.37	50.90	50.44	49.97	49.50	49.04	48.57	48.10	47.63	47.17
5	46.70	46.23	45.77	45.28	44.83	44.36	43.90	43.43	42.96	42.50
6	42.03	41.56	41.10	40.63	40.16	39.70	39.23	38.76	38.29	37.83
7	37.36	36.89	36.43	35.96	35.49	35.03	34.56	34.09	33.62	33.16
8	32.69	32.22	31.76	31.25	30.82	30.36	29.89	29.42	28.95	28.49
9	28.02	27.55	27.09	26.62	26.15	25.68	25.22	24.75	24.28	23.82
10	23.35	22.88	22.42	21.95	21.48	21.01	20.55	20.08	19.61	19.15
11	18.60	18.21	17.75	17.28	16.81	16.35	15.88	15.41	14.94	14.48
12	14.01	13.54	13.08	12.61	12.14	11.68	11.21	10.74	10.27	9.81
13	9.34	8.87	8.41	7.94	7.47	7.00	6.54	6.07	5.60	5.14
14	4.67	4.20	3.74	3.27	2.80	2.34	1.87	1.40	0.93	0.47

Determination of Uric Acid

(Benedict, S. R., Jour. Biol. Chem., 1922, li, 187)

Principle.—The tungstic acid blood filtrate is heated in a water-bath with a special uric acid reagent. A deep blue color is developed, the intensity of which is compared with that of a standard uric acid solution similarly treated.

Reagents.—

1. BENEDICT'S URIC ACID REAGENT.—One hundred grams of pure sodium tungstate are placed in a liter flask and dissolved in about 600 c.c. of water. Fifty grams of pure arsenic pentoxide are now

added, followed by 25 c.c. of 85 per cent phosphoric acid and 20 c.c. of hydrochloric acid. The mixture is boiled for 20 minutes, cooled and diluted to 1 liter. The reagent appears to keep indefinitely.

2. Standard Uric Acid Solution.—Dissolve 9.0 grams of pure crystalline hydrogen disodium phosphate and 1.0 gram of dihydrogen sodium phosphate in 200 to 300 c.c. of hot distilled water. Pour this warm, clear solution on 200 mg. of pure uric acid suspended in a few c.c. of water in a 1000 c.c. volumetric flask. Agitate until completely dissolved. Add at once *exactly* 1.4 c.c. of glacial acetic acid. Make up to 1000 c.c. Mix and add 5 c.c. of chloroform. One c.c. of this solution contains 0.2 mg. uric acid. This stock solution should be freshly prepared every two months.

The standard solution for use in the determination of uric acid in blood is made from the stock solution by measuring 10 c.c., containing 2 mg. of uric acid, into a 500 c.c. volumetric flask half filled with distilled water. Twenty-five c.c. of dilute hydrochloric acid (1 volume concentrated acid diluted to 10 volumes with water) are added and the flask filled to the mark with distilled water. This solution contains 0.02 mg. uric acid in 5 c.c. The solution should be made fresh once in two weeks.

3. Five per cent sodium cyanide solution containing 2 c.c. of concentrated ammonia per liter. This solution should be made up fresh once in 2 months.

Procedure.—The blood is precipitated with tungstic acid as described under the preparation of protein-free blood filtrates (page 129). The blood should be allowed to stand at least 10 to 20 minutes after adding the tungstate and sulphuric acid before filtration. The use of excess acid in the precipitation is to be avoided. Five c.c. of the water clear filtrate are transferred to a test tube and 5 c.c. of water added. Five c.c. of the standard solution containing 0.02 mg. uric acid are placed in another tube and the volume likewise made up to 10 c.c. To both standard and unknown are added 4 c.c. of the 5 per cent sodium cyanide solution containing 2 c.c. of concentrated ammonia per liter. The cyanide should always be measured from a burette. To each tube is then added 1 c.c. of the uric acid reagent. The contents of the tube should be mixed by one inversion after the addition of the reagent and placed immediately in boiling water where the

tubes should be left 5 minutes after the inversion of the last tube, but the time elapsing between the immersion of the first and last tubes should not exceed one minute. Exactly three minutes after the last tube is immersed the tubes are removed and placed in a large beaker of cold water for three minutes and read in a colorimeter against the standard as soon as may be convenient. It is best to read within 5 minutes.

$\frac{S}{R} \times 4 =$ mg. uric acid per 100 c.c. blood, when S represents the height of the standard and R the reading of the unknown solution.

Remarks.—The normal figure by this method is about 3 mg. uric acid per 100 c.c. blood. No evidence of definite uric acid retention is evident before a figure of 4.0 mg. or over per 100 c.c. is reached.

TABLE XX

DETERMINATION OF URIC ACID IN BLOOD

Take 5 c.c. or 2.5 c.c. of the protein-free blood filtrate and make up to a final volume of 15 c.c.

Use 5 c.c. of the standard uric acid solution containing 0.02 mg. uric acid and make up to a final volume of 15 c.c.

Set standard at 20, or fill 20 mm. Bock-Benedict cell.

Table shows the uric acid in milligrams per 100 c.c. blood corresponding to the different colorimeter readings using a plunger type instrument.

	USING 5 C.C. OF BLOOD FILTRATE (Uric acid in mg. per 100 c.c. blood)					USING 2.5 C.C. OF BLOOD FILTRATE (Uric acid in mg. per 100 c.c. blood)				
COLORIMETER READING	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8
12	6.7	6.5	6.4	6.3	6.2	13.3	13.1	12.9	12.7	12.5
13	6.1	6.1	6.0	5.9	5.8	12.3	12.1	11.9	11.8	11.6
14	5.7	5.6	5.6	5.5	5.4	11.4	11.3	11.1	10.9	10.8
15	5.3	5.3	5.2	5.1	5.1	10.7	10.5	10.4	10.2	10.1
16	5.0	4.9	4.9	4.8	4.8	10.0	9.9	9.8	9.6	9.5
17	4.7	4.7	4.6	4.5	4.5	9.4	9.3	9.2	9.1	9.0
18	4.4	4.4	4.4	4.3	4.2	8.9	8.8	8.7	8.6	8.5
19	4.2	4.2	4.1	4.1	4.0	8.4	8.4	8.3	8.2	8.1
20	4.0	4.0	3.9	3.9	3.8	8.0	7.9	7.8	7.7	7.6
21	3.8	3.8	3.7	3.7	3.7	7.6	7.5	7.5	7.4	7.3
22	3.6	3.6	3.6	3.5	3.5	7.3	7.2	7.2	7.1	7.0
23	3.5	3.4	3.4	3.4	3.4	6.9	6.9	6.8	6.8	6.7
24	3.3	3.3	3.3	3.3	3.3	6.7	6.6	6.6	6.5	6.4
25	3.2	3.2	3.2	3.1	3.1	6.4	6.4	6.3	6.2	6.2
26	3.1	3.1	3.0	3.0	3.0	6.2	6.1	6.1	6.0	6.0
27	2.9	2.9	2.9	2.9	2.9	5.9	5.9	5.8	5.8	5.8

If the reading is high it is best to repeat the determination using 2.5 c.c. of blood filtrate instead of 5 c.c.

It is essential that the volume of the unknown and of the standard be the same during the period of the reaction. Hence, if only 2.5 c.c. of filtrate be used, distilled water must be added to bring the volume up to 10 c.c. before the addition of the cyanide and reagent.

A method for checking uric acid determinations is given on page 251.

Determination of Creatinine

(Folin and Wu: Jour. Biol. Chem., 1919, xxxviii, 81)

Principle.—On adding picric acid and sodium hydroxide to a solution containing creatinine, a deep red color is produced. The intensity of this in the protein-free blood filtrate is compared with that of a standard creatinine solution similarly treated.

Reagents.—(1) Alkaline picrate solution made by adding 5 c.c. of 10 per cent sodium hydroxide to 25 c.c. of saturated picric acid solution. The picric acid should be tested as described under "Determination of Creatinine in Urine," page 52.

(2) Standard creatinine solution prepared as follows:

Transfer to a liter flask 6 c.c. of a standard creatinin solution containing 1 mg. of creatinine per cubic centimeter (see page 52); add 10 c.c. of normal hydrochloric acid, dilute to mark with water and mix. Transfer to a bottle, add 4 to 5 drops xylol. Five c.c. of this solution contain 0.03 mg. of creatinine and this amount plus 15 c.c. of water represents the standard needed for most bloods, as it covers the range of 1 to 2 mg. per 100 c.c. If needed 10 c.c. of this standard and 10 c.c. of water may be used for the range of 2 to 4 mg. per 100 c.c.; 15 c.c. of the standard and 5 c.c. of water for the range of 4 to 6 mg. per 100 c.c.; or 20 c.c. of the standard for the range of 6 to 8 mg. per 100 c.c. of blood.

Procedure.—Transfer 10 c.c. of protein-free blood filtrate (see page 129) to a small flask or test tube, transfer 5 centimeters of the standard creatinine solution to another flask and dilute the standard to 20 c.c. Then add 5 c.c. of the freshly prepared alkaline picrate solution to the blood filtrate and 10 c.c. to the

Table XXI

Determination of Creatinine in Blood

Use 10 c.c. of protein-free blood filtrate made up to a final volume of 15 c.c.

Make final volume of the standard up to 30 c.c.

Set standard at 20, or fill 20 mm. Bock-Benedict cell.

The following table shows the creatinine in mg. per 100 c.c. blood corresponding to the different readings with 5, 10, 15 or 20 c.c., of the standard creatinine solution, using a plunger type colorimeter.

	USING 5 C. C. STANDARD (Creatinine in mg. per 100 c. c. blood)					USING 10 C. C. STANDARD (Creatinine in mg. per 100 c. c. blood)					USING 15 C. C. STANDARD (Creatinine in mg. per 100 c. c. blood)					USING 20 C. C. STANDARD (Creatinine in mg. per 100 c. c. blood)				
Colorimeter Reading	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8
10	3.0	2.9	2.9	2.8	2.8	6.0	5.9	5.8	5.6	5.5	9.0	8.9	8.7	8.5	8.3	12.0	11.8	11.6	11.3	11.1
11	2.7	2.7	2.6	2.6	2.5	5.5	5.4	5.3	5.2	5.1	8.2	8.0	7.9	7.8	7.6	10.9	10.7	10.5	10.4	10.2
12	2.5	2.5	2.4	2.4	2.3	5.0	4.9	4.8	4.8	4.7	7.5	7.4	7.3	7.1	7.0	10.0	9.8	9.7	9.5	9.4
13	2.3	2.3	2.3	2.2	2.2	4.6	4.6	4.5	4.4	4.4	6.9	6.8	6.8	6.7	6.5	9.2	9.1	9.0	8.9	8.7
14	2.1	2.1	2.1	2.1	2.0	4.3	4.2	4.2	4.1	4.1	6.4	6.3	6.2	6.2	6.1	8.6	8.5	8.3	8.2	8.1
15	2.0	2.0	2.0	1.9	1.9	4.0	3.9	3.9	3.9	3.8	6.0	5.9	5.9	5.8	5.7	8.0	7.9	7.8	7.7	7.6
16	1.9	1.9	1.8	1.8	1.8	3.8	3.7	3.7	3.6	3.6	5.6	5.6	5.5	5.4	5.4	7.5	7.4	7.3	7.2	7.1
17	1.8	1.8	1.7	1.7	1.7	3.5	3.5	3.5	3.4	3.4	5.3	5.3	5.2	5.1	5.1	7.1	7.0	6.9	6.8	6.7
18	1.7	1.7	1.6	1.6	1.6	3.3	3.3	3.3	3.2	3.2	5.0	5.0	4.9	4.9	4.8	6.7	6.6	6.5	6.5	6.4
19	1.6	1.6	1.6	1.5	1.5	3.2	3.1	3.1	3.1	3.0	4.7	4.7	4.7	4.6	4.6	6.3	6.2	6.2	6.1	6.1
20	1.5	1.5	1.5	1.5	1.5	3.0	3.0	2.9	2.9	2.9	4.5	4.5	4.4	4.4	4.4	6.0	5.9	5.9	5.8	5.8
21	1.4	1.4	1.4	1.4	1.4	2.9	2.8	2.8	2.8	2.8	4.3	4.2	4.2	4.2	4.1	5.7	5.6	5.6	5.5	5.5
22	1.4	1.4	1.3	1.3	1.3	2.7	2.7	2.7	2.7	2.6	4.1	4.1	4.0	4.0	4.0	5.5	5.4	5.4	5.3	5.3
23	1.3	1.3	1.3	1.3	1.3	2.6	2.6	2.6	2.5	2.5	3.9	3.9	3.9	3.8	3.8	5.2	5.2	5.1	5.1	5.0
24	1.3	1.2	1.2	1.2	1.2	2.5	2.5	2.5	2.4	2.4	3.8	3.7	3.7	3.7	3.6	5.0	5.0	4.9	4.9	4.8
25	1.2	1.2	1.2	1.2	1.2	2.4	2.4	2.4	2.3	2.3	3.6	3.6	3.5	3.5	3.5	4.8	4.8	4.7	4.7	4.7
26	1.2	1.2	1.1	1.1	1.1	2.3	2.3	2.3	2.3	2.3	3.5	3.5	3.4	3.4	3.4	4.6	4.6	4.6	4.5	4.5
27	1.1	1.1	1.1	1.1	1.1	2.2	2.2	2.2	2.2	2.2	3.4	3.3	3.3	3.3	3.2	4.5	4.4	4.4	4.4	4.3
28	1.1	1.1	1.1	1.1	1.1	2.1	2.1	2.1	2.1	2.1	3.2	3.2	3.2	3.2	3.1	4.3	4.3	4.2	4.2	4.2
29	1.0	1.0	1.0	1.0	1.0	2.1	2.1	2.0	2.0	2.0	3.1	3.1	3.1	3.1	3.0	4.1	4.1	4.1	4.1	4.0

diluted creatinine solution. Let stand for eight to ten minutes and compare in colorimeter. Never omit to first ascertain that the two fields of this colorimeter are equal when both cups contain this standard creatinine picrate solution. The color comparison should be completed within 15 minutes from the time the alkaline picrate was added.

Calculation.—Set standard at 20. If R is the reading of the unknown, $\frac{20 \times 1.5}{R}$ = milligrams of creatinine in 100 c.c. of blood provided 5 c.c. of the standard has been used. If 10 c.c. of standard be taken multiply by 3 instead of 1.5; if 15 c.c., multiply by 4.5; if 20 c.c., multiply by 6.

Remarks.—The blood of a normal individual contains 1 to 2 mg. creatinine per 100 c.c.

If the creatinine content is near normal the colors are so light it is difficult to match them well. If the standard solution is made up in saturated picric acid solution and the protein-free blood filtrate is saturated with picric acid by shaking with a few crystals for 5 minutes before adding the other reagents the color will be darker and the readings made easier.

A method for checking creatinine determinations is given on page 251.

If the readings are high the test should be repeated using a larger amount of standard.

Table XXI shows the creatinine corresponding to the different readings with a plunger type colorimeter.

Determination of Creatine

(Folin and Wu: Jour. Biol. Chem., 1919, xxxviii, 81)

Principle.—On heating creatine with dilute mineral acids, it is dehydrated and its anhydride creatinine formed. The creatinine is then determined as for preformed creatinine.

Procedure.—Transfer 5 c.c. of the protein-free blood filtrate to a test tube graduated at 25 c.c. Add 1 c.c. of normal hydrochloric acid. Cover the mouth of this test tube with tin foil and heat in autoclave to 130° C. for 20 minutes, cool, and add 5 c.c. of alkaline picrate solution prepared by adding 5 c.c. of 10 per cent sodium hydroxide to 25 c.c. saturated picric acid solution.

Let stand for 8 to 10 minutes, then dilute to 25 c.c. The standard solution required is 30 c.c. of creatine solution made as described under creatinine determination in a 50 c.c. volumetric flask. Add to this 2 c.c. of normal acid and 10 c.c. of the alkaline picrate solution, and after 10 minutes' standing, dilute to 50 c.c.

Calculation.—Set standard at 20.

If R be the reading of the unknown solution.

$$\frac{20 \times 6}{R} = \text{creatine plus creatinine in milligrams per 100 c.c. of blood.}$$

Remarks.—The normal value for total creatinine by this method is about 6 mg. per 100 c.c. of blood.

Determination of Sugar

(Folin and Wu: Jour. Biol. Chem., 1919, xxxviii, 81; xli, 367)

Principle.—A weakly alkaline copper tartrate solution on heating with the tungstic acid blood filtrate is reduced by the glucose contained therein to cuprous oxide. On adding a special molybdate-phosphate reagent an intense blue color is developed by the action of the reagent on the cuprous oxide. This is compared with a standard solution of glucose similarly treated.

Reagents.—

1. Standard sugar solution. Dissolve one gram of pure anhydrous dextrose in water and dilute to a volume of 100 c.c. Mix, add a few drops of xylol or toluol, and bottle. Dilute 5 c.c. of this stock solution to 500 c.c., giving a solution containing 1 mg. of dextrose per 10 c.c. Similarly dilute 10 c.c. to 500 c.c., giving a solution containing 2 mg. of dextrose per 10 c.c. The stock solution is permanent; the dilutions from the stock solution should be made each month.

2. Alkaline copper solution. Dissolve 40 grams of anhydrous sodium carbonate in about 400 c.c. of water and transfer to a liter flask. Add 7.5 grams of tartaric acid and when the latter has dissolved add 4.5 grams of crystallized copper sulphate; mix and make up to a volume of one liter. If the carbonate used is impure, a sediment may be formed in the course

of a week or two. If this happens, decant the clear solution into another bottle. Two c.c. of this solution when mixed with 2 c.c. of the molybdate phosphate reagent, should show no color. Two c.c. should be decolorized by 1.4 c.c. normal acid.

3. Molybdate-phosphate solution. Transfer to a liter beaker 35 grams of molybdic acid and 5 grams of sodium tungstate. Add 200 c.c. of 10 per cent sodium hydroxide and 200 c.c. of water. Boil vigorously for 20 to 40 minutes so as to remove nearly the whole of the ammonia present in the molybdic acid. (Test with litmus paper.) Cool, dilute to about 350 c.c. and add 125 c.c. of concentrated (85 per cent) phosphoric acid. Dilute to 500 c.c.

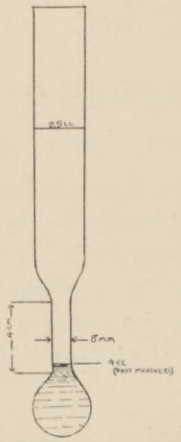


Fig. 44.—Folin blood sugar tube.

Procedure.—Transfer 2 c.c. of the tungstic acid blood filtrate (see page 129) to a special Folin blood sugar tube (Fig. 44), graduated to 25 c.c. To two other similar tubes add 2 c.c. of standard sugar solution containing 0.2 and 0.4 mg. of dextrose respectively. To each tube add 2 c.c. of the alkaline copper tartrate solution. The surface of the solution must now have reached the restricted portion of the tube. If the bulb is too large, a little but not more than 0.5 c.c. of 1:1 dilution of the copper solution may be added. Heat the tubes in boiling water-bath for six minutes. Cool in water-bath without shaking for 2 to 3 minutes. Add 2 c.c. of the phosphate molybdate solution. After the cuprous oxide is dissolved, dilute to 25 c.c. mark. Insert a

rubber stopper in the tube and mix. Compare the unknown solution in the colorimeter with the standard set at 20 millimeters.

Calculation.—

If R is reading of unknown:

$\frac{20}{R} \times 100 =$ milligrams of glucose per 100 c.c. of blood when the weaker standard is used.

$\frac{20}{R} \times 200 =$ milligrams of glucose per 100 c.c. of blood when the stronger standard is used.

TABLE XXII

DETERMINATION OF GLUCOSE IN BLOOD

Use 2 c.c. of the protein-free blood filtrate made up to 25 c.c. and 2 c.c. of the standard made up to a similar volume.

Set standard at 20 or fill 20 mm. Bock-Benedict cell.

Table shows the glucose in mg. per 100 c.c. blood corresponding to colorimeter readings with the two different standards, using an instrument of the plunger type.

COLORIMETER READING	STANDARD NO. 1 CONTAINING 1 MG. GLUCOSE IN 10 C.C. (Glucose in mg. per 100 c.c. blood).					STANDARD NO. 2 CONTAINING 2 MG. GLUCOSE IN 10 C.C. (Glucose in mg. per 100 c.c. blood).				
	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8
5	400	385	370	357	345	800	769	740	714	689
6	333	323	313	303	294	667	645	625	606	588
7	286	278	270	263	256	571	555	540	526	512
8	250	244	238	233	227	500	487	476	465	454
9	222	217	213	209	204	444	434	425	417	408
10	200	196	192	189	185	400	392	384	377	370
11	182	179	175	172	169	363	357	350	344	338
12	167	164	161	159	156	333	327	322	317	312
13	154	152	149	147	145	307	303	298	295	290
14	143	141	139	137	135	286	282	278	273	270
15	133	132	130	128	127	267	263	260	256	254
16	125	124	122	121	119	250	247	244	241	238
17	118	116	115	114	112	236	233	230	227	224
18	111	110	109	108	106	222	220	218	215	212
19	105	104	103	102	101	211	209	206	204	202
20	100	99	98	97	96	200	198	196	194	192
21	95	94	93	93	91	189	188	187	185	183
22	91	90	89	88	87	182	180	179	177	175
23	87	86	86	85	84	173	172	171	169	168
24	83	83	82	81	81	167	165	164	163	161
25	80	79	79	78	78	160	159	158	156	155
26	77	76	76	75	75	154	153	152	150	149
27	74	74	73	73	72	147	147	146	145	144
28	71	71	71	70	69	142	142	140	140	139
29	69	69	68	68	67	137	137	136	135	134

Remarks.—The blood of normal fasting individuals contains 80 to 120 mg. glucose per 100 c.c.

Table XXII shows the blood sugar values corresponding to the different colorimeter readings, with a plunger type of instrument.

A method for checking blood sugar determinations is given on page 250.

Blood Sugar Tolerance Test

(Janney and Isaacson: Jour. Amer. Med. Assn., 1918, lxx, 1131)

Principle.—Glucose is taken by mouth and the blood sugar is determined at regular intervals. The patient takes no food after the evening meal of the day preceding the test.

Procedure.—Five c.c. of blood are obtained by venipuncture and run into a test tube, containing a few crystals of potassium oxalate. One and five-tenths grams of glucose are given for each kilogram of body weight. The glucose is best given in a 50 per cent solution flavored with lemon. Five c.c. of blood are obtained at 30 minute intervals for the next hour, and at the end of two hours, three hours and of four hours. A blood sugar determination is made on each specimen by the Folin method (page 141). A specimen of urine is collected each time the blood is taken and tested for sugar by Benedict's solution. The results are recorded as follows:

Sugar Tolerance Test (	grams glucose given by mouth)
Blood sugar per 100 c.c.	Urine sugar per cent
Fasting Mgms.	per cent
30 Min. "	"
60 Min. "	"
2 Hrs. "	"
3 Hrs. "	"
4 Hrs. "	"

Remarks.—The typically normal blood sugar tolerance curve shows the following:

1. Blood sugar before the glucose is taken 70-120 mg. per 100 c.c.

2. Blood sugar within an hour after taking the glucose rises to 130 to 180 mg. per 100 c.c., this representing the peak of the curve.

3. Blood sugar returns to normal fasting level within 2 to 2½ hours.

The urine shows no sugar normally.

Fig. 45 shows several types of curves obtained in blood sugar tolerance tests.

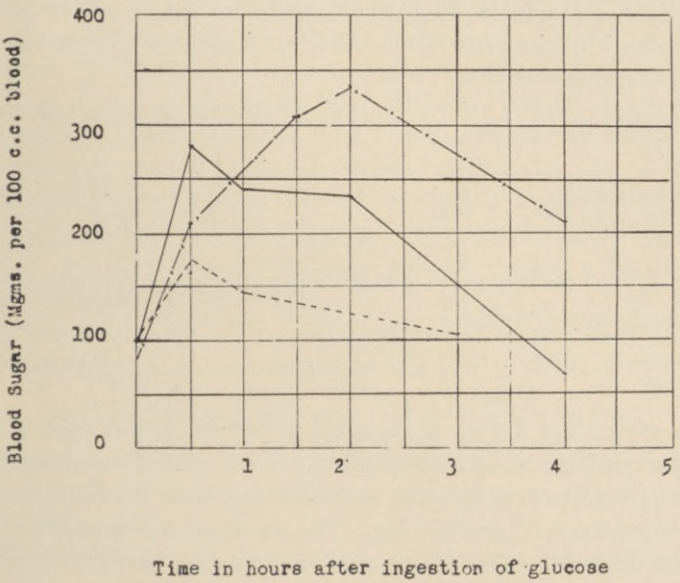


Fig. 45.—Types of curves obtained in blood sugar tolerance test.
---------- Curve of a normal individual.
———————— Curve of a mild diabetic.
—.—.—.—.— Curve of a severe diabetic.

Determination of Chlorides

(McLean and Van Slyke, Jour. Biol. Chem., 1915, xxi, 361; Gettler, A.O., Jour. Am. Med. Assn., 1921, lxxvii, 1652)

The chlorides may be determined in whole blood or in plasma. The chlorides are not uniformly distributed in blood and plasma, the whole blood containing a smaller amount. Hence the variation in plasma chlorides is more marked. It is more accurate, however, to make the determination on whole blood since unless the plasma is separated from the red cells almost immediately after withdrawal, its chlorides increase at the expense of the

corpuscles due to the passage of carbon dioxide from plasma to the corpuscles or its escape into the air.

Principle.—The proteins of the blood or plasma are precipitated with sodium tungstate and sulphuric acid as described under the preparation of protein-free blood filtrates (page 129). The chlorides in the filtrate are precipitated with silver nitrate, the silver chloride is filtered off, and the excess silver nitrate determined by titration with potassium iodide in the presence of nitrous acid and starch.

Reagents.—(1) $AgNO_3$ solution (1 c.c. equivalent to 2 mg. NaCl).

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

(2) M/117 potassium iodide solution (4 c.c. equivalent to 1 c.c. of the silver nitrate).

Potassium iodide	1.419	grams
Water to	1000.0	c.c.

The potassium iodide crystals are dissolved in less than a liter of water and standardized by adding 5 c.c. of the starch solution to 5 c.c. of the silver solution and titrating with the iodide to the first appearance of green color. The solution of potassium iodide is then diluted so that 20 c.c. are exactly equivalent to 5 c.c. of the silver solution.

(3) Starch solution:

Sodium citrate ($Na_3C_6H_5O_7$ plus $5\frac{1}{2}$ H_2O)	446.0	grams
Soluble starch	2.5	grams
Sodium nitrite	20.0	grams
Water to	1000.0	c.c.

Dissolve the starch in 500 c.c. of warm water, add the salts, heat to the boiling point, and boil vigorously for ten minutes. While still hot, filter through cotton, wash the filter with hot water, and after cooling, make filtrate up to 1000 c.c. The citrate solution becomes cloudy on standing, but keeps indefinitely. It is desirable to add a few cubic centimeters of chloroform to prevent the growth of moulds in the solution. The citrate is necessary to regulate the acidity for the endpoint, the nitrite to liberate the iodine from the iodide.

Procedure.—Pipette 20 c.c. of the protein-free filtrate from whole blood or plasma, prepared as described on page 129, into a dry beaker. If the determination is made on plasma, for each volume of plasma eight volumes of water and one-half volume each of 10 per cent sodium tungstate and ⅔ normal sulphuric acid are added; that is, for 4 c.c. of plasma, 32 c.c. of water, and 2 c.c. each of the tungstate and acid would be added. Add 10 c.c. of the silver nitrate solution. Shake and allow to stand at least ten minutes. It is preferable to allow it to stand overnight. Centrifuge or filter through a dry filter paper. Pipette 15 c.c. into a casserole or beaker, add 5 c.c. of the starch citrate solution and titrate with the M/117 potassium iodide to the first appearance of green color. As the endpoint is approached it is advisable, in order to avoid overrunning it, to pause after the addition of each drop of iodide, because the green color requires several seconds to develop.

Calculation.—The 10 c.c. of filtrate used in the determination are equivalent to 1 c.c. of plasma or blood:

$$\text{NaCl in mg. per 100 c.c.} = \left(10 - \frac{\text{c.c.KI}}{2}\right) 100$$

Remarks.—Plasma normally contains 570 to 620 mg. of chlorides calculated as sodium chloride per 100 c.c.; whole blood contains 470 to 520 mg.

In the titration the final acidity is the most important factor. The green color is best developed in slightly acid solution. Too much acid abolishes the blue or green color leaving only the brown color of the iodine liberated by the nitrous acid. If the solution is not acid enough no color at all will be formed because it is necessary for nitrous acid to be present to liberate hydrogen iodide from the potassium iodide. When a blue color is formed after the addition of each drop of iodide during the titration it is an indication that the optimum acidity is approximated.

The sodium tungstate solution used in the determination should be checked against contamination by chloride. This is done by mixing one volume of the 10 per cent sodium tungstate with two volumes of concentrated nitric acid and filtering into a test tube containing silver nitrate. There should be no cloud.

The tungstic acid filtrate should give no precipitate with an equal volume of nitric acid.

Table XXIII shows the chloride corresponding to the amount of potassium iodide used in titrating the excess of silver nitrate.

A method for checking chloride determinations is given on page 252.

TABLE XXIII

DETERMINATION OF CHLORIDES IN BLOOD

Take 15 c.c. of the filtrate after the addition of silver nitrate.

Table shows the number of milligrams of NaCl per 100 c.c. of plasma corresponding to the number of c.c. of KI used to titrate the excess of silver nitrate.

c.c. KI USED	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
4	800	795	790	785	780	775	770	765	760	755
5	750	745	740	735	730	725	720	715	710	705
6	700	695	690	685	680	675	670	665	660	655
7	650	645	640	635	630	625	620	615	610	605
8	600	595	590	585	580	575	570	565	560	555
9	550	545	540	535	530	525	520	515	510	505
10	500	495	490	485	480	475	470	465	460	455
11	450	445	440	435	430	425	420	415	410	405
12	400	395	390	385	380	375	370	365	360	355
13	350	345	340	335	330	325	320	315	310	305
14	300	295	290	285	280	275	270	265	260	255

Determination of Cholesterol

(Myers and Wardell: Jour. Biol. Chem., 1918, xxxvi, 147)

Principle.—The blood plasma is mixed with plaster of Paris, dried, and extracted with chloroform. The depth of color produced on mixing the chloroform extract with acetic anhydride and concentrated sulphuric acid is compared with that of a standard solution of cholesterol similarly treated.

Reagents.—Standard stock solution of cholesterol made by dissolving 1 gram of chemically pure cholesterol in 1,000 c.c. of anhydrous chloroform.

Dilute with chloroform 100 c.c. of this stock solution to 1,000 c.c., giving a solution 5 c.c. of which contains 0.5 mg. cholesterol.

Procedure.—One c.c. of blood plasma is pipetted into a porcelain crucible, or small beaker containing 4 to 5 grams of plaster of Paris, stirred, and dried, preferably in a drying oven. It is now emptied into a small extraction shell (4 cm. long) and then inserted in a short test tube in the bottom and top of which are

a number of small holes. This is attached to a large cork on a small reflux condenser, and the tube and cork are inserted in the neck of a 150 c.c. extraction flask (Fig. 46) containing

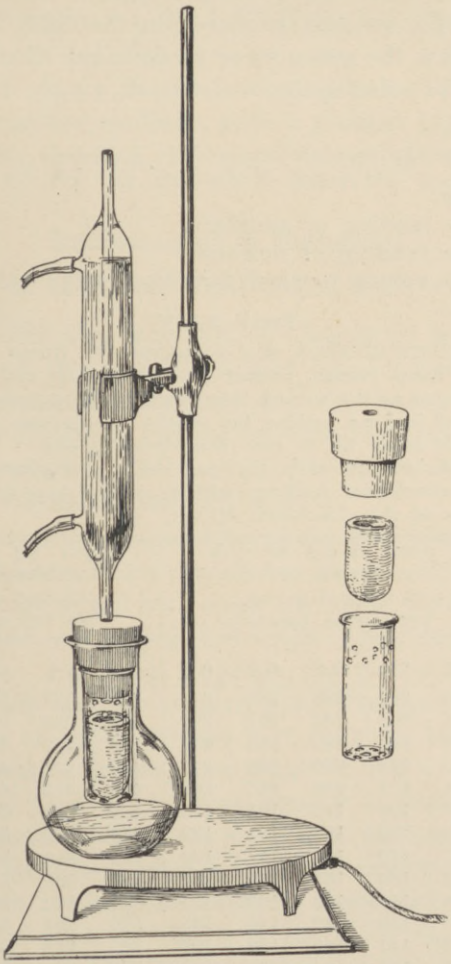


Fig. 46.—Cholesterol extraction apparatus. (*After Myers.*)

about 20 to 25 c.c. of chloroform. Extraction is continued for 30 minutes on an electric hot plate, the chloroform made up to some suitable volume such as 15 c.c., filtered if necessary, and colorimetric estimation carried out as follows: 5 c.c. of the

chloroform extract are pipetted into a dry test tube and into a second tube 5 c.c. of the standard solution containing 0.5 mg. of cholesterol. Add to each tube, 2 c.c. of acetic anhydride, and 0.1 c.c. of anhydrous concentrated sulphuric acid. After thorough mixing, the solution is placed in the dark for exactly 10 minutes to allow the green color to develop. Compare the two solutions in the colorimeter.

Calculation.—

$$\frac{S}{R} \times 0.5 \times \frac{V}{5} \times 100 = \text{mg. cholesterol per 100 c.c. of plasma.}$$

Where S = reading of standard
R = reading of unknown
V = volume to which the chloroform extract is made up.

TABLE XXIV

DETERMINATION OF CHOLESTEROL IN BLOOD

Use 1 c.c. of blood plasma, extract with chloroform and make up to 15 c.c. Compare 5 c.c. or 2.5 c.c. of this extract with a standard containing 0.5 gm. cholesterol in 5 c.c. making the volume in each case up to 7.1 c.c.

Set standard at 20 or fill 20 mm. Bock-Benedict cell.

Table shows the cholesterol in mg. per 100 c.c. of plasma corresponding to the different colorimeter readings with a plunger type instrument, using different amounts of the chloroform extract.

	USING 5 C.C. OF THE CHLOROFORM EXTRACT (Cholesterol in mg. per 100 c.c. plasma).					USING 2.5 C.C. OF THE CHLOROFORM EXTRACT (Cholesterol in mg. per 100 c.c. plasma).				
COLORIMETER READING	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8
10	300	294	289	282	277	600	588	577	564	554
11	273	268	263	259	254	546	536	526	518	508
12	250	246	242	238	234	500	492	484	476	468
13	230	228	225	222	218	460	456	450	444	436
14	214	211	208	205	203	428	422	416	410	406
15	200	198	195	193	190	400	395	389	385	380
16	188	185	183	181	179	375	370	366	361	357
17	177	175	173	171	169	353	349	345	341	337
18	167	165	163	162	159	333	330	326	323	319
19	158	156	155	153	152	316	312	309	306	303
20	150	149	147	146	145	300	297	294	291	289
21	143	141	140	139	138	285	282	280	277	275
22	137	135	134	133	132	273	270	268	265	263
23	131	130	129	127	126	261	259	257	255	252
24	125	124	123	122	121	250	248	246	244	242
25	120	119	118	117	117	240	238	236	234	233
26	115	115	114	114	113	230	229	228	227	225
27	111	111	110	109	108	224	222	220	218	216
28	107	107	106	105	104	215	213	211	210	208
29	103	103	102	102	101	207	205	204	203	202

Remarks.—Normal blood contains 140 to 170 mg. cholesterol per 100 c.c. (plasma).

For the proper color development it is necessary that the acetic anhydride, the chloroform, and the sulphuric acid be anhydrous. The acetic anhydride may be redistilled using the distillate between 134° C. and 140° C.

Table XXIV shows the cholesterol values corresponding to different colorimeter readings, with a plunger type instrument.

A method for checking cholesterol determinations is given on page 252.

Determination of Phosphates

(Bloor, W. R.: Jour. Biol. Chem., 1918, xxxvi, 31)

Principle.—The determination of phosphates is based on the precipitation of the phosphoric acid by strychnine molybdate as strychnine phosphomolybdate and the measurement of the amount of precipitate by comparing it nephrometrically with the precipitate produced, under conditions as nearly identical as possible, in a standard phosphate solution.

Reagents.—1. Strychnine Molybdate Solution.—A solution of sodium molybdate is first prepared as follows: 72 grams of pure molybdic acid are mixed with about 300 c.c. of water and neutralized with 40 per cent sodium hydroxide (free from all but traces of phosphate). Pure acid requires the theoretical amount of 100 c.c.; impure samples require less. The molybdate, now in clear solution, is boiled for one half an hour, adding water to keep the volume constant, and alkali if the solution becomes turbid. About 1 gram of talcum powder is added, and after a further 5 minutes' boiling, the solution is filtered, and the filter washed once with hot water, adding the washings to the main filtrate. After cooling, the solution containing approximately 100 grams of sodium molybdate is ready for use. Sufficient of the sodium molybdate solution is taken to contain 30 to 35 gm. of sodium molybdate (or this amount of dry sodium molybdate dissolved in a small amount of water) is measured into a precipitating jar or large beaker (2 liters), and 250 c.c. of a mixture of equal parts of concentrated HCl and water are added with stirring. Five hundred c.c. of water are mixed with the solution

and 40 to 50 c.c. of saturated (about 3 per cent) strychnine sulphate solution slowly added with stirring. Two hundred c.c. more of the dilute acid and 500 c.c. more water are added, and after mixing, the turbid solution is allowed to stand overnight or longer if convenient. After the precipitate has settled, most of the liquid may be poured off clear. The remainder is filtered through a hardened phosphorus-free filter. For use in the determination, 25 c.c. of this solution are taken without further additions.

2. Acid Ammonium Sulphate.—Saturated ammonium sulphate, free from all traces of phosphate, to which has been added 15 c.c. of glacial acetic acid per liter.

3. Standard Phosphate Solution.—

(a) Stock standard, containing in 100 c.c. 0.0834 gram of pure acid potassium phosphate.

(b) Standard for use: Dilute 25 c.c. of the above to 500 c.c. Each 5 c.c. of this standard contains 0.15 mg. of H_3PO_4. The dilute solution deteriorates in hot weather and should be made at least once a month.

4. Sodium Hydroxide (from sodium) 10 to 20 per cent.

5. Concentrated Sulphuric Acid and Nitric Acid free from all but traces of phosphates.

6. Dilute Sulphuric Acid.—One part of the concentrated acid and 3 parts of water.

Blanks run with the acids, alkalies, and ammonium sulphate should show only a slight cloudiness on standing for one hour with the reagent. The water should be stored in glass.

(1) **Procedure for Determination of Total Phosphates in Plasma.**—One half c.c. of plasma is measured with a 0.5 c.c. Ostwald pipette into a large (200 × 20 mm.) test tube, four glass beads about 3 mm. in diameter, and 1.5 c.c. of a mixture of concentrated sulphuric acid and nitric acid are added, and the whole heated with a microburner in the hood. The heating is carried out in three stages. In the first stage the mixture is raised to boiling and then the flame is turned down until only a slow but continuous bubbling takes place. Heating is continued at this rate until red fumes cease to come off. This ordinarily does not require more than fifteen minutes. In the second stage of heat-

ing the flame is increased until the water is driven off and strong heating with volatilization of a part of the sulphuric acid is continued for 8 to 10 minutes, taking care not to heat so strongly that the tube approaches dryness, in which case loss of phosphoric acid may occur. The sulphuric acid solution should be now clear and colorless. If it is brownish in color a drop of HNO_3 should be added and the heating continued for one minute. In the third stage the mixture is allowed to cool somewhat (say for about 2 minutes) and then one or more drops of one per cent cane sugar are added. (The amount added should be enough to produce a deep browning of the hot solution and the color should disappear when it is boiled. If too much sugar has been added, and the brown or yellow color persists after a half minute of cooling, a trace of nitric acid should be added and the boiling continued). The solution is then boiled until the moisture is gone—about one minute—then cooled and about 10 c.c. of water are added, rinsing down the sides of the tube. The solution in the tube is neutralized by approximate titration with 10 per cent NaOH (from sodium) using one drop of 0.3 per cent phenolphthalein as indicator, noting the amount of alkali added, then just made acid with a drop or two of dilute sulphuric acid (25 per cent). It is then cooled, transferred quantitatively to a 25 c.c. glass-stoppered, graduated flask, and the mixture made up to mark with water, and the whole mixed.

Five c.c. of the standard acid potassium phosphate solution (containing 0.15 mg. of H_3PO_4) are measured into a 25 c.c. glass-stoppered, graduated flask, a drop of phenolphthalein added, and the amount of alkali used in neutralizing the digestion mixture above run in. The solution is then made just acid with the 25 per cent H_2SO_4, cooled, made up to the mark with water, and well mixed. Twenty-five c.c. portions of the strychnine molybdate reagent are measured into each of two 50 c.c. glass-stoppered, graduated flasks. Five c.c. of the standard solution are run into one of the flasks, and 5 c.c. of the test solution are similarly added to the other. When the solutions are well mixed they are allowed to stand at least three minutes, then filled up to the mark with water, and mixed by inverting several times, after which

they are ready to be compared in the nephelometer. (See page 246.)

The nephelometer tubes are filled with the solutions to the same height and to the point at which, when the tubes are in position in the nephelometer, the meniscus is just out of reach of light.

Calculation.—If R be the reading of the test solution and the standard be set at 20:

$$\frac{20}{R} \times 30 = \text{mg. phosphoric acid per 100 c.c. plasma.}$$

(2) **Procedure for the Determination of Lipoid Phosphoric Acid.**—Two c.c. of plasma are measured into a 50 c.c. volumetric flask containing 35 to 40 c.c. of a mixture of 3 parts alcohol and 1 part ether (both redistilled). The blood is made to enter in a slow stream of drops and the liquid in the flask kept rotating rather rapidly so as to prevent the formation of large aggregates of precipitate which are difficult to extract. The flask and contents are then immersed in boiling water with frequent and strong rotation (to prevent superheating) until the liquid begins to boil, cooled to room temperature, made up to volume, mixed and filtered. For the determination 15 c.c. (equals 0.6 c.c. plasma) are measured into one of the large test tubes, glass beads added, and the whole evaporated to dryness in a boiling water-bath. The tube should be shaken frequently until boiling begins, after which the solution will proceed quietly to dryness. It should be left in the bath a few minutes after it is apparently dry to remove traces of alcohol which would interfere with the subsequent oxidation. One and one-half c.c. of sulphuric-nitric acid mixture are added, distributed by shaking to the material on the sides of the tube, and the mixture is digested in the same way as directed for total phosphates.

Calculation.—If R be the reading of the test solution and the standard be set at 20:

$$\frac{20}{R} \times 25 = \text{mg. lipoid phosphoric acid per 100 c.c. plasma.}$$

(3) **Procedure for Inorganic, Acid Soluble, and Other Forms of Phosphoric Acid.**—Three c.c. of plasma are run slowly into 20 c.c. of acid ammonium sulphate solution in a 25 c.c. glass-

stoppered, graduated flask, the volume made to the mark with water, the whole mixed, and let stand with occasional shaking for at least 10 minutes. It is then filtered. After the filter has drained it should be folded in the funnel and pressed out with a clean stirring rod to get as much filtrate as possible. The filtrate is clear and colorless and contains no detectable protein. With this filtrate determine:

(1) INORGANIC.—(a) Ten c.c. of the filtrate (=1.2 c.c. of plasma) are measured into one of the 25 c.c. flasks and made to the mark with water. A standard is prepared by adding to another flask 3 c.c. of the standard phosphate (0.09 mg. of H_3PO_4) and acid ammonium sulphate equal to that present in the test solution (8 c.c. is a sufficiently close approximation). The flask is then filled to the mark with water. The determination should be made promptly after filtering.

Twenty-five c.c. portions of the strychnine molybdate reagent are measured into each of two 50 c.c. glass-stoppered, volumetric flasks, and 5 c.c. of the standard solution are run into one of the flasks and 5 c.c. of the test solution are similarly added to the other. When the solutions are well mixed they are allowed to stand at least 3 minutes, then filled to the mark with water, and mixed by inverting several times, after which they are ready to be compared in the nephelometer.

CALCULATION.—If R be the reading of the unknown solution and the standard be set at 20:

$$\frac{R}{20} \times 7.5 = \text{mg. inorganic phosphoric acid per 100 c.c. plasma.}$$

(b) Where many determinations are to be made it is advisable to make a special standard—when determinations can be made more simply as follows:

STANDARD.—Made by measuring 2 c.c. of the strong stock standard phosphate solution (= 1.2 mg. of H_3PO_4) into a 100 c.c. flask, adding 80 c.c. of the acid ammonium sulphate and making up to 100 c.c. with water. Of this standard 5 c.c. contain 0.06 mg. of H_3PO_4. For the determination 5 c.c. of the filtrate (= 0.6 c.c. of plasma) are measured directly into 25 c.c. of the strychnine molybdate reagent in one 50 c.c. graduated flask, 5 c.c. of the standard solution into another, and after mixing and stand-

ing 3 minutes, the flasks are filled to the mark, the solution mixed, and the determination made.

Calculation.—If R be the reading of the unknown solution and the standard be set at 20:

$\frac{R}{20} \times 10 =$ mg. inorganic phosphoric acid per 100 c.c. plasma.

(2) Acid Soluble.—Ten c.c. of the filtrate are measured into one of the large test tubes, glass beads and 1.5 c.c. of the sulphuric-nitric acid mixture are added and the mixture digested. The digestion presents some difficulties because of the large amount of ammonium sulphate present. The first stage is passed over quickly, then in the second stage when the mixture thickens and begins to foam, the heat is moderated, and so continued until foaming ceases and the salt fuses to a small volume in the tube. Heating is then carried on at a rate just sufficient to prevent loss of ammonium sulphate from the tube by volatilization for 10 minutes. The tubes are then cooled, treated with one drop of 0.3 per cent cane sugar solution, and reheated in the regular way. After dissolving in water the solution is titrated with alkali, noting the amount used. Transfer to 25 c.c. volumetric flask and make up to mark with water. The standard is prepared by adding to another 25 c.c. flask, 3 c.c. of the standard phosphate solution (= 0.09 mg. H_3PO_4), 8 c.c. of the acid ammonium sulphate solution, and the amount of alkali used to neutralize the test solution. The mixture is then neutralized and made up to the mark with water. Twenty-five c.c. portions of the strychnine molybdate reagent are measured into each of two 50 c.c. glass-stoppered flasks. Five c.c. of the standard solution are run into one of the flasks and 5 c.c. of the test solution are similarly added to the other. When the solutions are well mixed they are allowed to stand at least three minutes, then filled to the mark with water, and mixed by inverting several times, after which they are compared in the nephelometer.

Calculation.—If R be the reading of the unknown and the standard be set at 20:

$\frac{R}{20} \times 7.5 =$ mg. acid soluble phosphoric acid per 100 c.c. plasma.

Table XXV

Determination of Phosphoric Acid in Blood

In determining total phosphates use 0.5 c.c. oxalated plasma made up to 25 c.c.; and compare with a standard containing 0.15 mg. of phosphoric acid in a similar volume; for lipoid phosphoric acid use the equivalent of 0.6 c.c. plasma and compare with a standard containing 0.15 mg. phosphoric acid; for inorganic or acid soluble phosphates employ the equivalent of 1.2 c.c. plasma and compare with standard containing 0.09 mg. phosphoric acid.

In each determination set standard at 20.

Table shows the amount of different forms of phosphoric acid in mg. per 100 c.c. of plasma corresponding to the different nephelometer readings.

	TOTAL PHOSPHORIC ACID (H_3PO_4 in mg. per 100 c.c. plasma).					LIPOID PHOSPHORIC ACID (H_3PO_4 in mg. per 100 c.c. plasma).					ACID SOLUBLE OR INORGANIC PHOSPHORIC ACID (H_3PO_4 in mg. per 100 c.c. plasma).				
NEPHELOMETER READING	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8
15	40.0	39.5	38.9	38.5	38.0	33.3	32.9	32.4	32.1	31.7	10.0	9.9	9.7	9.6	9.5
16	37.5	37.0	36.6	36.1	35.7	31.3	30.9	30.5	30.1	29.8	9.4	9.3	9.2	9.0	8.9
17	35.3	34.9	34.5	34.1	33.7	29.4	29.0	28.7	28.4	28.1	8.8	8.7	8.6	8.5	8.4
18	33.3	33.0	32.6	32.3	31.9	27.8	27.5	27.2	26.9	26.6	8.3	8.2	8.1	8.1	8.0
19	31.6	31.2	30.9	30.6	30.3	26.3	26.0	25.7	25.5	25.3	7.9	7.8	7.7	7.6	7.6
20	30.0	29.7	29.4	29.1	28.9	25.0	24.7	24.5	24.3	24.1	7.5	7.4	7.3	7.3	7.2
21	28.5	28.2	28.0	27.7	27.5	23.7	23.5	23.4	23.1	22.9	7.1	7.0	7.0	6.9	6.8
22	27.3	27.0	26.8	26.5	26.3	22.8	22.5	22.3	22.1	21.9	6.8	6.7	6.7	6.6	6.6
23	26.1	25.9	25.7	25.4	25.2	21.7	21.6	21.4	21.1	20.9	6.5	6.5	6.4	6.4	6.3
24	25.0	24.8	24.6	24.4	24.2	20.8	20.7	20.5	20.3	20.2	6.2	6.2	6.1	6.1	6.0
25	24.0	23.8	23.6	23.4	23.3	20.0	19.8	19.7	19.5	19.4	6.0	6.0	5.9	5.9	5.8

(3) Other Forms of Phosphoric Acid.—Obtained by subtracting inorganic from acid soluble phosphoric acid.

Remarks.—The average amount of the different forms of phosphoric acid found in normal individuals, per 100 c.c. plasma is as follows: (1) Total, 32 mg. (2) Acid soluble, 10.4 mg. (3) Inorganic, 8.7 mg. (4) Lipoid, 22.1 mg. (5) Other forms, 1.73 mg.

The quantities of plasma taken are based on average values for normal human plasma. Variations in amount of blood, or strength of standard may be necessary in special cases.

The readings obtained in the nephelometer are not exactly proportional to the amount of phosphoric acid in the solution. Solutions up to about 25 per cent stronger or weaker than the standard may be used, however, without correction.

Table XXV shows the phosphoric acid values corresponding to different nephelometer readings.

Determination of Calcium

(Lyman, H.: Jour. Biol. Chem., 1917, xxix, 169)

Principle.—The calcium in the blood plasma is precipitated as the oxalate under conditions to minimize the occlusion of magnesium; the precipitate is centrifuged, washed, and dissolved in nitric acid, and treated with a solution of ammonium stearate. The resulting cloud of calcium soap is read in a nephelometer against a standard solution of calcium oxalate similarly treated.

Collection of Blood Sample.—Withdraw 10 c.c. of blood by venipuncture and run into a tube containing 0.1 gram of solid sodium citrate. Centrifuge and use plasma for the test.

Reagents.—(1) Trichloracetic acid, 6.5 per cent aqueous solution.

(2) Indicator, methyl orange, 0.1 per cent. Dissolve 0.1 gram of methyl orange in 10 c.c. of alcohol and make up to 100 c.c. with water.

(3) Oxalic acid, 4 per cent solution.

(4) Sodium acetate. Dissolve 20 gm. of the crystallized sodium acetate in 100 c.c. of water.

(5) Ammonium oxalate, 0.5 per cent solution.

(6) Ammonium stearate reagent. Dissolve 4 grams of stearic acid and 0.5 c.c. of oleic acid in 400 c.c. of hot alcohol. Add 20 grams of ammonium carbonate dissolved in 100 c.c. of hot water and allow the mixture to boil a few minutes. Cool. Add 400 c.c. of alcohol, 100 c.c. of water, and 2 c.c. of ammonium hydroxide (sp.gr. 0.9). Filter. This solution should be as clear as freshly distilled water and perfectly colorless. If well stoppered it keeps indefinitely. Before using for analysis test as follows: Into two flasks pipette respectively 5 and 10 c.c. of the calcium oxalate standard and to the 5 c.c. add 5 c.c. of nitric acid, 0.05 N. Treat both with 25 c.c. of the ammonium stearate reagent and read in the nephelometer. If they do not read exactly 2 to 1 there is some impurity present in the chemicals used. The alcohol, if it has stood in a wooden barrel, will give a yellowish coloration with ammonia and will contain suspended particles which reflect light in the nephelometer. It should be redistilled with calcium carbonate. Stearic acid may be purified by recrystallizing from boiling alcohol. Ammonium carbonate may be resublimed.

(7) Calcium oxalate standard.—Dissolve 72.9 mg. of pure calcium oxalate ($CaC_2O + 1H_2O$) in 25 c.c. of nitric acid, 2N, and make up to 1,000 c.c. with distilled water.

10 c.c. of this standard contains 0.2 mg. of calcium; 7.5 c.c. contains 0.15 mg.; 5 c.c. contains 0.10 mg.

(8) Nitric acid, 0.1 N and 0.05 N. These do not have to be exact. They are conveniently made from the 2N acid.

(9) Ammonium Hydrate, 2N. Thirteen and one-half c.c. of ammonium hydrate, sp.gr. 0.9, made up to 100 c.c. of water will serve the purpose.

Use calcium-free filter paper. Baker and Adamson's paper "A" will answer the purpose. For filtering the reagents absorbent cotton washed first with 10 per cent hydrochloric acid, and then with water until the wash water is no longer acid to litmus, and finally dried, may be used.

Procedure.—Run 5 c.c. of blood plasma into a small flask containing 15 c.c. of trichloracetic acid, 6.5 per cent, while agitating the flask. Mix and let stand for a few minutes. Filter through a calcium-free filter paper. Pipette 10 c.c. of the filtrate

into an Erlenmeyer flask of about 50 c.c. capacity. Add one drop of methyl orange, 0.1 per cent. Add 2 N ammonium hydrate drop by drop until just yellow. Add nitric acid, 0.05 per cent, dropwise until just pink, and then 1 c.c. more. Add 1 c.c. of oxalic acid, 4 per cent. Add 1 c.c. of sodium acetate, 20 per cent, dropwise. Cool under the water tap until a faint cloud appears. Shake ten minutes or let stand overnight, at room temperature, as convenient. Rinse the stopper with ammonium oxalate, 0.5 per cent. Pour into a centrifuge tube and centrifuge. Pipet off the supernatant liquor. Rinse the flask with 5 c.c. of ammonium oxalate, 0.5 per cent, pour into a centrifuge tube, stir, rinsing the rod with 0.5 per cent ammonium oxalate, and again centrifuge. Pipet off the supernatant liquor. Dissolve the precipitate in 5 c.c. of 0.1 N nitric acid by means of stirring, and pour into the original flask. Agitate a moment to dissolve any precipitate adhering to the walls. Rinse the rod and centrifuge tube with 5 c.c. of water, and pour into the flask.

Prepare three standards in Erlenmeyer flasks of 100 c.c. capacity as follows: No. 1, 20 c.c. of the standard calcium oxalate solution; No. 2, 15 c.c. of the standard and 5 c.c. of distilled water; No. 3, 10 c.c. of the standard and 10 c.c. of water. Pipette 50 c.c. of the ammonium stearate solution into each of three dry beakers, and 25 c.c. into a fourth. Pour the three standard solutions respectively into the beakers containing the 50 c.c. of reagent and the unknown into the 25 c.c. and pour back twice. Stopper and let stand ten minutes. Compare the unknown in the nephelometer with the standard it most nearly matches.

Calculation.—Set the standard at 32. If R be the reading of the unknown, $\frac{32}{R} \times 8$ equals mg. of calcium (as Ca) per 100 c.c. of plasma if standard No. 1 be used.

$\frac{32}{R} \times 6$ equals mg. of calcium per 100 c.c. if standard No. 2 be used.

$\frac{32}{R} \times 4$ equals mg. of calcium per 100 c.c. if standard No. 3 be used.

Table XXVI

Determination of Calcium in Blood

Make volume of the unknown solution up to 35 c.c.; that of the standards up to 70 c.c.
Set standard at 32.
Table shows the calcium as Ca in milligrams per 100 c.c. plasma, corresponding to the different nephelometer readings.

	USING STANDARD NO. 1 (Calcium as Ca per 100 c.c. plasma)					USING STANDARD NO. 2 (Calcium as Ca per 100 c.c. plasma)					USING STANDARD NO. 3 (Calcium as Ca per 100 c.c. plasma).				
NEPHELOMETER READING	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8	0.0	0.2	0.4	0.6	0.8
24	10.6	10.6	10.5	10.4	10.3	8.0	8.0	7.9	7.8	7.8	5.3	5.3	5.3	5.2	5.2
25	10.2	10.1	10.0	10.0	9.9	7.7	7.6	7.6	7.5	7.5	5.1	5.0	5.0	5.0	5.0
26	9.8	9.8	9.7	9.7	9.6	7.4	7.4	7.3	7.2	7.2	4.9	4.9	4.8	4.8	4.8
27	9.5	9.4	9.3	9.3	9.2	7.1	7.1	7.0	6.9	6.9	4.7	4.7	4.7	4.6	4.6
28	9.0	9.0	9.0	8.9	8.8	6.9	6.8	6.8	6.7	6.6	4.5	4.5	4.5	4.5	4.4
29	8.8	8.7	8.7	8.6	8.5	6.6	6.6	6.5	6.5	6.4	4.4	4.4	4.4	4.3	4.3
30	8.5	8.4	8.4	8.3	8.3	6.4	6.3	6.3	6.3	6.2	4.3	4.2	4.2	4.2	4.2
31	8.3	8.2	8.1	8.1	8.0	6.2	6.2	6.1	6.1	6.0	4.2	4.1	4.1	4.0	4.0
32	8.0	7.9	7.9	7.8	7.8	6.0	5.9	5.9	5.9	5.8	4.0	4.0	3.9	3.9	3.9
33	7.8	7.7	7.6	7.6	7.5	5.8	5.8	5.7	5.7	5.7	3.9	3.9	3.8	3.8	3.8
34	7.5	7.5	7.4	7.4	7.4	5.6	5.6	5.6	5.5	5.5	3.8	3.7	3.7	3.7	3.7
35	7.3	7.3	7.2	7.2	7.2	5.5	5.5	5.4	5.4	5.4	3.7	3.6	3.6	3.6	3.6
36	7.1	7.1	7.0	7.0	6.9	5.3	5.3	5.3	5.2	5.2	3.5	3.5	3.5	3.5	3.5
37	6.9	6.9	6.8	6.8	6.8	5.2	5.2	5.1	5.1	5.1	3.5	3.4	3.4	3.4	3.4
38	6.7	6.7	6.6	6.6	6.6	5.0	5.0	5.0	4.9	4.9	3.4	3.4	3.3	3.3	3.3
39	6.5	6.5	6.5	6.5	6.4	4.9	4.9	4.9	4.8	4.8	3.3	3.3	3.2	3.2	3.2
40	6.4	6.4	6.3	6.3	6.3	4.8	4.8	4.7	4.7	4.7	3.2	3.2	3.2	3.2	3.1

Remarks.—Using this method the average normal calcium per 100 c.c. of plasma is 10 mg.

Table XXVI shows the calcium values corresponding to the different nephelometer readings.

Determination of Acetone Bodies in Blood

(Van Slyke, D.D., and Fitz, R.: Jour. Biol. Chem., 1917, xxxii, 495)

The same procedure and reagents used in the determination of acetone bodies in urine (see page 62) may be applied to blood, after the proteins of the blood have been removed.

Removal of Proteins.—1. Whole Blood.—Dilute 10 c.c. of whole blood with 100 c.c. of water in a 250 c.c. flask, and add 10 c.c. of the 10 per cent mercuric sulphate. Shake for a moment until the protein coagulates, and fill to the mark. After 15 minutes or longer filter through a dry folded filter.

2. Plasma or Serum.—Dilute 8 c.c. of plasma or serum in a 20 c.c. flask with 30 to 40 c.c. water and add 15 c.c. of the mercuric sulphate. Shake gently until the precipitate coagulates in floccules and then fill to mark with water. After standing 15 minutes or longer the solution is filtered through a dry folded filter.

Determination.—For the determination of acetone plus acetoacetic acid, of beta-hydroxybutyric acid, or of the total acetone bodies together, 125 c.c. of the filtrate corresponding to 5 c.c. of blood may be treated exactly as the 25 c.c. of urine filtrate plus 100 c.c. water in urine analysis. If it is desired to determine separately in a single sample of blood both the acetone plus acetoacetic acid, and the beta-hydroxybutyric acid the preformed acetone and that from the acetoacetic acid are precipitated as described for urine and the beta-hydroxybutyric acid determined in the filtrate.

The filtrate from the mercury-acetone precipitate is received in a dry flask. One hundred sixty c.c., equivalent to $^{160}/_{170} \times 5$ c.c. blood of the filtrate is measured into an Erlenmeyer flask and 10 c.c. water added. The mixture is heated to boiling under a reflux condenser, 5 c.c. of the dichromate solution added, and the

determination continued as described for total acetone bodies in urine.

The procedure for beta-hydroxybutyric acid followed in urine cannot be used in blood because the excess mercury used in removing the proteins previous to boiling off the acetone, would partly precipitate the latter before it escaped.

TABLE XXVII

FACTORS FOR CALCULATING RESULTS WHEN FILTRATE EQUIVALENT TO 5 C.C. OF BLOOD IS USED FOR DETERMINATION

DETERMINATION PERFORMED	ACETONE BODIES CALCULATED AS GRAM OF ACETONE PER LITER OF BLOOD, INDICATED BY:	
	1 gm. of precipitate	*1 c.c. of 0.2 KI Sol.*
Total acetone bodies	12.8	0.161
Beta-hydroxybutyric acid	13.2	0.72
Acetone plus acetoacetic acid	10.0	0.130

Remarks.—Normal blood when analyzed for total acetone bodies yields by this method 13 to 26 mg. acetone bodies calculated as acetone.

Determination of the Bicarbonate Content of the Blood Plasma Under Constant Carbon Dioxide Tension

(Van Slyke and Cullen: Jour. Biol. Chem., 1917, xxx, 289; Van Slyke: Jour. Biol. Chem., 1917, xxx, 347)

Principle.—A given volume of blood plasma is saturated with carbon dioxide under normal tension by shaking it in a separatory funnel filled with air whose carbon dioxide tension is approximately equal to that of normal arterial blood. The plasma is then run into a special apparatus, acid added, and carbon dioxide liberated by the production of a Torricellian vacuum. The carbon dioxide is measured at atmospheric pressure, and the volume corresponding to 100 c.c. of plasma calculated.

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

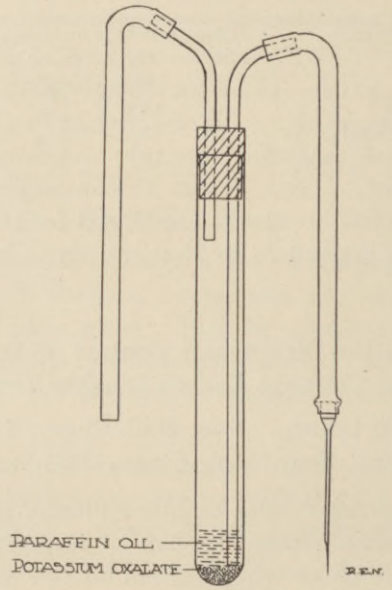


Fig. 47.—Tube used in collecting blood for the determination of the carbon dioxide combining power. (*After Van Slyke and Cullen.*)

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 for this purpose (Fig. 47). 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 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 (Fig. 48) of about 300 c.c. capacity and the funnel is filled with

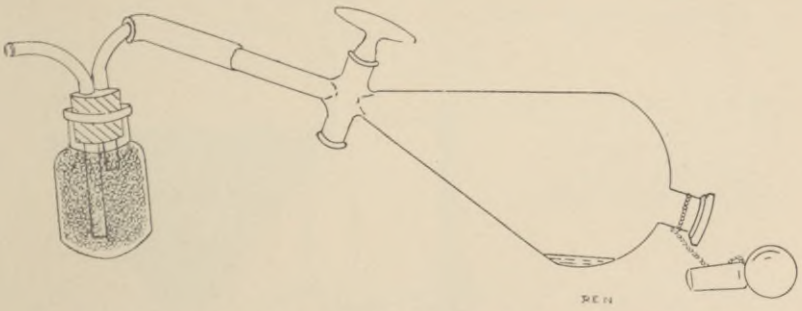


Fig. 48.—Separatory funnel, used in saturating blood plasma with carbon dioxide. (*After Van Slyke and Cullen.*)

alveolar air from the lungs of the operator. The air is passed through a bottle full of glass beads before it enters the funnel, in order to bring the moisture content down to saturation at room temperature. If one blows directly into the separatory funnel, enough moisture condenses on the walls to dilute the plasma appreciably. The lungs are completely emptied through the funnel by a quick, forced expiration. The stopper is inserted just before the stream of breath stops. The funnel is then rotated for two minutes in such a manner that the plasma is distributed as completely as possible about the walls, forming a thin layer, which quickly approaches equilibrium with the CO_2 in the air.

Analysis.—The determination of the carbon dioxide content of the saturated plasma is performed as follows: The CO_2 appa-

ratus (Fig. 49) held in a strong clamp on a ringstand is completely filled with mercury, which should fill both capillaries above the upper stopcock "e." The mercury leveling bulb is placed about on a level with the lower cock. The cup "b" at the top of the apparatus is washed out thoroughly with dilute ammonia, followed by water, medicine droppers being convenient for

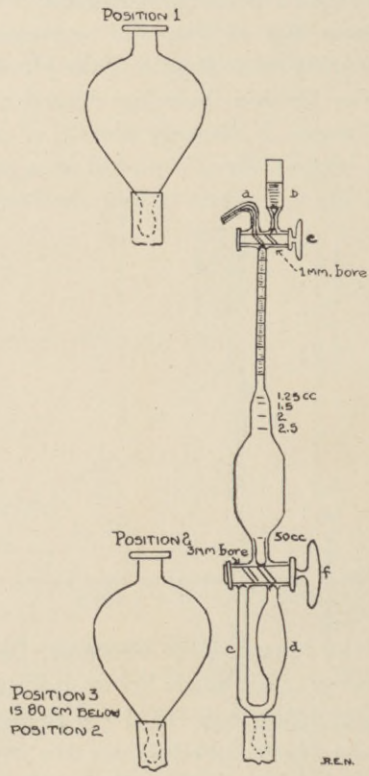


Fig. 49.—Van Slyke carbon dioxide apparatus. (*After Van Slyke.*)

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 stopcock in order to seal the same and make it capable of holding an absolute vacuum.

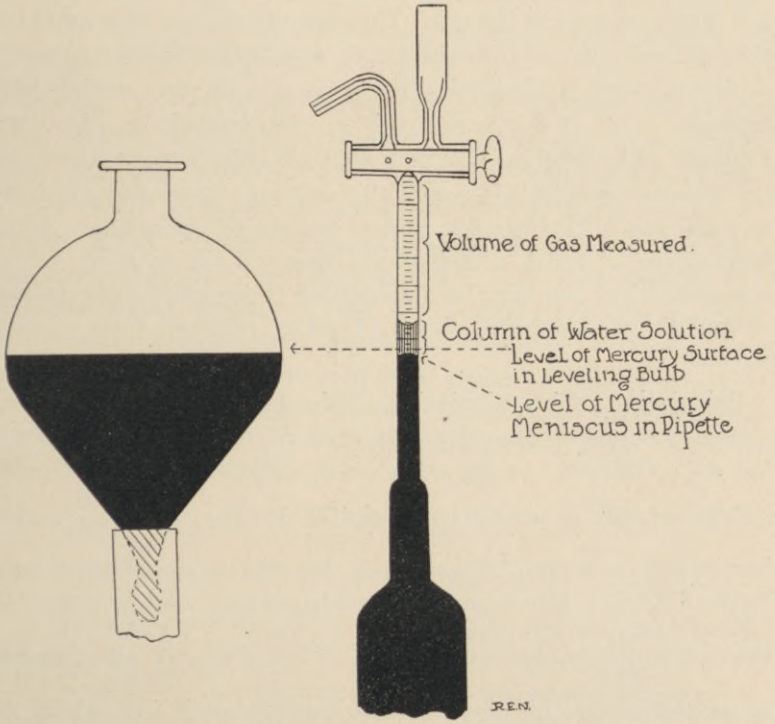


Fig. 50.—Showing position of bulb when the gas volume in the pipette of the Van Slyke carbon dioxide apparatus is read.

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 stopcock "f," 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 "d" below the lower stopcock. The water solution is drained out of the portion of the apparatus above the stopcock as completely as possible, but without removing any of the gas. The mercury bulb is now raised in the left hand and the lower stopcock is turned with the right hand so that mercury is admitted to the apparatus through the left-hand entrance, "c," 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 (Fig. 50) 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.

Calculation.—The calculation of the CO_2 combining power is made from the volume of gas as read off from the burette with the aid of the two tables given by Van Slyke and Cullen. The volume of gas observed is multiplied by the factor $\frac{B}{760}$. The barometric pressure is read and this factor ascertained from Table XXVIII. For example, if the barometer reading were 744, the factor $\frac{B}{760}$ is seen to be 0.979. The volume of gas observed is multiplied by this and the resultant figure is located in the first column of Table XXIX, the column headed "Observed volume of gas $\times \frac{B}{760}$." The line is carried across horizontally to the column which is headed by the temperature at which the reading was made, and the result read off. This is expressed in terms of c.c. of carbon dioxide bound by 100 c.c. of plasma, reduced to 0 degrees, and a barometric pressure of 760 mm.

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 the 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.

Table XXIX facilitates calculation of the results. It contains corrections for the air (about 0.05 c.c.) dissolved by the 2.5 c.c. of water introduced into the apparatus, for an approximately equal volume of CO_2 physically dissolved by the 1 c.c. of plasma in addition to that chemically bound as bicarbonate, also corrections for temperature, pressure, and vapor tension necessary to reduce the gas volume to standard conditions; viz., temperature of 0° C. and pressure of 760 mm.

TABLE XXVIII

SHOWING BAROMETER COEFFICIENTS

Values are given below for the ratio $\frac{B}{760}$ over the range usually encountered.

BAROMETER	$\frac{B}{760}$	BAROMETER	$\frac{B}{760}$
732	0.963	756	0.995
734	0.966	758	0.997
736	0.968	760	1.000
738	0.971	762	1.003
740	0.974	964	1.006
742	0.976	766	1.008
744	0.979	768	1.011
746	0.981	770	1.013
748	0.984	772	1.016
750	0.987	774	1.018
752	0.989	776	1.021
754	0.992	778	1.024

TABLE XXIX

FOR CALCULATION OF CARBON DIOXIDE COMBINING POWER OF PLASMA

Observed vol. gas $X \frac{B}{760}$	C.c. of CO_2 reduced to O° 760 mm. bound as bicarbonate by 100 c.c. of plasma				Observed vol. gas $X \frac{B}{760}$	C.c. of CO_2 reduced to O° 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	41.4
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	23.2	24.0	24.5	4	61.3	61.4	61.7	61.6
5	23.6	24.2	24.9	25.4	5	62.3	62.4	62.6	62.5
6	24.6	25.2	25.8	26.3	6	63.2	63.3	63.6	63.4
7	25.5	26.2	26.8	27.3	7	64.2	64.3	64.5	64.3
8	26.5	27.1	27.7	28.2	8	65.2	65.3	65.5	65.3
9	27.5	28.1	28.7	29.1	9	66.1	66.2	66.4	66.2
0.40	28.4	29.0	29.6	30.0	0.80	67.1	67.2	67.3	67.1
1	29.4	30.0	30.5	31.0	1	68.1	68.1	68.3	68.0
2	30.3	30.9	31.5	31.9	2	69.0	69.1	69.2	69.0
3	31.3	31.9	32.4	32.8	3	70.0	70.0	70.2	69.9
4	32.3	32.8	33.4	33.8	4	71.0	71.0	71.1	70.8
5	33.2	33.8	34.3	34.7	5	71.9	72.0	72.1	71.8
6	34.2	34.7	35.3	35.6	6	72.9	72.9	73.0	72.7
7	35.2	35.7	36.2	36.5	7	73.9	73.9	74.0	73.6
8	36.1	36.6	37.2	37.4	8	74.8	74.8	74.9	74.5
9	37.1	37.6	38.1	38.4	9	75.8	75.8	75.8	75.4
0.50	38.1	38.5	39.0	39.3	0.90	76.8	76.7	76.8	76.4
1	39.1	39.5	40.0	40.3	1	77.8	77.7	77.7	77.3
2	40.0	40.4	40.9	41.2	2	78.7	78.8	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

Determination of the Oxygen Binding Capacity of Blood (Gasometric Hemoglobin Estimation)

(Van Slyke, D. D., Jour. Biol. Chem., 1918, xxxvi, 127; Van Slyke, D. D., and Stadie, W. C., Jour. Biol. Chem., 1921, l, 49)

Principle.—The Van Slyke apparatus (Fig. 49) used for determining the carbonic acid binding power of the blood plasma may be used with equal facility for determining the oxygen binding power of blood. The oxygen in a measured amount of blood is set free from combination with the oxyhemoglobin by the addition of potassium ferricyanide. The oxygen is then extracted in the Van Slyke apparatus and measured at atmospheric pressure.

Reagents.—(1) 1 per cent saponin (Merck) solution.

(2) 20 per cent potassium ferricyanide. The ferricyanide solution is made air-free by boiling or by shaking in an evacuated flask and is kept in a bottle or burette under a layer of paraffin oil 2 or 3 cm. thick to exclude air.

(3) 0.5 N sodium hydroxide.

Procedure.—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 five or more minutes on a mechanical shaker. The blood is then transferred to a cylinder or heavy walled tube. The blood gas apparatus is washed twice with distilled water before each analysis in order to remove the alkali used to absorb CO_2 in any former analysis. For 2 c.c. of blood 6 c.c. of water 0.3 c.c. of 1 per cent saponin (Merck), and 2 to 3 drops of caprylic alcohol are introduced into the cup "b" of the apparatus and the apparatus is evacuated by lowering the leveling bulb until the level of the mercury in the apparatus is just above the lower stopcock. The air is extracted by shaking for about 15 seconds. The extracted air is expelled and the process repeated to make sure that no air is left in the solution. Nearly the entire 6 c.c. is then forced up into the cup of the apparatus, the blood is stirred to assure even distribution of the corpuscles and drawn into a pipette calibrated to deliver 2 c.c. between two marks of which the lower is 3 to 4 cm. from the tip.

The pipette is introduced under the water solution in the cup so that the tip rests on the bottom near the capillary. As the blood flows out of the pipette held in the left hand, the upper stopcock is partially opened with the right, so that the blood accompanied by some of the water flows at once into the chamber of the apparatus. The layer of blood need never rise more than 1 to 2 mm. above the bottom of the cup and the slight amount adhering is washed completely into the chamber by the water which follows after all the blood has been delivered.

Before the last c.c. of water is readmitted, 0.10 to 0.12 c.c. of a solution of potassium ferricyanide containing 20 grams per 100 c.c. is added and thereby introduced into the chamber after the blood. A mercury seal is made by placing a drop of mercury in the cup and allowing it to run down to the upper stopcock. The blood and water in the apparatus are thoroughly mixed and allowed to stand until the blood is completely laked. This requires from 30 seconds to a minute. The apparatus is now evacuated until only a few drops of mercury remain above the upper stopcock, and is shaken preferably with a rotatory motion to whirl the blood in a thin layer about the wall of the chamber. The shaking should be continued for three minutes. Usually two minutes, frequently less, are sufficient for the complete extraction but three minutes are sometimes required before the last traces are extracted. The extracted solution is trapped in the bulb below the lower stopcock as in the determination of CO_2 and the vacuum is released; 0.5 c.c. of 0.5 N. sodium hydroxide solution previously saturated with air or oxygen is admitted from the cup of the apparatus and allowed to trickle slowly down the inner wall to absorb the CO_2 from the gas mixture. If any of the alkali solution remains just below the stopcock it may be dislodged by running in a little mercury from the cup above. After the absorption of CO_2 is complete two minutes must be allowed for drainage of the alkali solution before the reading is taken.

Calculation.—The volume of oxygen obtained as read off from the burette of the apparatus is reduced to 0° C. and 760 mm. pressure by multiplying by the proper factor as indicated in Table XXX.

TABLE XXX

Temperature (t°)	Factor by which gas measured moist at t°C. and B mm. pressure is reduced to O°C. and 760 mm.
O°C.	
15	$0.932 \times \frac{B}{760}$
16	0.928 x "
17	0.924 x "
18	0.919 x "
19	0.915 x "
20	0.910 x "
21	0.906 x "
22	0.901 x "
23	0.897 x "
24	0.892 x "
25	0.888 x "
26	0.883 x "
27	0.878 x "
28	0.873 x "
29	0.868 x "
30	0.863 x "

The result obtained is then multiplied by 50 (when 2 c.c. of blood are used in the determination) to bring to a volume per cent basis.

The gas as measured in the burette includes the nitrogen and oxygen which is in physical solution in the blood as well as the oxygen in chemical combination with the hemoglobin. In order to find the oxygen bound by hemoglobin in blood saturated in air the nitrogen and dissolved oxygen are subtracted as indicated below:

15° C.	2.14	volume	per	cent
20° C.	2.10	"	"	"
25° C.	2.06	"	"	"
30° C.	2.02	"	"	"

The figure obtained gives the oxygen binding capacity of the blood per 100 c.c. This figure may be converted into grams of hemoglobin per 100 c.c. by multiplying by the factor 0.746 since 1 gram of hemoglobin combines with 1.34 c.c. of oxygen.

Example.—Blood sample 2.00 c.c.

O_2 and N_2 measured = 0.495 c.c. at 20° C., 767 mm. $0.495 \times 0.910 \times \frac{767}{760}$ = O_2 and N_2 = 0.455 c.c. at 0° C., 760 mm. 50×0.455 = O_2 and N_2 per 100 c.c. blood = 22.75 c.c. at 0° C. and 760 mm. Physically dissolved O_2 and N_2 per 100 c.c. blood = 2.10 c.c. at 0° C. and 760 mm. 22.75 – 2.10 = combined O_2 per 100 c.c. = 20.65 cc. at 0° C. and 760 mm. 20.65×0.746 = hemoglobin per 100 c.c. = 15.40 grams. $\frac{15.40}{15.60} \times 100$ = per cent of normal standard = 99 per cent.

CHAPTER VIII

SEROLOGICAL TECHNIC

Technic of the Wassermann Reaction

General Considerations

The essential features of the technic of the Wassermann reaction as outlined on the following pages may be summarized as follows:

The test is based on the "quarter unit" amount, the total volume of the reagents in each tube being 1.25 c.c., one-fourth of that originally suggested by Wassermann.

An antisheep hemolytic system is employed.

The dose of amboceptor is two units, the unit being determined daily with 0.25 c.c. complement diluted 1:10 in a water-bath at 37° C. for one hour.

The complement dose is two units, the unit being determined daily by titration with two units of amboceptor.

The antigen dose is not greater than one-fourth of the anticomplementary dose. Two doses are used in the antigen control with the same preliminary incubation as in the tests.

The patient's serum is diluted 1:5. 0.25 c.c. and 0.125 of the diluted serum are used in the test, and 0.5 c.c. in the serum control tube.

The preliminary incubation is four hours in the ice box at 8° C. with plain antigen and ¾ hour in the water-bath at 37° C. for the cholesterin fortified antigen.

The quantitative readings are made on Citron's scale.

Natural hemolysin is disregarded. The pooled guinea pig serum is tested for hemolysin and rejected if any is present.

Preparation of Glassware

All glassware used in the test must be chemically clean and free of all traces of acid or alkali. Acidity or alkalinity may give rise to false reactions. The tubes, pipettes and flasks are

thoroughly washed in soapy water; well washed in running tap water; dried in metal baskets and sterilized in the hot air oven. Very dirty glassware may be cleaned by boiling in dilute acid.

The test tubes should be of uniform bore. The most desirable size is 100 × 12 millimeters. The pipettes and flasks should be accurately calibrated.

Preparation and Standardization of Reagents

The reagents used in the complement-fixation test for syphilis are:

1. Physiological salt solution.
2. Sheep's red blood cells.
3. Amboceptor (antisheep immune rabbit serum).
4. Complement (guinea pig serum).
5. Patient's serum.
6. Antigen (alcoholic extract of heart muscle).

Physiological Salt Solution.—Physiological salt solution is used in performing the test, in diluting the reagents and in washing the sheep's blood. It is prepared by dissolving 8.5 grams of chemically pure sodium chloride in a liter of distilled water and autoclaving the solution for fifteen minutes at fifteen pounds pressure.

The Sheep's Blood.—Sheep's blood may be obtained from a slaughter house or from a sheep kept for the purpose. The blood is collected directly from the sheep into a sterile bottle containing glass beads and immediately defibrinated by shaking or allowed to run into a wide-mouth bottle half full of a 1.5 per cent solution of sodium citrate in physiological salt solution.

The sheep's cells are washed and prepared in the following manner:

A centrifuge tube is filled one-fourth with defibrinated or citrated blood, thoroughly mixed with physiological salt solution and centrifuged rapidly for a sufficient time to throw the cells to the bottom of the tube. The supernatant fluid is decanted; the sedimented cells again mixed with salt solution and centrifuged. This process is repeated until the cells have been washed four times. After the final washing the level of the cells is noted, the

supernatant fluid discarded and the packed cells diluted with salt solution to twenty times their volume. This gives the 5 per cent suspension of sheep's cells as used in the test. The speed and duration of centrifugation should always be the same for the final washing in order that the packing of the cells may be uniform from day to day.

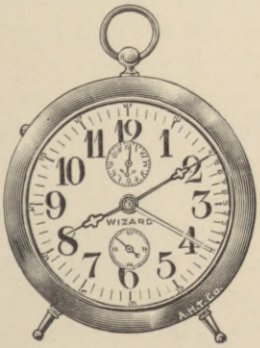


Fig. 51.

Fig. 52.

Fig. 51.—Interval timer. This is a valuable aid in chemical and serological work.
Fig. 52.—The most desirable type of burette for general laboratory use.

Amboceptor.—The amboceptor is prepared by injecting intravenously a full grown healthy rabbit with washed sheep's red blood cells at five day intervals. The corpuscles must be freshly obtained and well washed. A 50 per cent suspension is used for the injection, 4 c.c. being given the first time; 6 c.c. the second and 8 c.c. the third. The second and third

injections must be made very slowly to prevent sudden death from agglutination of the corpuscles.

The rabbit is bled from the ear vein seven to nine days after the last injection, and the serum after inactivating is titrated for hemolytic power. The serum is satisfactory to use if 0.25 c.c. of a 1:2000 dilution hemolyzes completely 0.25 c.c. of a 5 per cent suspension of sheep's corpuscles in the presence of 0.25 c.c. of guinea pig serum diluted 1:10 after incubation in the water-bath at 37° C. for one hour.

The animal is then bled either from the carotid artery or by puncturing the heart directly. The blood is allowed to clot. The clear serum is removed with a sterile pipette and heated in the water-bath at 55° C. for ½ hour.

This heated immune rabbit serum constitutes the amboceptor. It is mixed with an equal quantity of glycerine and distributed in 2 c.c. quantities into small sterile ampoules or test tubes.

The stock amboceptor keeps indefinitely in the refrigerator.

The unit of amboceptor is defined as the smallest amount which will give complete hemolysis of 0.25 c.c. of a 5 per cent suspension of sheep's corpuscles in the presence of an excess of complement after an incubation of one hour in the water-bath at 37° C. The unit is roughly determined for each new batch of amboceptor as follows: Into each of seven test tubes pipet 0.25 c.c. physiological salt solution. To the first add 0.25 c.c. of the amboceptor diluted 1:100. Mix and transfer 0.25 c.c. to the second tube. Mix and transfer 0.25 c.c. to the third tube. Proceed in this manner up to and including the seventh tube. Discard the last 0.25 c.c. A series of dilutions of the amboceptor ranging from 1:200 to 1:12800 is thus obtained. Add to each tube 0.25 c.c. of guinea pig serum diluted 1:10, 0.25 c.c. 5 per cent suspension of sheep's corpuscles, and 0.5 c.c. salt solution to bring the volume to 1.25 c.c. Incubate in water-bath at 37° C. for one hour. Table XXXI shows the scheme for this titration.

After the incubation the titration is read to determine the highest dilution in which hemolysis is complete. This shows the dilution in which a unit of amboceptor is contained in a volume of 0.25 c.c. The amboceptor is used in the dilution so determined in

TABLE XXXI

PRELIMINARY AMBOCEPTOR TITRATION

NUMBER OF TUBES	AMBOCEPTOR		CORPUSCLE SUSPENSION	COMPLEMENT 1:10	SALT SOLUTION	
	c.c.	Dilution	c.c.	c.c.	c.c.	
1	0.25	1:200	0.25	0.25	0.5	
2	0.25	1:400	0.25	0.25	0.5	
3	0.25	1:800	0.25	0.25	0.5	Incubate one
4	0.25	1:1600	0.25	0.25	0.5	hour in water-
5	0.25	1:3200	0.25	0.25	0.5	bath at 37° C.
6	0.25	1:6400	0.25	0.25	0.5	
7	0.25	1:12800	0.25	0.25	0.5	

the daily titration of the amboceptor preliminary to the Wassermann test.

Complement.—Complement is obtained from guinea pig's blood. Large male pigs furnish the most active complement. The blood is aspirated from the heart with a Luer syringe. The chest wall should be punctured close to the sternum through the second or third right interspace. The needle is directed downward and backward for 1 to 2 centimeters. A large pig will withstand the loss of 7 to 8 c.c. of blood. The interval of bleedings should not be less than three weeks.

It is preferable to bleed at least three pigs for making any test or titration and to pool the blood obtained. The blood is allowed to clot at room temperature, placed in ice box overnight and is then centrifuged. The clear serum is pipetted off and diluted as indicated by the preliminary complement titration. The complement should be kept in the ice box as it deteriorates rapidly at room temperature.

The Patient's Serum.—The patient's serum is obtained from blood withdrawn by venipuncture. The blood is delivered into a small test tube and allowed to stand at room temperature until the clot retracts. For use in the test the clear serum is removed with a pipette, diluted with four parts of physiological salt solution and heated in a water-bath at 55° C. for 15 minutes to inactivate.

Spinal fluids are not diluted or inactivated.

Antigen.—Antigen is prepared from fresh human or beef heart muscle. The fat and connective tissue are carefully removed with scissors. The muscle is then finely ground in a meat chop-

per. Five hundred c.c. of absolute alcohol are added to 100 grams of the ground heart. The suspension is shaken for twenty-four hours in a shaking machine or placed in the incubator at 37° C. and left from 10 to 14 days, shaking by hand daily. The mixture is allowed to sediment at room temperature. The supernatant fluid is plain antigen. Guinea pig heart makes a very satisfactory antigen, when extracted with alcohol for four months in the ice box. The alcohol is added in the proportion of 25 c.c. to one heart. To make cholesterinized antigen add 0.2 gram of cholesterin to 100 c.c. of the plain antigen and place in incubator at 37° C. until completely dissolved. The stock antigens will keep almost indefinitely at room temperature.

In diluting the stock antigens for use in the test, the antigen is transferred to a dry graduate with a dry pipette. Physiological salt solution is added drop by drop shaking well after the dilution of each drop.

There are two requirements for a good antigen—(1) a long range and (2) specificity. The range is the difference between the anticomplementary dose, (the smallest dose of antigen that is in itself inhibitory), and the minimum fixing dose, the antigen unit.

The antigen is titrated for anticomplementary property by incubating varying amounts of antigen with two units of complement, and adding amboceptor and corpuscle suspension. This titration is illustrated in the protocol in Table XXXII.

TABLE XXXII

TITRATION OF ANTIGEN FOR ANTICOMPLEMENTARY PROPERTY

NUMBER OF TUBE	ANTIGEN DILUTED 1:5	COMPLEMENT 2 UNITS	SALT SOLUTION	Incubate 4 hours in ice box at 8° C.	AMBOCEPTOR 2 UNITS	CORPUSCLE SUSPENSION	
	c.c.	c.c.	c.c.		c.c.	c.c.	Incubate
1	0.5	0.25	0		0.25	0.25	in water-
2	0.4	0.25	0.1		0.25	0.25	bath at
3	0.3	0.25	0.2		0.25	0.25	37° C.
4	0.2	0.25	0.3		0.25	0.25	for ½ hr.
5	0.1	0.25	0.4		0.25	0.25	

The tube containing the largest amount of antigen in which hemolysis is complete shows the largest amount of antigen which is not anticomplementary.

The natural hemolytic property of the antigen is determined by incubating varying dilutions of antigen with complement and corpuscle suspension as outlined in Table XXXIII.

TABLE XXXIII

TITRATION OF ANTIGEN FOR HEMOLYTIC PROPERTY

NUMBER OF TUBE	ANTIGEN DILUTED 1:5	SALT SOLUTION	COMPLEMENT 2 UNITS	CORPUSCLE SUSPENSION	
	c.c.	c.c.	c.c.	c.c.	
1	0.5	0	0.25	0.25	Incubate
2	0.4	0.1	0.25	0.25	in water-
3	0.3	0.2	0.25	0.25	bath at
4	0.2	0.3	0.25	0.25	37° C.
5	0.1	0.4	0.25	0.25	for ½ hour.

Note the tube containing the largest amount of antigen causing no hemolysis. There should be little if any hemolysis even in the tubes containing the largest amount of antigen.

The best way to standardize an antigen either qualitatively or quantitatively is by testing its complement-fixing power against a large number of known positive and known negative specimens of blood serum and spinal fluids. The unknown antigen should be run in parallel with a known satisfactory antigen for comparison.

Some idea of the inhibitory properties of an antigen may be obtained by running a regular Wassermann test using serum pooled from at least five positive specimens of blood and employing successive dilutions of the antigen.

Daily complete titrations of the antigen are unnecessary. However, antigen control tubes are included in each series to show that the antigen is not anticomplementary in double the dose used in the test and to show that the antigen fixes complement completely in the presence of a positive serum.

The largest amount of antigen which may be safely employed in the diagnostic test is one-fourth of the largest dose which is not anticomplementary but should be 8 to 10 times as great as the smallest dose which gives complete fixation with a pooled positive serum.

Daily Titration of Reagents Preliminary to the Diagnostic Test

The correct adjustment of the hemolytic system is the crucial factor in the technic of the Wassermann reaction. The most

variable factor is the strength of the complement as obtained from different guinea pigs. Each day the hemolytic system is standardized by titrating the amboceptor in the presence of fixed amounts of complement; and by titrating the complement in the presence of two units of amboceptor.

1. **Titration of Amboceptor.**—A dilution of the amboceptor is prepared in which the unit of amboceptor is contained in a volume of 0.25 c.c. as determined roughly in the preliminary titration of the amboceptor. Varying amounts of this dilution are then incubated with complement and corpuscle suspension for one hour in the water-bath at 37° C.

Table XXXIV shows the scheme for the amboceptor titration.

TABLE XXXIV

AMBOCEPTOR TITRATION

	NUMBER OF TUBE	COMPLEMENT 1:10	CORPUSCLE SUSPENSION	SALT SOLUTION	HEMOLYTIC SERUM IN DILUTION TO BE TESTED FOR UNIT	
		c.c.	c.c.	c.c.	c.c.	Incubate for one hour in water-bath at 37° C.
To determine the unit of amboceptor	1	0.25	0.25	0.25	0.50	
	2	0.25	0.25	0.30	0.45	
	3	0.25	0.25	0.35	0.40	
	4	0.25	0.25	0.40	0.35	
	5	0.25	0.25	0.45	0.30	
	6	0.25	0.25	0.50	0.25	
	7	0.25	0.25	0.55	0.20	
	8	0.25	0.25	0.60	0.15	
	9	0.25	0.25	0.65	0.10	
	10	0.25	0.25	0.70	0.05	
To show that each reagent has no hemolyzing effect	11	0	0.25	0.50	0.50	
	12	0.50	0.25	0.50	0	
	13	0	0.25	1.00	0	

The reagents are added in the order given.

The tubes are shaken thoroughly and incubated one hour in the water-bath at 37° C.

The tube containing the smallest amount of amboceptor in which hemolysis is complete contains the unit. For instance if in titrating a dilution of 1:1000, tubes 1 to 8 show complete hemolysis, then 0.15 is a unit. From this we may calculate that dilution in which 0.25 c.c. is a unit as follows:

$$X : 0.25 :: 1000 : 0.15$$

$$X = \frac{25000}{15}$$

$$X = 1600 \text{ (about)}$$

If a dilution of 1:1600 is now made and titrated, it will be found that 0.25 c.c. contains one amboceptor unit. In the test two units are employed and the dilution is so made that 0.25 c.c. contains two units. In the illustration given this would be a 1:800 dilution.

Tubes are included in the amboceptor titration to show that each reagent has no hemolyzing effect.

2. **Titration of Complement.**—A 1:20 dilution of the guinea pig serum to be used in the diagnostic test is prepared. Varying amounts of the diluted serum are incubated for ½ hour in the water-bath at 37° C. with 0.25 c.c. of the corpuscle suspension and 0.25 c.c. of the amboceptor containing two units as determined in the preliminary amboceptor titration.

The unit of complement is defined as the smallest amount in the presence of which two units of amboceptor will completely hemolyze 0.25 c.c. of a 5 per cent suspension of sheep's corpuscles.

TABLE XXXV

TITRATION OF COMPLEMENT

	NUMBER OF TUBE	COMPLEMENT 1:20	SALT SOLUTION	CORPUSCLE SUSPENSION	AMBOCEPTOR 2 UNITS	
		c.c.	c.c.	c.c.	c.c.	
To determine the unit of complement	1	0.50	0.25	0.25	0.25	Incubate in water-bath at 37° C. for ½ hour
	2	0.45	0.30	0.25	0.25	
	3	0.40	0.35	0.25	0.25	
	4	0.35	0.40	0.25	0.25	
	5	0.30	0.45	0.25	0.25	
	6	0.25	0.50	0.25	0.25	
	7	0.20	0.55	0.25	0.25	
	8	0.15	0.60	0.25	0.25	
	9	0.10	0.65	0.25	0.25	
	10	0.05	0.70	0.25	0.25	
To show that each reagent has no hemolyzing effect.	11	0.0	0.50	0.25	0.25	
	12	0.50	0.50	0.25	0.25	
	13	0.0	1.00	0.25	0.25	

Table XXXV shows the arrangement of the tubes for the complement titration, and the order in which the reagents are to be added.

The tubes are thoroughly shaken and incubated in the water-bath at 37° C. for ½ hour. The tube containing the smallest amount of complement in which hemolysis is complete contains one unit. For instance if Tubes 1 to 7 show complete hemolysis then 0.20 c.c. of the 1:20 dilution is a unit. From this we may calculate that dilution in which 0.25 c.c. is a unit as follows:

$$X : 0.25 :: 20 : 0.20$$

$$X = \frac{5.0}{0.20}$$

$$X = 25$$

If a dilution of 1:25 is now made and titrated it will be found that 0.25 c.c. contains one unit. In the diagnostic test two units are employed and the dilution is so made that 0.25 c.c. contains two units. In the illustration given two units would be contained in 1:12.5 dilution.

The Diagnostic Test

Reagents.—(1) *Patient's serum* diluted with four parts of salt solution and heated in the water-bath at 55° C. for 15 minutes.

(2) *Complement*—guinea pig serum free from natural hemolysin and so diluted that 0.25 c.c. contains 2 units.

(3) *Antigen*—alcoholic extract of heart muscle which is not hemolytic and is so diluted that four times the amount used (0.25 c.c.) is not anticomplementary.

(4) *Amboceptor*—immune rabbit serum so diluted that 0.25 c.c. contains two units.

(5) *Sheep's corpuscles*—5 per cent suspension in salt solution.

Procedure.—Into the first of three tubes pipette 0.50 c.c. of the diluted patient's serum; into the second 0.25 c.c. and into the third 0.125 c.c. Add to the second and third tubes 0.25 c.c. of the antigen. Finally add to each of the three tubes 0.25 c.c. of complement.

Fix for 45 minutes in the water-bath at 37° C. if cholesterin-

TABLE XXXVI

TEST OF SERUM FOR DIAGNOSIS.

NUMBER OF TUBE	KNOWN NEGATIVE SERUM	KNOWN POSITIVE SERUM	PATIENT'S SERUM	ANTIGEN IN STANDARD DILUTION	COMPLEMENT 2 UNITS	AMBOCEPTOR 2 UNITS	5 PER CENT SUSPENSION SHEEP'S CELLS
	c.c.	c.c.		c.c.	c.c.	c.c.	c.c.
1	0.50	0	0	0	0.25	0.25	0.25
2	0.25	0	0	0.25	0.25	0.25	0.25
3	0.125	0	0	0.25	0.25	0.25	0.25
4	0	0.50	0	0	0.25	0.25	0.25
5	0	0.25	0	0.25	0.25	0.25	0.25
6	0	0.125	0	0.25	0.25	0.25	0.25
7	0	0	0.50	0	0.25	0.25	0.25
8	0	0	0.25	0.25	0.25	0.25	0.25
9	0	0	0.125	0.25	0.25	0.25	0.25
10	0	0	0	0.50	0.25	0.25	0.25

Incubate 45 min. in water-bath at 37° C. if cholesterinized antigen is used; or 4 hrs. in ice box at 8° C. if plain antigen is used.

Incubate in water-bath at 37° C. until serum control tubes (Nos. 1, 4, and 7) and antigen control tube (No. 10) show complete hemolysis.

ized antigen is used, or in ice box at 8° C. for four hours if plain antigen is used.

Remove from the water-bath or ice box and add to each tube 0.25 c.c. of a 5 per cent suspension of sheep's cells and 0.25 c.c. of amboceptor. It is convenient to mix equal parts of corpuscles and amboceptor just before using and to add 0.50 c.c. of the mixture.

Incubate in water-bath at 37° C. until hemolysis is complete in the serum control tube. A test is also made with a known positive and a known negative serum as control of the fixability and specificity of the antigen. A tube containing a double dose of antigen is included in the series to show that the antigen is not anticomplementary.

Tables XXXVI and XXXVII show the arrangement of the tubes in the diagnostic test:

TABLE XXXVII

TEST OF SPINAL FLUID FOR DIAGNOSIS

NUMBER OF TUBE	PATIENT'S SPINAL FLUID UNDILUTED	ANTIGEN IN STANDARD DILUTION	COMPLEMENT 2 UNITS		AMBOCEPTOR 2 UNITS	5 PER CENT SUSPENSION SHEEP CELLS	
	c.c.	c.c.	c.c.		c.c.	c.c.	
1	1.0	0	0.25	Fix 4 hrs. in ice box at 8° C. for plain, or ¾ hr. in water-bath at 37° C. for cholesterinized antigen.	0.25	0.25	Incubate in water-bath at 37° C. until hemolysis is complete in tube 1.
2	1.0	0.25	0.25		0.25	0.25	
3	0.50	0.25	0.25		0.25	0.25	
4	0.25	0.25	0.25		0.25	0.25	
5	0.125	0.25	0.25		0.25	0.25	

Reading the Diagnostic Test

Citron's standard is used in reading all tests on serum after the final incubation. (Plate V.)

Complete absence of hemolysis in tubes 2 and 3 equals positive 4 plus or very strongly positive.

Complete absence of hemolysis in tube 2 and faint hemolysis in tube 3 equals positive 3 plus or strongly positive.

Complete absence of hemolysis in tube 2 and complete or nearly complete hemolysis in tube 3 equals positive 2 plus or positive.

Partial hemolysis in tube 2, complete or nearly complete hemolysis in tube 3 equals positive one plus or suggestive.

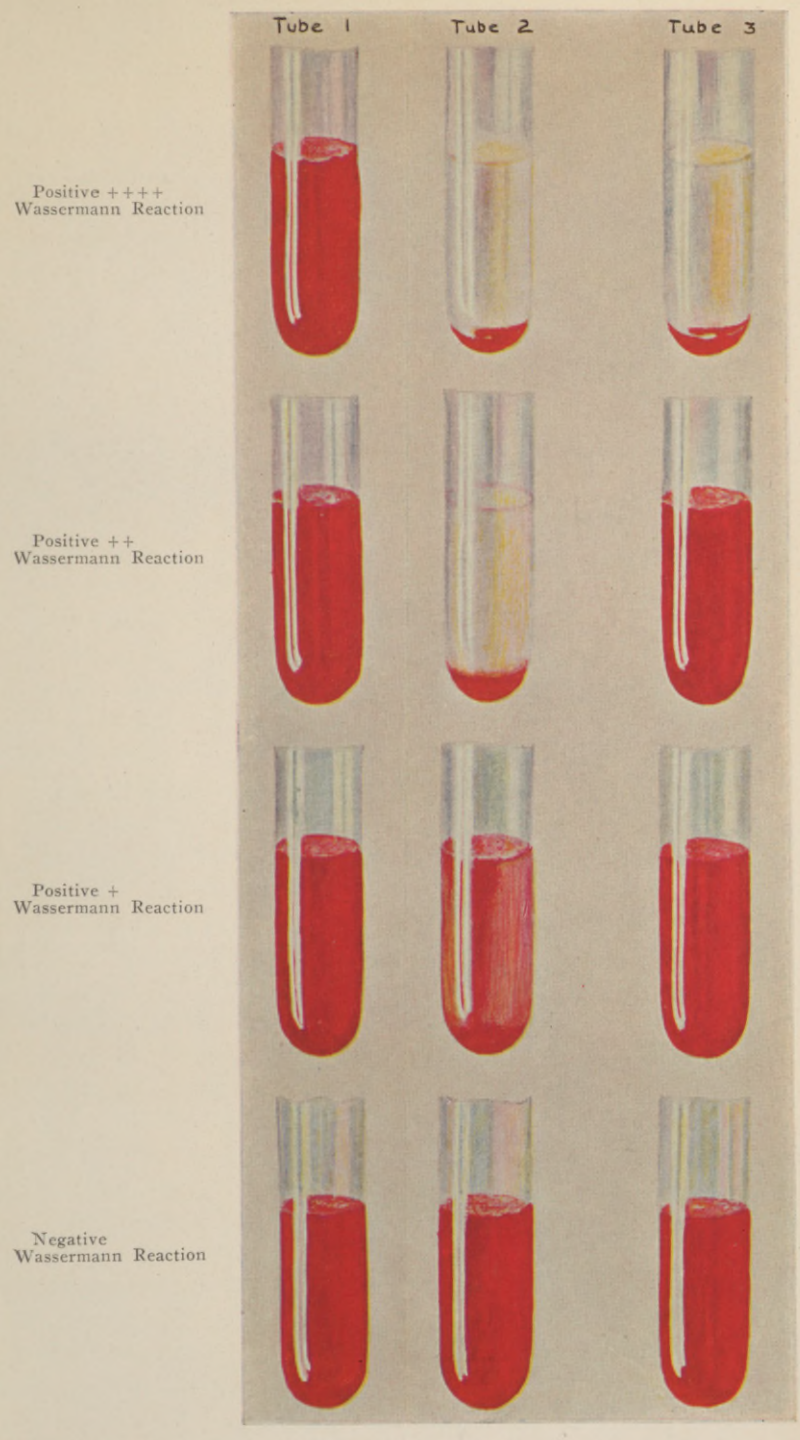


PLATE V.

Citron's scale for reading the Wassermann Reaction.

Complete hemolysis in all tubes equals negative.

This standard cannot be used in reading the tests on spinal fluids. Complete fixation in any amount of spinal fluid as used in the test is considered a positive test. A report is made of the degree of fixation with the varying amounts of spinal fluid used.

Widal Reaction

(Modified Dreyer Technic)

Principle.—The Widal reaction is a procedure by which the agglutinating power of a serum against any organism may be tested. Serum in varying dilutions is mixed with a standardized bacterial suspension. The tubes containing the mixtures are incubated for 2 hours at 55° C., and examined for agglutination.

The most essential part of the procedure is the use of a standardized bacterial suspension. Having this, quantitative fluctuations in the agglutinin content of the blood can be accurately determined.

Preparation of Bacterial Suspension.—In making a bacterial suspension, the organism is subcultured daily in broth for about ten days to increase its agglutinability and to diminish any tendency to spontaneous agglutination. It is then planted on agar slants in Kolle flasks or in large bottles and incubated overnight. The growth is washed off with 0.85 per cent salt solution to which 0.1 per cent formalin has been added. It is then placed in an ice box for 4-5 days and shaken repeatedly. It is tested for sterility. The suspension is diluted with salt solution to make a suspension containing 2,000 million organisms per c.c. This may be done by counting, using Wright's method or by contrifuging and diluting according to the table of Hopkins (see page 230).

The suspension is then ready to be standardized for agglutinability. A rabbit immune serum of known agglutinin unit content is set up in two parallel series in varying dilutions, the variations not to be excessive. To one series a standardized suspension is added, to the other the suspension to be standardized. The tubes are then shaken, incubated at 55° C. for two hours

and read for the highest dilutions showing agglutination visible to the naked eye.

That dilution of the standardized suspension is to the dilution of the new suspension as the known factor of the standardized suspension is to x (the factor of the new suspension).

Example.—If the dilution in which the standardized suspension is agglutinated is 1 to 6400 while that of the new suspension is 1 to 3200 and the factor of the standardized suspension is 1.0, then—

$$\frac{6400}{3200} = \frac{1}{x} \text{ or } x = \frac{3200}{6400} = \frac{1}{2}$$

The factor of the new bacterial suspension is therefore one-half.

The suspension should be bottled in small sterile bottles and kept in an ice box.

Procedure.—Blood is withdrawn by venipuncture, allowed to clot, and the serum separated off. Select ten small test tubes about 1 cm. in diameter and of uniform bore. In the first tube place 1.8 c.c. 0.85 per cent salt solution and in each of the others 1 c.c. Transfer 0.2 c.c. of blood serum to the first tube, mix thoroughly and carry forward 1 c.c. to the next tube. This procedure is carried out through the series of ten tubes thus making serum dilutions ranging from 1 to 10, to 1 to 5120.

To each tube in the series 1.5 c.c. of the standardized bacterial suspension is added. The tubes are next thoroughly shaken.

The tubes are then placed in a water-bath at from 50° to 55° C. for two hours, are removed and cooled for fifteen minutes at room temperature, and read. The highest dilution showing agglutination without sedimentation visible to the naked eye gives the reading. The dilution of the serum in this tube, divided by the factor of agglutinability gives a final reading expressed in the number of standard agglutinin units per cubic centimeter of serum. It must be kept in mind that the addition of the suspension has increased the dilution of the serum one and a half times, thus the first tube represents a dilution of 1 to 25, the last a dilution of 1 to 12,800.

Remarks.—The Widal reaction is applied principally in diagnosing the existence of enteric fevers. In noninoculated persons

who have not had typhoid or paratyphoid fever agglutination in a dilution of 1 in 25 justifies a strong suspicion of typhoid or paratyphoid infection. Marked agglutination in 1 to 50 or more is nearly always diagnostic of active typhoid or paratyphoid infection.

In inoculated persons the test is done at intervals of one week, and if an active enteric infection exists, there will be a definite rise in agglutinin titer.

The agglutinin titer in active typhoid increases for the first three weeks after which it falls, at first rapidly and then slowly.

Determination of Types of Pneumococcus

(Blake, F. S.: Jour. Exp. Med., 1917, xxxvi, 67)

1. **Mouse Method.**—If mice are available, proceed as follows:

A small portion of the sputum is selected and washed through three or four changes of sterile salt solution in sterile Petri dishes. The washed sputum is then transferred to a sterile mortar, ground up and emulsified with about 1 c.c. of sterile bouillon, added drop by drop, until a homogeneous emulsion is obtained that will readily pass through the needle of a small syringe. One-half to 1 c.c. of this emulsion is inoculated intraperitoneally into a white mouse with a sterile syringe. 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, and examined microscopically for pneumococcus. 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 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.

As soon as the mouse is killed the peritoneal cavity is opened with sterile precautions, and cultures are made on plain broth and on one-half of a blood agar plate. Smears are made and stained by gram and for capsules.

The peritoneal exudate is washed out by means of a sterile glass pipette with 4 to 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 side of the blood agar plate.

The peritoneal washings are centrifugalized at low speed for a few moments until the cells and fibrin are thrown down. Decant the supernatant bacterial suspension with a centrifuge tube and centrifuge at high speed. The bacterial sediment is taken up in sufficient sterile salt solution to make a moderately heavy suspension.

The suspension is then mixed with diluted immune serum of the three fixed types, and with undiluted Type II serum, in equal quantities of 0.5 c.c. each.

Table XXXVIII shows the possible reactions after incubation in a water-bath at 37° C. for 1 hour.

TABLE XXXVIII

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	–	–	–	–

A fifth tube should be added containing 0.1 c.c. sterile bile and 0.3 to 0.5 c.c. of bacterial suspension to determine the bile solubility of the stain for differentiation from the streptococcus. Gentle agitation of the tubes will hasten clumping. An organism which agglutinates in undiluted Type II serum alone should be incubated with undiluted Type I and Type II serum also to rule out a Group IV stain that shows cross agglutination in all three types of immune serum. If no agglutination occurs in any tube and the organism is bile soluble it is reported as pneumococcus Group IV.

The determination of type on the peritoneal washings should be confirmed by agglutination tests with a pure bouillon culture of the pneumococcus obtained from culture of the heart's blood of the mouse.

Bouillon cultures from blood, spinal fluid or empyema fluid

showing a pneumococcus may be centrifuged, the sediment mixed with salt solution, and the type determined by the method outlined above.

2. **Avery Cultural Method.**—If mice are not available the typing of pneumococcus may be done by the cultural method of Avery. Wash sputum as indicated under mouse method. Grind in sterile mortar with 9 c.c. of meat infusion broth with an H-ion concentration of 7.6, adding the broth drop by drop. Pour in centrifuge tube, add 0.5 c.c. each of sterile 20 per cent dextrose and blood.

Incubate at 37° C. for 5 to 6 hours. The tube is now centrifuged at low speed long enough to throw down the red cells but not enough to bring down the bacteria. A smear is then made, stained by gram, and a blood agar plate is inoculated.

The supernatant suspension is used for agglutination tests as indicated under mouse method.

Blood Tests Preliminary to Transfusion

Before a blood transfusion is done, it is necessary to show that the serum of the donor does not agglutinate or hemolyze the cells of the recipient, or vice versa.

Since hemolysis never occurs when agglutination is absent, the selection of a proper donor from the point of view of agglutination determines the other also. Two tests should be done. First, the blood group of both the donor and the recipient are determined. If these are found to be the same, the corpuscles of the recipient are matched against the serum of the donor and the serum of the recipient against the corpuscles of the donor. The two tests act as a check on each other.

1. **Determination of Blood Group.**— (A) Preparation of Diagnostic Sera.—Blood is withdrawn by venipuncture from a person whose group is known to be II. The serum is separated off and put in small sterile tubes or ampoules in 1 c.c. amounts. Similarly serum is procured from a person who belongs to Group III. It is important that the sera selected be high in agglutinin content.

(B) Procedure.—Several drops of blood from the person to be grouped are collected in a small test tube containing 3 to 5 c.c. 1½ per cent of sodium citrate in physiological salt solution.

With a capillary pipette one drop of the corpuscle suspension and one drop of Group II serum are placed on a porcelain color mixing plate (Fig. 23) and thoroughly mixed.

A similar preparation is made with the corpuscle suspension and Group III serum.

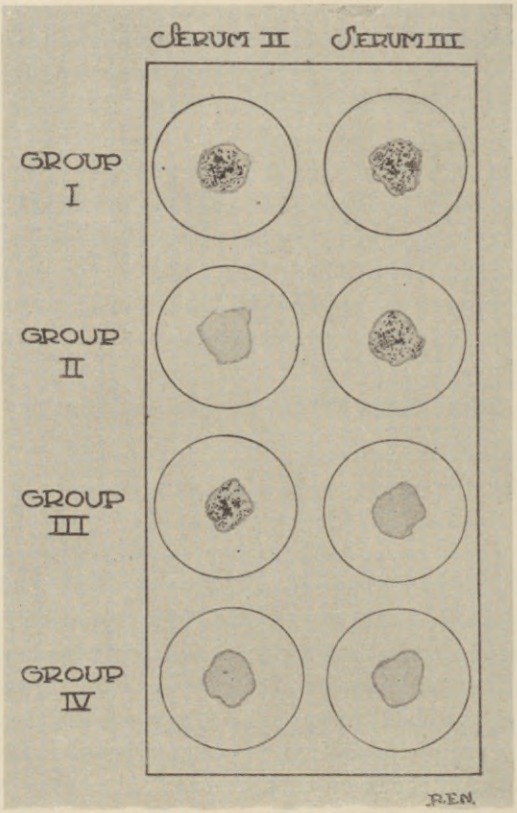


Fig. 53.—Showing the reaction of corpuscles of various groups with Group II and Group III sera.

Cover the mixing plate with a moist blotter and examine the preparations at intervals. If agglutination takes place the mixture of serum and corpuscles takes on a "brick-dust" appearance (Fig. 53).

The following diagram shows the agglutination reactions between the corpuscles and serum of different groups (Moss classification).

	CORPUSCLE GROUP			
SERUM GROUP	1	2	3	4
1	0	0	0	0
2	×	0	×	0
3	×	×	0	0
4	×	×	×	0

Table XXXIX gives all the possible combinations of reactions which can be obtained by mixing the corpuscles of an unknown group with the serum of Group II and Group III and indicates the group to which the unknown blood belongs (Moss classification):

TABLE XXXIX

	THE UNKNOWN BLOOD BELONGS TO GROUP
X Corpuscles plus Group II Serum equals 0 X Corpuscles plus Group III Serum equals 0	IV
X Corpuscles plus Group II Serum equals plus X Corpuscles plus Group III Serum equals 0	III
X Corpuscles plus Group III Serum equals plus X Corpuscles plus Group II Serum equals 0	II
X Corpuscles plus Group II Serum equals plus X Corpuscles plus Group III Serum equals plus	I

Report results as follows:

Determination of Blood Group The blood belongs to Group....

2. **Transfusion Test.**—Having determined that the donor and recipient belong to same group, make a preparation as described above using the suspension of the donor's corpuscles, and the recipient's serum, and another from the donor's serum and the recipient's corpuscles. There should be no agglutination or hemolysis.

Report as follows: Donor.....

Transfusion Test ..... No agglutination or hemolysis.

CHAPTER IX

PREPARATION OF BACTERIOLOGICAL SOLUTIONS, STAINS, AND MEDIA

Sodium Citrate Solution for Blood Culture

Sodium chloride ..... 8.5 grams
Sodium citrate .....15.0 grams
Water (distilled) .....1000 c.c.

Fill in 60 c.c. Erlenmeyer flasks, in 20 c.c. amounts. Sterilize in autoclave.

Physiological Salt Solution

Sodium chloride, chemically pure .....8.5 grams
Water .....1000 c.c.

Stock Staining Solutions (Wood)

	Saturation Strengths
Basic fuchsin in alcohol	3.0 per cent
Gentian violet in water	1.5 per cent
Gentian violet in alcohol	4.8 per cent
Methylene blue in water	6.7 per cent
Methylene blue in alcohol	7.0 per cent
Safranin in water	4.0 per cent

The saturated alcoholic solutions can be kept and aqueous staining solutions made from them by adding 5 per cent of the saturated alcoholic solution to water.

Carbol-Thionin

Saturated solution of thionin in 50% alcohol.... 10 c.c.
2% phenol .....100 c.c.

Stain two minutes.

Andrade Indicator

0.5% solution of acid fuchsin	100 c.c.
Normal sodium hydroxide	16 c.c.

Gram Stain

Reagents.—

1. Sterling's Gentian Violet Solution.—

Gentian violet	5.0 gm.
Anilin oil	2 c.c.
95% alcohol	10 c.c.
Water	88 c.c.

Mix the anilin oil and the 95 per cent alcohol. Shake and add the distilled water. Add the anilin solution to the gentian violet while grinding in a mortar. Filter through paper.

This solution has intense staining power and will keep indefinitely.

2. Gram's Solution.—

Iodine	1 gm.
Potassium iodide	2 gm.
Water	300 c.c.

3. Counter Stain.—

Use either Bismarck brown 0.5 per cent aqueous solution; or safranin, 0.1 per cent aqueous solution.

Procedure.—1. Stain the fixed smear in the Sterling's gentian violet for a minute.

2. Wash in water.

3. Cover with Gram's solution for one minute.

4. Decolorize in 95 per cent alcohol until the smear has a grayish color.

5. Wash in water.

6. Counterstain one minute with the 0.5 per cent Bismarck brown, or the 0.1 per cent safranin.

7. Wash, dry and examine.

The gram positive organisms are stained purple; the gram negative ones are brown if Bismarck brown be used as counter stain, or red if safranin be used.

Neisser's Stain for Diphtheria Bacillus

Reagents.—

1. Neisser Stain Number 1 (Methylene Blue Solution).—

   Methylene blue ..... 1 gram
   Alcohol—95% .....20 c.c.

   Dissolve and add

   Glacial acetic acid .....30 c.c.
   Distilled water .....950 c.c.

   Filter.

2. Neisser Stain Number 2 (Bismarck Brown Solution).—

   Bismarck brown .....2 grams
   Boiling distilled water .....1000 c.c.

   Dissolve—filter after the solution cools.

Procedure.—

1. Stain in solution Number 1—one to three seconds.
2. Wash quickly in water.
3. Stain in solution Number 2—three to five seconds.
4. Wash quickly, dry and examine.

The diphtheria bacilli appear a light brown, rods containing one to three dark blue granules (polar bodies).

Ziehl-Neelson Stain for Tubercle Bacilli

Reagents.—

1. Carbol-Fuchsin.—

   Basic fuchsin, saturated alcoholic solution ..... 90 c.c.
   5 per cent carbolic acid to .....100 c.c.

2. Acid Alcohol.—

   Hydrochloric acid, conc ..... 20 c.c.
   80 per cent alcohol to .....1000 c.c.

3. Loeffler's Methylene Blue.—

   Methylene blue, saturated alcoholic solution..... 30 c.c.
   Potassium hydroxide, 0.01 per cent solution .....100 c.c.

Procedure.—1. Fix the smear by passing it through the flame of a Bunsen burner.

2. Cover the smear with carbol-fuchsin and warm it till the stain steams. Maintain this temperature for 3 to 5 minutes.

3. Wash in running water.

4. Decolorize in acid alcohol until only the thickest part of the smear remains a faint pink.

5. Wash in water.

6. Stain in Loeffler's methylene blue, 30 seconds to one minute.

7. Wash dry and examine under the oil immersion objective.

The tubercle bacilli appear red; all else is blue.

Ponder's Stain

Prepare as follows:

Toluidin blue	0.02	gram
Glacial acetic acid	1	centimeter
Absolute alcohol	2	centimeters
Distilled water	100	centimeters

The film is made on a cover glass and fixed in the usual way. A small quantity of the stain is spread on the film and the cover glass turned over and mounted as a hanging drop preparation. The metachromatic granules of the diphtheria bacilli stain with striking intensity. With diphtheroids the more intense staining sharply differentiates from ordinary cocci and bacilli, which show in the preparation only as faint light blue bodies.

Capsule Stain (Modified Hiss Stain)

Smear the organisms in a drop of animal serum, preferably beef blood serum, and make film preparation on a glass slide.

Dry in air and fix by heat.

Stain for a few seconds with Sterling's gentian violet. The preparation is flooded with the dye and held for a second over a free flame until it steams.

Wash off stain with 20 per cent copper sulphate solution.

Decolorize quickly with 95 per cent alcohol.

Wash again with the copper sulphate solution.

Dry and mount.

Huntoon Capsule Stain

(Huntoon, F. M.: Jour. Bact., 1917, ii, 241)

Solutions.—

1. Nutrose Diluent Solution.—

Sift three grams of nutrose into 100 c.c. of distilled water and heat to 100° C. in the Arnold sterilizer for one hour. Add 5 c.c. of 2 per cent aqueous solution of carbolic acid to serve as a preservative. Decant into test tubes and allow to settle. Employ the supernatant fluid as the diluent. Since the supernatant fluid tends to become thinner by constant precipitation of the nutrose the solution should be occasionally reboiled.

2. Fixing and Staining Solution.—

2 per cent aqueous solution of carbolic acid..	100	c.c.
Lactic acid 75 per cent.....	0.25-0.5	c.c.
1 per cent acetic acid.....	1	c.c.
Saturated alcoholic solution of basic fuchsin..	1	c.c.
Carbol-fuchsin (old)	1	c.c.

Technic.—1. Employ the nutrose solution as a diluent, emulsifying the bacteria in one or two loopfuls and then spreading in as thin a film as possible with the loop.

2. Allow to dry in the air.

3. Cover the film with the fixative and staining solution and allow to act for thirty to forty-five seconds.

4. Wash quickly in water, dry and examine.

Remarks.—If nutrose is not available, the following solution may be used as a substitute for the 3 per cent nutrose solution: Render milk as nearly fat free as possible by means of centrifugation, add 1 per cent of 2 N sodium hydrate and bring to a boil. After cooling add ether and shake. After a few minutes decant off the ether. The remaining opalescent fluid may be used in place of the nutrose solution.

Stain for Spirocheta Pallida (Medalia)

(Medalia: Jour. Am. Med. Assn., 1918, lxx, 914)

The surface of the lesion is cleaned with a wad of sterile absorbent cotton, soaked in sterile saline solution, and all the dried

exudate is removed. The patient is then told to squeeze the lesion between the thumb and forefinger until serous exudate appears. The appearance of the exudate may be hastened by scraping the deeper parts or the ragged edges of the lesion with a broken wooden applicator, or with a stiff platinum wire. The exudate thus obtained from the deeper parts of the lesion should be mostly a serous exudate; real bleeding should be avoided. A good smear is one in which only an occasional red blood cell is found. The smears may be made on clean slides by placing a drop of deep exudate obtained with a broken applicator, a sterile toothpick, or a platinum loop, on one slide and spreading it out thinly with the other slide. The slides should not be pulled out but slid apart.

The smears are then dried in the air and stained with Wright's blood stain in the ordinary way, except that instead of plain distilled water to dilute the stain, one per cent solution of sodium carbonate in distilled water is used. The film is covered with Wright's stain, to fix the smear, a sufficient quantity being used, a little in excess of what is used in staining blood smears. At the end of from one to two minutes, to the staining fluid are added from 45 to 50 drops of one per cent sodium carbonate solution in distilled water to a slide, or as much as the film will hold without running over. The diluted stain is allowed to remain on the film for fifteen to twenty minutes, and is gently steamed all the while with the flame of an alcohol lamp, as in the staining of sputum for tubercle bacilli by carbol fuchsin. The slide is then lightly washed with water, dried between filter paper and examined with the oil immersion lens.

The spirochetes appear intensely violet on a pale blue background and are easily found.

Sterilization

All dry glassware should be sterilized in a hot air sterilizer for one hour at 150° C. Petri dishes and pipettes are placed in copper boxes or wrapped in paper. Tubes and flasks are plugged with absorbent cotton.

Media is sterilized by steam, using either an autoclave or Arnold sterilizer. Media containing sugar is heated in the Arnold

on three successive days for thirty minutes. The temperature cannot rise above 100° C. since no pressure is used. All other media is sterilized by heating in the autoclave at 15-20 pounds pressure for 15-30 minutes. Fifteen pounds of pressure is equivalent to a temperature of 121.3° C., 30 pounds is equivalent to 134.6° C.

Titration of Culture Media to Definite Hydrogen-ion Concentration

The reaction of culture media should be accurately adjusted to a definite hydrogen-ion concentration. This may be quickly and simply done by the colorimetric method.

Reagents.—(1) Phenolsulphonephthalein (phenol red), 0.02 per cent water solution.

(2) N/10 NaOH and N/1 NaOH.

(3) Standard phosphate mixtures of varying hydrogen-ion concentration prepared according to Sorensen's directions as follows:

One-fifteenth mol. acid or primary potassium phosphate. 9.078 grams of the pure recrystallized salt (KH_2PO_4) is dissolved in freshly distilled water and made up to 1 liter.

One-fifteenth mol. alkaline or secondary sodium phosphate. The pure recrystallized salt ($Na_2HPO_412H_2O$) is exposed to the air for from ten days to two weeks, protected from dust. Ten molecules of water of crystallization are given off and a salt of the formula $Na_2HPO_42H_2O$ is obtained. 11.876 grams of this is dissolved in freshly distilled water and made up to 1 liter. The solution should give a deep rose-red color with phenolphthalein. If only a faint pink color is obtained, the salt is not sufficiently pure.

The solutions are mixed in the proportions indicated in Table XL to obtain the desired P_H.

Procedure.—Measure 10 c.c. of the media to be titrated into a test tube that has been rinsed with a portion of the media. Add 0.5 c.c. of the 0.02 per cent phenolsulphonephthalein. Run in from a burette a measured quantity of N/10 NaOH until the color matches that of the standard chosen.

To make the color standard measure 10 c.c. of the phosphate

TABLE XL

P_H	6.4	6.6	6.8	7.0	7.1	7.2	7.3	7.4	7.5	7.6	7.7	7.8	8.0	8.2	8.4
Primary potassium phosphate c.c.	73	63	51	37	32	27	23	19	15.8	13.2	11.0	8.8	5.6	3.2	2.0
Secondary sodium phosphate c.c.	27	37	49	63	68	73	77	81	84.2	86.8	89.0	91.2	94.4	96.8	98.0

mixture corresponding to the hydrogen-ion concentration to which the media is to be adjusted and add 0.5 c.c. of the 0.02 per cent solution of phenol red. Compare in color comparator, holding four tubes. Place behind the media to be tested a tube of distilled water and behind the color standard a tube of the media containing no indicator (Fig. 61). In this way the native color of the media is compensated for.

Calculate the amount of normal alkali or acid to be added to the medium to give the proper reaction. For example, if it requires 2 c.c. of N/10 NaOH to bring 10 c.c. of the medium to P_H 7.6 then to bring 1 liter of the medium to the same reaction will require the addition of

$$\frac{1000}{10} \times 2 = 200 \text{ c.c. N/10 NaOH or } 20 \text{ c.c. N/1 NaOH.}$$

Media is made more acid by the process of autoclaving. The change is small and is much less with meat infusion than with meat extract media.

Table XLI shows the optimum reaction for the growth of various organisms and for the preparation of special media.

TABLE XLI

OPTIMUM H-ION CONCENTRATION OF MEDIA FOR CULTIVATION OF VARIOUS ORGANISMS, AND FOR PREPARATION OF SPECIAL MEDIA

(Fennel and Fisher: Jour. Infect. Dis., 1919, xxv, 418.)

B. Typhosus	6.2 – 7.2
B. Paratyphosus	6.2 – 7.2
Pneumococcus	7.8
B. Influenzae	7.8
Streptococcus Viridans	7.6 – 7.8
Streptococcus Hemolyticus	7.6 – 7.8
Meningococcus	7.6
Gonococcus	7.4 – 7.6
B. Dysenteriae, Shiga, Flexner, and "Y"	6.2 – 6.8
B. Diphtheriae for toxin production	8.0 – 8.2
Endo Agar	7.4 – 7.8
Russell Agar	7.4 – 7.6
Brilliant Green Agar	7.0 – 7.2

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.

II. Add 15 grams of thread agar, 10 grams peptone, and 5 grams sodium chloride to 500 c.c. water and dissolve in autoclave.

Cool to about 65° C.

III. Then to Solution I of meat infusion add Solution II of agar (at 65° 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 P_H 7.4, add normal sodium hydrate solution as required and place the mixture in the Arnold sterilizer for one-half hour.

Filter through gauze and absorbent cotton. Sterilize in the autoclave for 15 minutes at 15 pounds pressure.

Glucose Agar

Dissolve 10 grams of glucose in one liter of stock agar. Tube and autoclave at 15 pounds pressure for 15 minutes.

Meat Extract Agar

To 1 liter of tap water add:

Peptone	10 grams
Beef extract	3 grams
Sodium chloride	5 grams
Agar	15 grams

Dissolve all ingredients except agar first; add agar and autoclave for one hour at fifteen pounds pressure. Cool to 50° C. Add white of an egg and steam in Arnold for thirty minutes.

Adjust reaction to P_H 7.4. Steam for a few minutes. Filter through cotton, and autoclave for fifteen minutes at fifteen pounds pressure.

Red Blood Agar

To 100 c.c. of melted agar at a temperature of 50° C. add 5 c.c. of defibrinated blood. Pour plates using 12 to 15 c.c. of agar for each 100 mm. plate.

Brown Blood Agar

To 100 c.c. of melted agar at a temperature of 90° C., add 5 c.c. of defibrinated blood. Allow the mixture to stand until it assumes a chocolate brown color.

Pour into Petri dishes using 12-15 c.c. of blood for a 100 mm. plate.

Meat Infusion Broth

One pound of ground beef in one liter of water is heated to 55° C. for one hour. Filter through cotton. Add peptone, one per cent and sodium chloride, 0.5 per cent, and bring to boiling to dissolve the peptone. Adjust reaction to P_H 7.8-8.0. Filter through paper and autoclave in liter quantities.

For pneumococcus cultures add 0.5 per cent glucose.

Meat Extract Broth

Peptone	10 grams
Meat extract	5 grams
Salt	5 grams
Water	1000 c.c.

Dissolve by heat. Adjust reaction to P_H 7.4. Heat for a few minutes.

Filter through paper. Autoclave under 15 pounds pressure for 15 minutes.

Endo's Agar

To 1000 c.c. of water add:

Peptone	10 grams
Beef extract	3 grams
Sodium chloride	5 grams
Agar	15 grams

Dissolve all ingredients except agar first; add agar and autoclave for one hour at 15 pounds pressure. Cool to 50 degrees. Add the white of one egg and steam in autoclave for thirty minutes.

Adjust reaction to P_H 7.4. Boil over free flame for 5 to 6 minutes. Filter. Fill in flasks in 100 c.c. amounts and autoclave.

Before pouring the Endo plates the reaction is adjusted to 7.6 to 7.8. It usually takes 0.9 c.c. N NaOH for 100 c.c. agar. The amount required must be determined for each batch.

Add to each 100 c.c. flask one gram of chemically pure lactose, 0.5 c.c. of a saturated alcoholic solution of basic fuchsin and 1 c.c. of a 10 per cent solution of anhydrous sodium bisulphite.

Colonies of lactose fermenting bacteria when cultured on this media are red; others are colorless.

Russell's Agar

Make meat infusion agar as described on page 202 and adjust reaction to P_H 7.4.

Add 10 c.c. of Andrade indicator per liter, and tube in 5 c.c. amounts using Wassermann tubes (4 × ½ in.). Autoclave under 15 pounds pressure for 15 minutes. While still hot add to each tube 0.25 c.c. of a solution containing 20 per cent lactose and 2 per cent glucose. Cool in such a position that the slant will be 1½ inches long, and the butt ½ to ⅝ inches in depth.

In inoculating the tube the needle is drawn along the slant and then stabbed through to the bottom of the tube.

The following table shows the appearance of the media after incubation with the common gram negative bacilli.

	Butt	Slant
B. Typhosus	Acid	Unchanged
B. Paratyphosus "A"	Acid and gas	Unchanged
B. Paratyphosus "B"	Acid and gas	Unchanged
B. Dysenteriae	Acid	Unchanged
B. Coli and members of the colon group	Acid and gas	Acid

Krumwiede's Brilliant Green Media

Make stock agar as for Endo medium. Adjust reaction to 7.0-7.2. To each 100 c.c. of melted stock agar add 0.25 c.c. of a one per cent solution of neutral red, one per cent lactose and 0.1 per cent glucose. The optimum concentration of brilliant green must be determined for each batch of agar as follows:

Pour plates using three dilutions of the dye; namely, 1 to 200,000, 1 to 330,000, and 1 to 500,000, or expressed in terms

of 0.1 per cent dye solution per 100 c.c. agar the amounts are 0.5, 0.3, and 0.2 c.c. Add a loopful of a twenty-four hour broth culture to 10 c.c. of a stool suspension of moderate density, and inoculate the plates containing the different dilutions of brilliant green, and a plate of Endo medium as a control. Incubate 18 to 24 hours. Determine: (1) The concentration of dye which has little effect on the number and size of the typhoid colonies but which shows a marked restraint of the fecal flora; and (2) the concentration which shows a moderate reduction in number and size of the typhoid colonies and a still greater reduction of the fecal flora. The two optimum dilutions average 0.2 and 0.3 respectively of a 0.1 per cent solution of the dye to 100 c.c. of agar.

Dextrose Brain Broth

(Rosenow Formula)

Dehydrated Bacto nutrient broth (Digestive Ferments Co.)	8 gm.
Sodium chloride	8 gm.
Dextrose C. P.	2 gm.
Andrade indicator	10 c.c.
Distilled water	1000 c.c.

Dissolve the broth and salt by heating. When cool add the indicator and dextrose. Tube in large tubes (8 x 5/8 in.). Add three pieces of calf brain, about 1 cm. square, also 2 or 3 pieces of calcium carbonate, preferably as crushed marble, to each tube. Sterilize for 20 minutes in the autoclave at 20 pounds pressure.

If the broth is to be used for blood cultures, 5 gm. of sodium citrate is added to each liter, to prevent coagulation of the blood.

Dextrose Brain Agar

Dissolve 7 gm. of powdered agar in 1 liter of dextrose broth prepared as indicated above. Add the calf brain and calcium carbonate, tube and sterilize.

Potato Media

Select large potatoes. Remove skin and cut cylinders with an apple corer. Wedge-shaped pieces are obtained from the cylinders by an oblique cut.

Place in test tubes, add a little water and sterilize in autoclave under 20 pounds pressure for 15 minutes.

Dunham's Peptone Solution

Dissolve 10 grams peptone and 5 grams sodium chloride in 1,000 c.c. water.

Filter through paper until clear. Tube, and sterilize by autoclaving for 15 minutes at 15 pounds pressure.

Litmus Milk

Place perfectly fresh milk in ice box overnight. Syphon off the milk leaving the cream in the bottle. Add litmus solution until the milk is a light blue color. Tube, and sterilize for 20 minutes on three successive days in the Arnold sterilizer.

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 15 minutes. Adjust weight, filter through cotton, and sterilize by fractional sterilization. Gelatin should not be subjected to prolonged heating.

Loeffler's Blood Serum

Allow the serum to separate from clotted sheep or ox blood. Mix three parts of clear serum with one part of meat infusion broth containing 1 per cent dextrose. Fill into small culture tubes. Put tubes in slanting position in Koch serum inspissator and heat gradually until temperature reaches 90° Centigrade. Hold temperature at this point until medium is completely solidified. Sterilize on the two successive days by placing in Arnold sterilizer for 20 minutes. Raise temperature very slowly.

Petroff's Tubercle Bacillus Medium

Treat 500 grams of chopped-up meat with 500 c.c. of 15 per cent glycerine solution. Keep in ice chest for 24 hours and filter through gauze. Sterilize the shells of eggs by immersion in 70 per cent alcohol for ten minutes or by dipping them in boiling water for five seconds or so. Mix white and yolk of these eggs in a *sterile* mortar and add an equal volume of the glycerine meat infusion which should have had added to it before mixing 1 c.c. of 1 per cent alcoholic solution of gentian violet to each 100 c.c. of the glycerine meat infusion.

Put 3 to 4 c.c. of this medium in test tubes and inspissate as slants at 85° C. until the medium is solidified. Subject these slants to temperature of 75° C. on the second and third days for one hour.

In culturing sputum mix with an equal amount of 3 per cent sodium hydroxide and leave in incubator for one to two hours. Neutralize with normal hydrochloric acid and centrifugalize. Take up sediment and smear out on slants.

Sugar-Free Broth

Inoculate a liter of meat infusion broth with Bacillus coli. Incubate for two days. Heat in Arnold sterilizer for 20 minutes. Adjust reaction and heat thoroughly again. Put about 5 grams pure talc in a large mortar. Add the dead colon culture, stirring constantly. Filter through filter paper until perfectly clear. This is used as stock broth from which the carbohydrate media is made.

Carbohydrate Broth for Fermentation Reactions

Add sugar in proportion of one per cent to sugar free broth. Put in Dunham fermentation tubes (Fig. 54) and heat in Arnold sterilizer for 20 minutes on three successive days.

Glucose Broth for Blood Culture

Add 10 grams dextrose to 1,000 c.c. of meat infusion broth. Autoclave under 15 pounds pressure for 15 minutes.

Glucose Ascitic Fluid Broth

Add one part of sterile ascitic fluid to three parts of sterile glucose broth.

Carbohydrate Serum Water Fermentation Media (Hiss Media)

Collect sheep or ox blood in sterile wide-mouth bottles. Separate clot and place in ice box for 24 hours. Pipette off clear serum and add three volumes of distilled water.

Heat for 15 minutes in the Arnold Sterilizer.

Add 1 per cent Andrade indicator and the desired sugar in

Fig. 54.—Dunham fermentation tube.

proportion, 1 gram to 100 c.c. Dissolve, tube, and sterilize in Arnold sterilizer on three successive days.

In making inulin medium it is advisable to first dissolve the inulin in water and sterilize for 15 minutes in the autoclave before adding it to the diluted serum.

Lactose Bile Medium

To 1,000 c.c. of fresh ox bile add 10 grams peptone and 10 grams lactose. Dissolve and filter.

Place in bottles in 50 c.c. amounts. Autoclave 15 minutes under 15 pounds pressure.

CHAPTER X

GENERAL BACTERIOLOGICAL METHODS

Blood Culture

Before the venipuncture is done, clean up the antecubital space very thoroughly with tincture of iodine and alcohol. Withdraw blood with sterile syringe. Place 10 c.c. of blood in citrate solution (1.5 per cent sodium citrate in physiological salt solution) provided in 50 cubic centimeter Erlenmeyer flasks. Pour half of citrated blood into a bottle containing 50 c.c. of glucose meat infusion broth and incubate. Melt two tubes each of plain and of glucose agar, cool to 42°, and add some citrated blood. Pour into petri plates. If a typhoid infection is suspected, add some citrated blood to a bottle containing lactose bile medium.

At intervals place drops of broth and of the sediment at the bottom of the bottle on a slide, dry, stain with methylene blue and examine for organisms.

If bile cultures are made, transfer a loopful to a Russell agar slant on the second, third, and fifth days. If the typhoid bacillus is present the butt of this Russell tube will become red and the slant remain colorless if Andrade indicator is used, or remain blue, if litmus is used as indicator.

Sputum Culture

A small portion of sputum is selected and washed through three or four changes of sterile salt solution in sterile petri dishes.

The washed sputum is then thoroughly emulsified in a small amount of sterile salt solution or broth in a sterile test tube.

Smears are made from the washed sputum and stained by gram. A drop of the emulsion is transferred to a blood agar plate and spread over the surface of the plate with a glass rod.

Incubate for twenty-four hours. If the plate shows a pure culture of pneumococcus the growth may be washed off with sterile salt solution and the type identified as indicated under

the determination of types of pneumococcus. Single colonies may be picked and grown in broth for type determination. Hemolytic streptococcus colonies may be identified directly by the clear zone of hemolysis and by the Gram stain.

For the influenza bacillus the plate should be incubated forty-eight hours.

Influenza colonies are minute, colorless, translucent, and discrete. The influenza bacillus when stained is small, shows polar staining and is Gram negative.

Other organisms present may be identified by smear and further cultures.

Stool Culture

If typhoid or paratyphoid bacilli are to be searched for, use Endo and Krumwiede's brilliant green agar; for dysentery bacilli use freshly prepared Endo agar.

The stool should be fresh. Dilute fluid feces or emulsify solid feces in peptone water or broth to a density corresponding to one part of solid feces to fifteen of diluent. Allow the suspensions to stand fifteen to thirty minutes for sedimentation of the particles and then inoculate plates from the surface of the suspension.

The plates are conveniently inoculated by dipping a bent glass rod into the fecal suspension and passing it over three plates in succession using a clockwise motion.

In the case of dysentery, select fragments of bloody mucus, rinse free of fecal material and inoculate on Endo plate. Spread with a bent glass rod passing the rod over several plates in succession.

Incubate 18 to 24 hours and fish colonies having the appearance of the typhoid, paratyphoid, dysentery group to Russell medium. After incubation a tentative decision as to type can be made from the appearance of the tubes (see Russell medium, page 205).

The final identification is made by macroscopic agglutination with the appropriate specific immune serum depending upon the reaction obtained with the Russell tube, and by further fermentation reactions (Table XLII).

Urine Culture

If the urine is clear, pour a small amount on a plain agar plate, rotate or tilt plate so that the specimen is well spread over the surface, and drain off excess.

If the specimen is cloudy use only a few drops and spread over surface of plate with a sterile rod. Also pour several drops on a Russell agar slant, spread over surface and stab through to bottom of tube with platinum wire.

If the plate shows a growth after incubation, make smear and stain by Gram method. Identify a Gram negative bacillus as indicated under stool culture.

Nose and Throat Cultures

All diagnostic cultures are to be made on both Loeffler's blood serum and blood agar. Streak surface of a blood agar plate and a blood serum slant with the same swab.

The following morning, make smear from the Loeffler tube and stain with Neisser. Examine under oil immersion lens for diphtheria bacilli.

Examine the blood agar for colonies of B. influenzae, hemolytic streptococcus or other predominant organisms.

Eye and Ear Cultures

Make culture with swab directly on blood agar plate. Incubate 48 hours and identify organisms present.

After culture is made, smear swab over glass slide and stain with Gram's stain. Note types of organisms present.

Report findings of both smear and culture.

Miscellaneous Cultures

The most satisfactory medium for routine use is Loeffler's blood serum. Material obtained at surgical operations or from other sources in which the use of a special medium is not indicated are cultured directly on slants of the blood serum. If the presence of a hemolytic streptococcus is suspected a culture should be made on blood agar also.

Virulence Test for Diphtheria Bacillus

Streak the surface of a number of ascitic infusion agar plates with the growth from the Loeffler tube. Incubate for 16 hours at 37 degrees C. and examine the growth along the line of streak. Diphtheria colonies are most apt to be found at the edges of the streak. Fish suspicious colonies to ascitic infusion broth or Loeffler's medium. If very few bacilli are present it is well to inoculate several tubes of ascitic broth. As the diphtheria bacillus will grow mostly on the surface of the broth, this portion can be used with success when direct plates fail.

After the isolation of the bacillus in pure culture, inoculate a tube of ascitic fluid broth of P_H equal 8.0-8.2.

One cubic centimeter of the ascitic fluid broth culture (incubated 48 hours) is injected subcutaneously into a guinea pig weighing 250 grams. A second guinea pig is injected with the same amount of culture to which has been added 50-100 units of diphtheria antitoxin. If the first pig dies within two or three days and on postmortem examination shows typical engorgement of the adrenals, and the second pig lives, the bacillus is a true toxin-producing diphtheria bacillus. Otherwise, it is not.

Animal Inoculation for Tuberculosis

The centrifugalized sediment from urine, spinal fluid, etc., or the sediment from antiforminized material is emulsified in sterile 0.85 per cent salt solution and injected subcutaneously into the groin of a guinea pig.

The inoculation should be made into the loose tissue of the groin well out and away from the midline, avoiding the mammary gland. It is well to always inoculate two pigs.

A lesion developing in 3 to 4 days is always nontuberculous and usually subsides.

If the suspected material contains tubercle bacilli, the inguinal glands begin to swell in from twelve to twenty days; rarely at eight to twelve.

The guinea pig is killed after the inguinal glands show definite involvement, and autopsied. Smears are made from the glands and stained for tubercle bacilli by the Ziehl-Neelson method.

TABLE XLII

FERMENTATION REACTIONS OF THE COMMONER GRAM-NEGATIVE BACILLI

	DEXTROSE	LACTOSE	SACCHAROSE	MALTOSE	MANNITE	LEVULOSE	GALACTOSE	DEXTRIN	LITMUS MILK	INDOL FORMATION	MOTILITY	REMARKS
B. Typhosus	+*	0	0	+	+	+	+	+	±	0	+	
B. Paratyphosus A	++	0	0	++	++	++	++	0	+	0	+	
B. Paratyphosus B	++	0	0	++	++	++	++	0	±	0	+	
B. Dysenteriae (Shiga)	+	0	0	0	0	0	0	0	±	0	0	
B. Dysenteriae (Flexner)	+	0	+	+	+	+	+	+	±	+	0	
B. Dysenteriae (Hiss-Russell)	+	0	0	0	+	+	+	0	±	+	0	
B. Dysenteriae (Rosen)	+	+	0	+	+	+	+	+	+	+	+	
B. Enteritidis	++	0	0	++	++	++	++	++	0	0	+	
B. Coli Communis	++	++	0	++	++	++	++	++	+	+	+	Milk coagulated
B. Coli Communior	++	++	++	++	++	++	++	++	+	+	+	Milk coagulated
B. Paracolon (Day)	++	0	++	++	++	++	++	++	±	?	+	
B. Lactis Aerogenes	++	++	++	++	++	++	++	++	+	+	0	Milk coagulated
B. Acidi Lactici	++	++	0	++	++	++	++	++	+	+	0	Milk coagulated
B. Cloacae	++	++	++	++	++	++	++	++	+	0	+	Milk coagulated and peptonized
B. Fecalis Alkaligenes	0	0	0	0	0	0	0	0	0	0	+	
B. Mucosus Capsulatus	++	++	++	++	++	++	++	++	+	+	0	Milk coagulated
B. Proteus	++	0	++	0	++	++	++	?	+	+	+	Milk coagulated and peptonized

*0 —No fermentation
\+ —Acid formation
++—Acid and gas formation
±—Primary acidity with terminal alkalinity.

TABLE XLIII

FERMENTATION REACTIONS OF GRAM-NEGATIVE GROUPS OF COCCI ON CARBOHYDRATE SERUM-WATER MEDIA
(Elser, Wm. J., and Huntoon, Frank, M.: Jour. Med. Res., 1909, xx, 427.)

	DEXTROSE	MALTOSE	LEVULOSE	SACCHAROSE	LACTOSE	GALACTOSE	DEXTRIN	MANNITE	DULCITE	INULIN
Meningococcus	+*	+	0	0	0	0	0	0	0	0
Pseudomeningococcus	+	+	0	0	0	0	0	0	0	0
Gonococcus	+	0	0	0	0	0	0	0	0	0
Micrococcus Catarrhalis	0	0	0	0	0	0	0	0	0	0
Micrococcus Pharyngis Siccus	+	+	+	+	0	0	0	0	0	0
Chromogenic Group I	+	+	+	+	0	0	0	0	0	0
Chromogenic Group II	+	+	+	0	0	0	0	0	0	0
Chromogenic Group III	+	+	0	0	0	0	0	0	0	0

*+ Acid formation.
0 No fermentation.

Sections are made from the spleen and glands for histological examination.

If the pig shows no signs of tuberculosis, it should be killed at the end of six weeks and autopsied.

Examination of Stool for the Tubercle Bacillus

Select purulent or mucous particles and make film preparation. If no tubercle bacilli are found, dilute feces with three volumes of water, mix thoroughly and allow to stand one-half hour. Saturate the filtrate with dry sodium chloride and allow to stand one-half hour. Skim off the film from the surface, dilute material with distilled water and add antiformin to 20 per cent concentration. Treat sediment as in the case of sputum. (Page 80.)

All cases showing positive findings in stool should be controlled with a sputum examination.

Examination of Urine for the Tubercle Bacillus

Collection of Specimen.—(a) Men: Patient is cleaned up as for a catheterization and then voids into a sterile vessel. The first few drops voided are discarded. (b) Women: A specimen is collected by catheterization, using routine sterile technic. As much urine as possible should be obtained.

Procedure.—The total specimen is centrifuged at high speed for at least one-half an hour. Place a drop of blood serum on a slide, mix the sediment with it and spread.

Dry the smear in the air, fix in flame, and stain by the Ziehl-Neelson method.

If there is a large amount of pus present, mix the sediment with distilled water, add antiformin to 20 per cent concentration, place in incubator for several hours, centrifuge again and make preparation on slide as above.

Remarks.—In a properly collected specimen all acid-alcohol-fast bacilli can be safely considered as tubercle bacilli. Absolute differentiation can be made, however, only by guinea pig inoculation.

Examination of Smears for the Gonococcus

A satisfactory examination for the gonococcus can be made only on well prepared specimens. In men a preparation is made

by carefully spreading secretion from the urethra on a glass slide. Norris and Mickelberg suggest that specimens be obtained in women with a medicine dropper which has been drawn out in the flame to 6-8 cm. in length and the thickness of a coarse capillary tube. The drop of material desired for examination is drawn up into the pipette and spread evenly on a slide in weak mercury bichloride solution. In female children a soft eye syringe is used. Weak bichloride of mercury solution is sucked in and out of the vagina several times. The washings are centrifugalized and the sediment examined for organisms. In making slide preparations all pressure must be avoided since the pus cells are easily crushed.

The smear on the slide is stained by Gram's method. The gonococci are gram negative and appear as small biscuit-shaped cocci in pairs. In carefully made preparations they are always found largely in pus cells. In a positive case one nearly always finds a few pus cells containing a very large number of cocci.

In urethral smears one may be reasonably sure that diplococci morphologically similar to gonococci occurring outside pus cells are specific. In the vulvovaginal tract one may find other diplococci which stain as gonococci hence one cannot be sure here that organisms occurring outside the pus cells are really gonococci. Only those smears showing leucocytes filled with morphologically typical gonococci are reported positive.

Classification of Streptococci

(Holman, W. L.: Jour. Med. Research, 1916, xxxiv, 377)

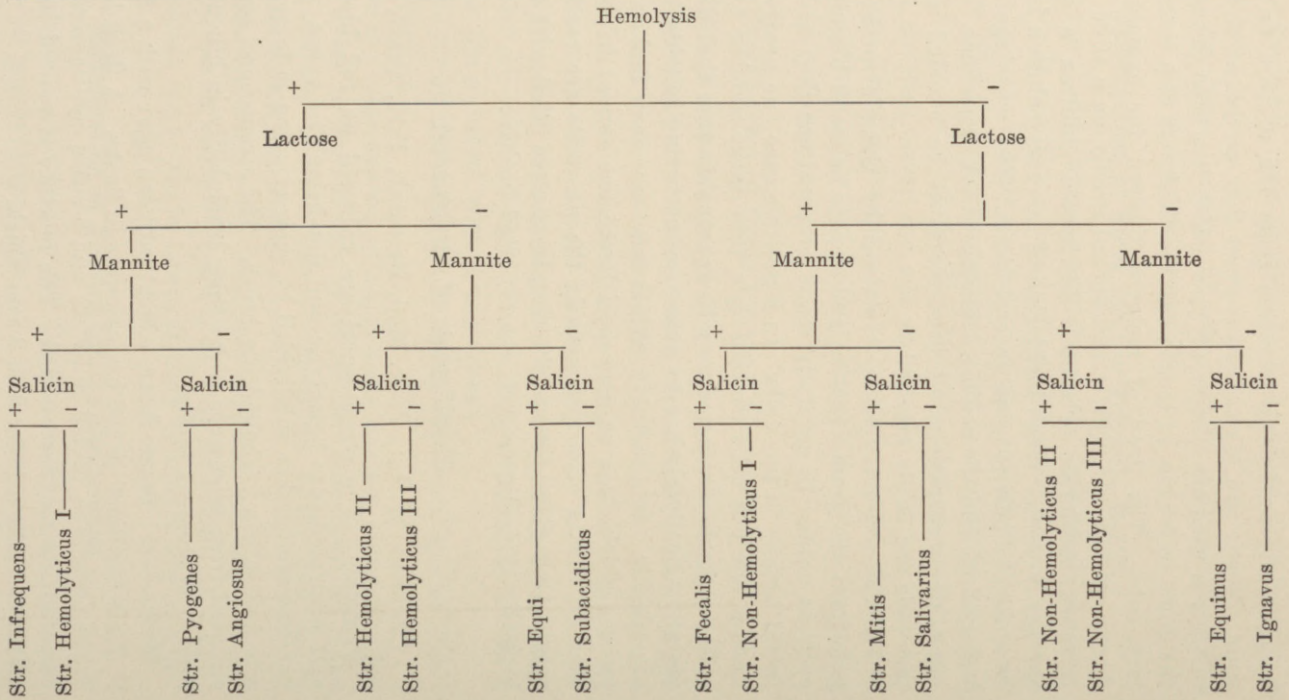
Principle.—The streptococci are cultured on red blood agar and separated into hemolytic and non-hemolytic types. The organisms are further subdivided by their reaction in lactose, mannite and salicin carbohydrate broth. The streptococci are differentiated from pneumococci by their insolubility in bile and their failure to ferment inulin.

Media.—(1) Blood Agar.—Meat infusion agar with a P_H equal to 7.4 is sterilized in 100 c.c. amounts in flasks. A flask is heated in the autoclave, cooled to 50° C. and 5 cubic centimeters of defibrinated human blood added. The mixture is poured into petri dishes to make a layer 2 to 3 mm. thick.

TABLE XLIV

CLASSIFICATION OF STREPTOCOCCI

(Gram-positive cocci in chains, no capsules, insoluble in bile)



(2) Carbohydrate Media.—Take 200 c.c. of double strength meat infusion broth with P_H equal 7.4, add to this 100 c.c. of water, 4 grams of the test substance and 4 c.c. of Andrade's indicator. This is sterilized in a large flask on three successive days in an Arnold sterilizer. Beef serum diluted one half with water is slowly filtered through a Berkefeld filter and 200 c.c. added to the above. The whole is tubed through a sterile funnel into sterile test tubes and the tubes incubated for two days to eliminate chance contaminations.

Procedure.—The blood agar plate is streaked with a loopful of the streptococcus culture. The surface is covered with as many parallel lines as it is possible to make in order to thoroughly distribute the material.

After growing the streptococcus on blood agar for 24 hours transfer to inulin, lactose, mannite and salicin serum broth tubes and incubate for at least a week at 37° C.

Colonies of hemolytic streptococcus on the blood agar plate will show well defined colorless zones of hemolysis.

There should be no pigmented corpuscles visible under the low power of the microscope remaining next to or under the colony.

If there is any question as to the hemolytic property it is well to mix 0.5 c.c. of a 24 hour bouillon culture with 0.5 c.c. of a 5 per cent suspension of washed human red blood cells in physiologic salt solution and incubate in water-bath at 37° C. for two hours. True hemolytic streptococci produce laking of blood under these conditions.

Pneumococci may be differentiated by solubility in bile when 1 c.c. of a bouillon culture is incubated at 37° C. for one hour with 0.2 c.c. of sterile ox bile.

Table XLIV shows the classification of the streptococci using the above procedure.

CHAPTER XI

MISCELLANEOUS CLINICAL PATHOLOGICAL EXAMINATIONS

Examination of Cerebrospinal Fluid

Reagents.—

(1) Pandy's solution—
Saturated aqueous solution of phenol.

(2) Ross-Jones solution—
Saturated (by heat) aqueous solution of ammonium sulphate.

(3) Fuchs-Rosenthal solution—

Glacial acetic acid	2 c.c.
Methyl violet	0.1 gram.
Water	50 c.c.

(4) Tsuchiya reagent (see page 41).

Procedure.—

1. Test for Excess Globulin.—

Qualitative.—The Pandy Test. To 1 c.c. of Pandy's reagent add one drop of spinal fluid. If an excess of globulin is present a white cloud will develop immediately.

Record results as follows:

Negative	0
Borderline	±
Positive	+
Strongly positive	++

Ross-Jones Test.—Layer the spinal fluid on a saturated solution of ammonium sulphate. If an excess of globulin is present a white ring develops at the line of contact.

Quantitative.—Fill a Felton precipitometer to line marked "Spinal Fluid" and add Tsuchiya solution to line marked "Re-

agent." Invert several times and let tube stand overnight. Read off directly in grams per liter.

2. **Cell Count.**—This should be done as soon after the withdrawal of the fluid as possible since the cells settle out rapidly.

If the fluid is clear draw up the Fuchs-Rosenthal reagent to 1 mark on white blood cell counting pipet and fill to the 11 mark with spinal fluid. Allow pipette to stand for a few minutes, shake and fill a Fuchs-Rosenthal counting chamber (Fig. 55). The white cells are stained violet; red cells if present are only faintly stained. The number of white cells in the entire ruled area divided by 3 gives the number per cubic centimeter.

If a counting chamber with Fuchs-Rosenthal ruling is not available, an ordinary blood cell counting chamber is employed. The

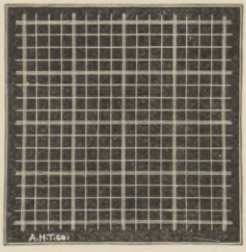


Fig. 55.—Fuchs-Rosenthal ruling of counting chamber for spinal fluids.

total number of cells in the ruled area (Neubauer ruling) multiplied by the factor $\frac{100}{81}$ gives the number of cells per cubic millimeter.

A cloudy fluid should be diluted as for a white blood count and counted in an ordinary counting chamber.

3. **Wassermann Test.**—(See page 175.)

4. **Colloidal Gold Test.**—(See page 222.)

5. **A Culture** should be made if the fluid is cloudy or if otherwise indicated. This is best done by centrifuging the fluid in a sterile tube and culturing the sediment on a blood agar plate and in dextrose bouillon.

6. **Smears.**—A cloudy fluid should be centrifuged, smears made from the sediment, and stained with gram and with methylene

blue. Make a different count of the white cells and look for bacteria.

7. **Examination for Tubercle Bacilli.**—If tuberculosis is suspected, allow tube to stand overnight in ice box. Float the film which forms onto a slide and stain for tubercle bacilli by Ziehl-Neelson method. Another good way of getting a film preparation is to place a cover glass in a conical test glass, add the spinal fluid and allow to stand overnight. The film will collect on the cover glass. The fluid is then pipetted off, the cover glass removed and the film stained by the Ziehl-Neelson Method.

Lange's Colloidal Gold Test on Spinal Fluids

(Miller, Brush, Hammers, and Felton: Bull. Johns Hopkins Hospital, 1915, xxvi, 391)

A. **Preparation of the Colloidal Gold Solution.**—

(a) Reagents.—

1. Merck's gold chloride (sealed in brown glass ampoules) ..... 1 gram.
   Water triply distilled up to..... 100 c.c.
2. Chemically pure potassium carbonate (desiccated) ..... 2 grams.
   Water triply distilled up to..... 100 c.c.
3. Chemically pure formaldehyde (40 per cent solution) ..... 1 c.c.
   Water triply distilled up to..... 40 c.c.
4. Chemically pure crystalline oxalic acid ..... 1 gram.
   Water triply distilled up to..... 100 c.c.

The reagents need not be made up fresh each day but may be kept as stock solutions.

(b) Cleaning the Glassware.—The beakers and pipettes are thoroughly brushed under hot tap water with ivory soap solution. They are then rinsed in running water, filled with bichromate cleaner, and left standing for at least one-half hour. When needed, the beakers are emptied, washed again in running water for five minutes, rinsed with ordinary distilled water, and finally with triply distilled water. The pipettes, flasks and graduates

are cleaned in the same manner. The glassware should not be allowed to dry in the air before use.

(c) Distillation of Water.—The first distillate is taken from an ordinary Stoke's still, and immediately poured into clean Pyrex distilling flasks. Five c.c. of a saturated solution of barium

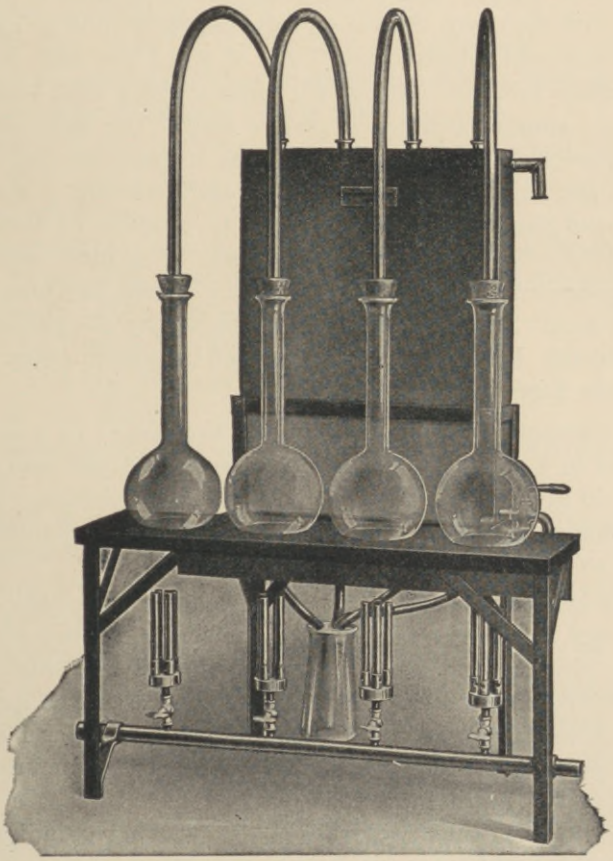


Fig. 56.—Block tin still used in distilling water for colloidal gold solution.

hydroxide for each liter of water is added. The water is then distilled twice, using a still (Fig. 56) with either Pyrex glass or block tin condensing tubes. Each distilling flask is washed out with some of the new lot of water that is to be put into it. The water should be used as soon as possible after its last distillation.

There should be no rubber connections in the distilling apparatus. If the tap water contains chlorine it is better to start with spring water instead of the distillate from the Stoke's still.

(d) Technic for the Preparation of the Solution.—Rinse out a beaker with a portion of the triply distilled water and fill to the liter mark. Raise the temperature gradually to about 50° C., and then turn the gas on full. At 60° C. add 10 c.c. of the 1 per cent gold solution, and 7 c.c. of the 2 per cent potassium bicarbonate solution. At 80° C., while stirring with a thermometer, add slowly 10 drops of oxalic acid. The solution should remain colorless and clear. When the temperature has reached 90° C., the flame is withdrawn and while stirring, 5 c.c. of the 1 per cent formaldehyde, or enough to produce an initial pink color, is slowly added a drop at a time. If a pink color makes its appearance before all the reducing agent has been added, stop at once. The final end point is marked by the production of a beautiful brilliant clear orange-red solution. The solution should be kept in a dark place.

(e) Testing the Solution for.—1. *Reaction:* To 5 c.c. of the solution in a test tube are added 2 drops of 1 per cent alizarin red in 50 per cent alcohol. With an alkaline solution a purplish red color is produced; the neutral point is of a brownish red tinge; an acid solution gives a lemon yellow color.

Ten test tubes are set up in a rack and 1 c.c. of freshly distilled water put into each. Depending upon whether the solution is acid or alkaline as shown by the preliminary test, add 1 c.c. of N/50 NaOH or N/50 HCL to the first tube. Mix well and transfer 1 c.c. to the second tube. Again mix and transfer 1 c.c. from the second to the third tube. Proceed in this manner up to and including the ninth tube. The tenth tube serves as a control. Now add to each tube two drops of the alizarin red indicator and 5 c.c. of the gold solution to be tested. The amount of acid or alkali in each successive tube will be as follows: 0.5 c.c., 0.25 c.c., 0.125 c.c., 0.0625 c.c., etc., each tube having exactly one-half of the preceding one. The tube showing the most typical brownish red is selected as neutral; the amount of acid or alkali in that tube is divided by 5 to determine the amount necessary to neutralize 1 c.c.; this is multiplied by the number of cubic centi-

meters of solution to be corrected and the resulting amount of fiftieth normal hydrochloric acid or sodium hydroxide added to the solution, which is then ready for use. It should be noted that a deep dark red with a purplish tinge in the upper layer when shaken, as well as a deep purple, is a definite sign of an alkaline solution, and that no red without a definite brownish tinge should be regarded as neutral.

2. *Protection.*—Add 1.7 c.c. of one per cent salt solution to 5 c.c. of the colloidal gold solution. The gold should be completely precipitated in one hour's time.

3. *Reaction Curves with Known Normal and Abnormal Fluids.*—Test the fluid with a known paretic cerebrospinal fluid, and four known normal fluids.

(f) Requirements for a Satisfactory Colloidal Gold Solution.—1. It must be clear to both direct and transmitted light and preferably of a brilliant red orange or salmon red color.

2. Five c.c. of the solution must be completely precipitated by 1.7 c.c. of a one per cent sodium chloride solution in the time interval of one hour.

3. The solution must be neutral in reaction on the day on which it is used.

4. It must give a typical reaction curve with a known paretic cerebrospinal fluid.

5. It must produce no reaction greater than a number one with four normal cerebrospinal fluids.

B. **Procedure in Making the Test.**—Into the first of 10 clean, dry test tubes reserved especially for the purpose put 1.8 c.c. of fresh sterile 0.4 per cent NaCl solution made from a stock 4 per cent NaCl solution. Into each of the remaining nine tubes put 1 c.c. of salt solution of the same strength. Now add to the first tube by means of a clean, dry, certified 1 c.c. pipette, 0.2 c.c. of the spinal fluid to be tested. Mix well. Transfer 1 c.c. of the resultant 1 to 10 solution of spinal fluid to the second tube and again mix thoroughly and transfer 1 c.c. of the solution to the third tube. Proceed in this manner up to and including the tenth tube. By this method a series of dilutions of the spinal fluid is secured in geometrical progression ranging from 1 to 10 to 1 to 5120. Now add to each tube 5 c.c. of the colloidal gold

solution. Shake each tube thoroughly. Read the tubes after standing overnight at room temperature. All readings must be done with direct daylight, holding the tubes up against the sky.

In recording the results of the test, numbers are used to denote the various color changes, as follows:

Unchanged	0
Bluish red	1
Reddish blue	2
Deep blue	3
Gray blue	4
Colorless	5

Remarks.—The reaction types are classified as follows:

1. *Normal* in which there is no reaction or a maximum color change of 1.

2. *Zone I* (so-called paretic zone): The greatest reaction is in the first three to six tubes and is of the 5 type. The color values rapidly fall in the succeeding two or three tubes to 0.

3. *Zone II* (so-called luetic type): The greatest color change is in the fourth and fifth tubes but is seldom greater than 4 or less than 3.

4. *Zone III* (so-called meningitic type): The maximum color change occurs in the higher dilutions beginning with the sixth tube.

The reaction types are illustrated in Fig. 57.

Satisfactory solutions cannot be uniformly made with the quantities of reagents directed. If trouble is experienced, it is best to vary the amounts until a successful combination is found. If the solutions are turbid, the oxalic acid should be omitted.

Examination of Ascitic and Pleural Fluids

Collection of Specimen.—Collect specimen in sterile container, diluting the fluid with an equal volume of sterile 1.5 per cent sodium citrate in normal salt solution to prevent clotting. Frankly purulent fluids need not be diluted.

Routine Examination.—

1. Specific Gravity.—Take specific gravity with urinometer.

2. Quantitative Albumin Determination.—Dilute one to twenty with distilled water and make quantitative albumin deter-

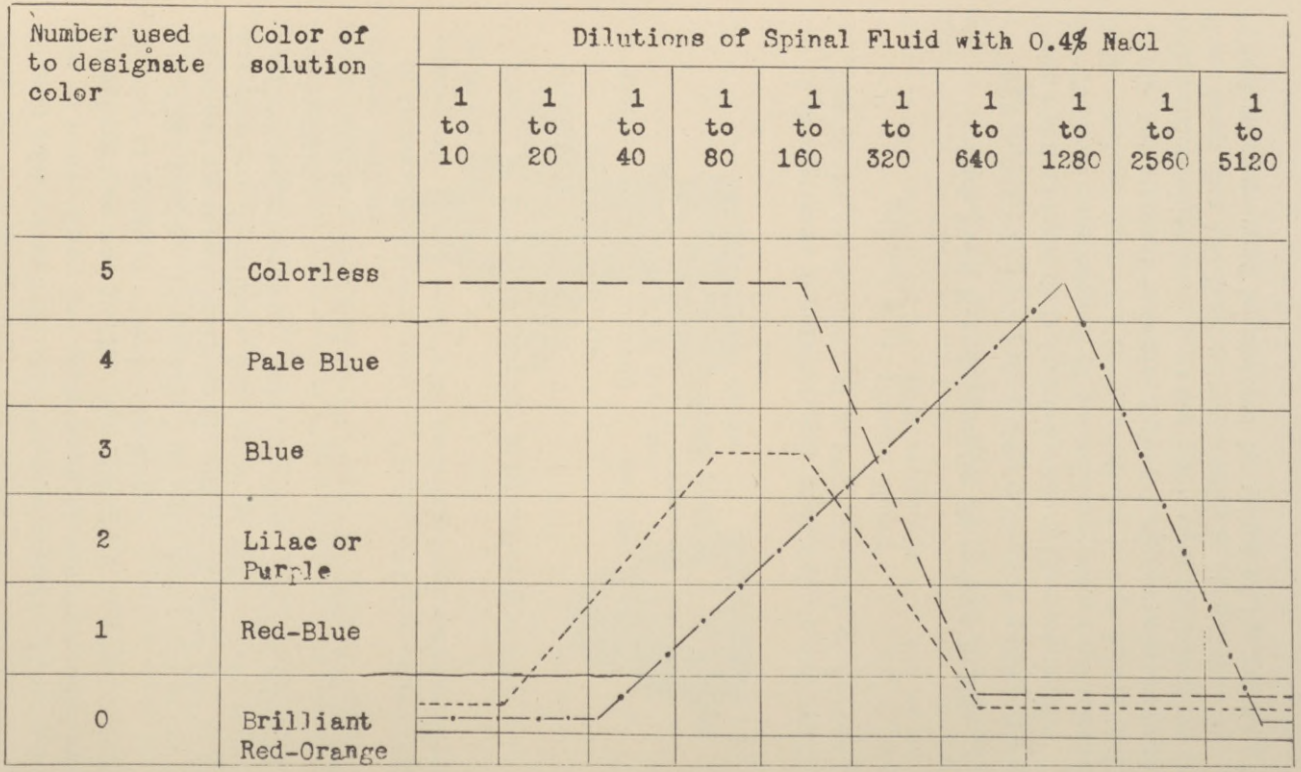


Fig. 57.—Reaction types of spinal fluid with colloidal gold solution.

———— No reaction—normal spinal fluid. . . . . Zone II reaction (luetic type).
– – – – – – – – Zone I reaction (paretic type). —.—.—.—.— Zone III reaction (meningitic type).

mination. Fill Esbach tube (Fig. 17) to point marked "W" with diluted fluid and to "R" with Tsuchiya reagent. Stopper, invert ten to twelve times, and allow to stand overnight. The amount of sediment as read on the scale multiplied by twenty gives the number of grams of albumin per liter.

3. Cell Count.—Dilute with 1 per cent acetic acid as for white blood cell count and determine the number of white cells in counting chamber. Centrifuge, make smear of sediment and stain with Loeffler's methylene blue. Make differential count of cells and look for bacteria.

4. Culture.—Make culture on blood agar plate and in tube of glucose bouillon.

5. Guinea Pig Inoculation.—If indicated inject guinea pig subcutaneously in the groin with centrifugalized sediment from 10 to 50 c.c. of fluid.

Report as follows if complete examination is made:

Pleural
: Fluid
Ascitic

Sp.Gr.
Albumin gms. per liter
WBC per cmm.
Differential – PMM
SM
Smear shows
Culture

If the specimen is purulent only make culture and smear. Report the examination of a purulent fluid as follows:

Pleural fluid { Smear shows
Culture shows

Dark-Field Examination for Spirocheta Pallida

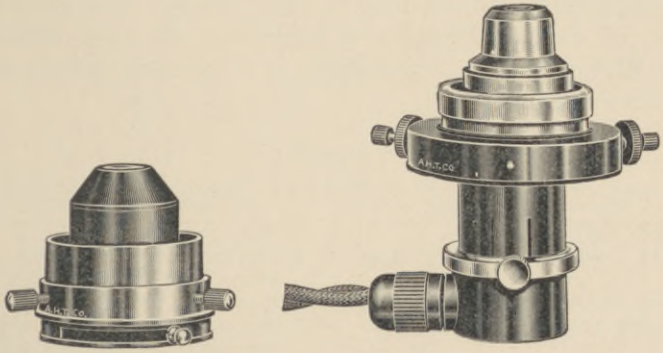
The simplest and most successful method of finding the spirocheta pallida in luetic lesions is by use of the dark-field.

The apparatus consists of an ordinary microscope from which the substage condensor is removed and a dark-field illuminator (Fig. 58) attached instead. A funnel stop is inserted in the oil immersion objective. A strong light, such as that given by an arc lamp or a 100 Watt nitrogen bulb, is necessary. The dark-field substage must be centered by means of the lateral adjust-

ment screws. This is accomplished by bringing the rings on the apex of the condensor to the center of the field as viewed through the low power lens of the microscope.

Recently an illuminator has been devised in which the light is placed in the substage (Fig. 56).

In obtaining the material for dark-field examination, the lesion should be superficially cleansed in order to remove the many contaminating organisms on the surface. In taking a specimen from a primary lesion, it is well to cleanse rather roughly, remove most of the surface layer, and to squeeze out a little serum from the deeper tissues. A drop of the secretion is caught in the



A. *B.*

Fig. 58.—*A.*—Old style dark-field illuminator. An external source of light is employed.

B.—New type dark-field illuminator. The light is an integral part of the instrument.

center or the side of a cover glass, which is then placed on a thin slide. Secretion from a gland can be obtained by inserting a needle into the gland and massaging the latter for a few minutes. A few drops of secretion may be obtained by aspiration.

A sufficient quantity of immersion oil is placed on the cover glass, also on the apex of the dark-field substage. The preparation is placed on the condensor gently so that the drop of oil on the condensor spreads evenly without bubbles.

If the condensor is properly centered the correct focus of light can be obtained by moving the condensor up and down until the center ring of light becomes a point of light in the middle of

the preparation. This adjustment is necessary because of variations in the thickness of slides.

With the proper adjustment the silvery appearance of the illuminated organic structures will stand out conspicuously in contrast with the background which should be of an intense brown color.

The Spirocheta pallida will appear as a silvery, very slender, highly flexible structure, which is spirally wound, having anywhere from ten to twenty turns. Its length is about twice the diameter of a red blood cell. The turns are closely set and of more or less equal size and shape. The organism will move slowly across the field, the movements being corkscrew-like.

The Spirocheta pallida must be differentiated from other spirochetes, of which Spirocheta refringens is the most important. Table XLV given by Dourmashkin is of value in differentiating the two organisms.

TABLE XLV

SPIROCHETA PALLIDA	SPIROCHETA REFRINGENS
1. Body very slender.	1. Body much coarser.
2. Has from ten to twenty turns.	2. The turns are much fewer, anywhere from five to eight.
3. The movements are regular.	3. Extremely irregular.
4. Moves slowly.	4. Movements very rapid; may be compared to lightning; difficult to follow it, as the organism dashes across the field.
5. Retains its turns.	5. Does not retain its turns.
6. Does not take up methylene blue stain.	6. Takes up the stain.

Preparation of Bacterial Vaccines

(Hopkins: Jour. Am. Med. Assn., 1913, lx, 1615)

Each organism to be used must be isolated in pure culture. Plain agar slants may be employed for the colon, typhoid and staphylococcus groups and dextrose broth for the pneumococcus, streptococcus and M. catarrhalis groups. It will be necessary to use blood agar slants or plates for B. influenzae and dextrose ascitic agar slants or plates for the gonococcus.

Inoculate medium and incubate for 24 hours. Wash the organisms off solid medium with sterile physiological salt solution and

place the suspension in a Hopkins vaccine centrifuge tube (Fig. 59). If the organisms have been grown in a fluid medium the material from the bottom of container is placed in the vaccine centrifuge tube. Fasten a sterile cotton plug in centrifuge tube with adhesive. Centrifuge for 30 minutes at 2800 revolutions per minute. Remove supernatant fluid with sterile pipette and add sterile physiological salt solution to make a 1 per cent suspension. Mix and transfer to sterile tube containing some beads. Stopper with sterile rubber stopper and shake until a homogeneous suspension is obtained.

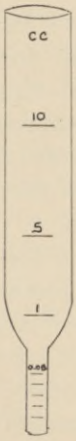


Fig. 59.—Hopkins vaccine tube.

Heat in water-bath at 60° C. for one hour. The level of the water in the tube must be well above the level of the vaccine.

Culture by adding one drop of the suspension to a blood agar plate and one drop to a tube of plain broth with a sterile pipette. Incubate for 72 hours. After culturing add 0.25 c.c. of 10 per cent tricresol mixture, or 0.5 c.c. of 10 per cent phenol, to each 10 c.c. of vaccine and shake thoroughly.

Below is shown the number of organisms per cubic centimeter in a 1 per cent suspension, as given by Hopkins:

Streptococcus hemolyticus—8 billions per cubic centimeter.

Staphylococcus aureus and albus—10 billions per cubic centimeter.

Gonococcus—8 billions per cubic centimeter.

Pneumococcus—2.5 billions per cubic centimeter.

B. Typhosus—8 billions per cubic centimeter.

B. Coli—4 billions per cubic centimeter.

Label with name, date, type of organism and number of organisms per cubic centimeter. The suspension may be diluted as desired. Keep in ice box.

Wound Bacterial Count

Principle.—In order to intelligently control the treatment of infected wounds with Dakin's solution it is necessary to determine frequently the number of bacteria in the exudate.

Procedure.—A smear is made from the infected area on a slide and spread uniformly with a platinum wire. The smear is stained two minutes with carbol-thionin, washed under the tap, and dried. Using the Bausch and Lomb No. 10 eyepiece and the 1.90 mm. objective, a standard field is selected in which the leucocytes just touch but do not overlap.

The bacteria in the standard field are counted and if over 60, are recorded as infinity. If the number falls between 10 and 60, five fields are counted and the average taken; with counts between 2 and 10, the average of ten fields is taken, and with those below 2, the average of 20 fields.

In examining the smear the predominant cell type—mononuclear or polymorphonuclear—and the predominant type of organism—bacillus, coccus, or streptococcus—are noted.

The findings are reported as follows:

WOUND	Bacterial count
	Predominant cell type
BACTERIAL COUNT	Predominant organism type

Detection of Mercury in Excretions

(Vogel and Lee: Jour. Am. Med. Assn., 1914, lxii, 532)

Principle.—The organic combination in which the mercury exists in the excretions is broken down by oxidation with nascent chlorine. Metallic copper is added to the solution containing the mercury. By electrolytic double decomposition the mercury is deposited on the copper and from this it is distilled on to a piece

of dentists' gold foil. The mercury is easily recognized as globules or as a silvery patch of discoloration on the gold.

Procedure.—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. The fluid becomes pale yellow or colorless when the organic material is completely oxidized. It is not advisable to use more acid or chlorate than is required for complete decolorization. Specimens containing a large amount of protein, such as stools, vomitus and blood, are first diluted with several volumes of water, and require more of the oxidizing materials. The excess of acid and chlorine is eliminated by evaporation and the solution concentrated to about 25 c.c. The solution is filtered to remove any solid matter. A short piece of clean copper wire, a number of small copper discs, or a small amount of copper dust, is dropped into the solution and allowed to remain for several hours, or overnight, preferably in a warm place. The copper is washed with distilled water, dried with alcohol and ether, and placed in the bottom of a small glass tube. It is then followed by a cylinder of gold foil pushed to within 2 centimeters of the copper.

The tube is held horizontally, and its closed end is carefully treated over a microburner almost to the softening point, care being taken to avoid heating the part of the tube containing the gold foil. The gold foil is examined for any trace of silvery discoloration, signifying the presence of mercury. A hand lens is useful in recognizing small amounts of the metal. If chlorine is still present in the concentrated oxidized solution the wire may be completely dissolved, in which case the solution should be diluted, again concentrated by boiling, and another wire dropped in.

If further confirmation of the identity of the mercury is required, the gold foil may be suspended in a tube containing a few crystals of iodine, which are then gently warmed. The mercury thus becomes converted into red mercuric iodide.

By this method 1 mg. of mercury in 100 c.c. can be detected in urine, stomach contents, and stools; the copper being allowed to remain in the oxidized mixture 2 hours.

Colorimetric Determination of the Hydrogen-Ion Concentration of Biological Fluids

(Clark and Lubs: Jour. Bact., 1917, ii, 1, 101, 191)

Principle.—The colorimetric method of determining hydrogen-ion concentration is based upon the fact that each indicator has a characteristic zone of hydrogen-ion concentration within which its color changes occur. The unknown solution is treated with a few drops of indicator and the color obtained compared with that produced with the same amount of indicator and a solution of known hydrogen-ion concentration.

The essentials are: First, a set of indicators which (1) cover the ranges of P_H to be studied; (2) are little affected by the presence of protein and neutral salts and, (3) do not fade rapidly; and second, a set of buffer solutions of known hydrogen-ion concentration.

Reagents.—1. Standard Buffer Solutions.—The various mixtures are made from the following stock solutions: M/5 potassium chloride (KCl), M/5 acid potassium phosphate (KH_2PO_4), M/5 acid potassium phthalate ($KHC_8H_4O_4$), M/5 boric acid with M/5 potassium chloride (H_3BO_3, KCl), M/5 sodium hydroxide (NaOH), and M/5 hydrochloric acid (HCl).

The water used in the crystallization of the salts and in the preparation of the stock solutions and mixtures should be redistilled and preferably "conductivity" water.

M/5 Potassium Chloride Solution.—(This solution will not be necessary except in the preparation of the most acid series of mixtures.) The salt should be recrystallized three or four times and dried in an oven at about 120° C. for two days. The fifth molecular solution contains 14.912 grams in 1 liter.

M/5 Acid Potassium Phthalate Solution.—Acid potassium phthalate may be prepared by the method of Dodge (1915) modified as follows: Make up a concentrated potassium hydroxid solution by dissolving about 60 grams of a high grade sample in about 400 c.c. of water. To this add 50 grams of the commercial *resublimed* anhydrid of orthophthalic acid. Test a cool portion of the solution with phenolphthalein. If the solution is still alkaline, add more phthalic anhydrid; if acid, add more KOH. When

roughly adjusted to a slight pink with phenolphthalein add as much more phthalic anhydrid as the solution contains and heat until all is dissolved. Filter while hot, and allow the crystallization to take place slowly. The crystals should be drained with suction and recrystallized at least twice from distilled water. Dry the salt at 110° C. to 115° C. to constant weight.

A fifth molecular solution contains 40.828 grams of the salt in 1 liter of the solution.

M/5 Acid Potassium Phosphate Solution.—A high grade commercial sample of the salt is recrystallized at least three times from distilled water and dried to constant weight at 110° to 115° C. A fifth molecular solution should contain in 1 liter 27.232 grams. The solution should be distinctly red with methyl red and distinctly blue with brom phenol blue.

M/5 Boric Acid M/5 Potassium Chloride.—Boric acid should be recrystallized several times from distilled water. It should be air dried in thin layers between filter paper and the constancy of weight established by drying small samples in thin layers in a desiccator over $CaCl_2$. Purification of KCl has already been noted. One liter of the solution should contain 12.4048 grams of boric acid and 14.912 grams of potassium chloride.

M/5 Sodium Hydroxide Solution.—This solution is the most difficult to prepare, since it should be as free as possible from carbonate. A solution of sufficient purity for the present purposes may be prepared from a high grade sample of the hydroxide in the following manner. Dissolve 100 grams NaOH in 100 c.c. distilled water in a Jena or Pyrex glass Erlenmeyer flask. Cover the mouth of the flask with tin foil and allow the solution to stand overnight till the carbonate has mostly settled. Then prepare a filter as follows. Cut a "hardened" filter paper to fit a Buchner funnel. Treat it with warm strong (1 : 1) NaOH solution. After a few minutes decant the sodium hydroxide and wash the paper first with absolute alcohol, then with dilute alcohol, and finally with large quantities of distilled water. Place the paper on the Buchner funnel and apply gentle suction until the greater part of the water has evaporated but do not dry so that the paper curls. Now pour the concentrated alkali upon the middle of the paper, spread it with a glass rod making sure

that the paper, under gentle suction, adheres well to the funnel, and draw the solution through with suction. The clear filtrate is now diluted quickly, after rough calculation, to a solution somewhat more concentrated than N/1. Withdraw 10 c.c. of this dilution and standardize roughly with an acid solution of known strength, or with a sample of acid potassium phthalate. From this approximate standardization calculate the dilution

Fig. 60.—Showing H-ion comparison standards, comparator, and sodium hydroxide bottle with soda lime guard tubes.

required to furnish an M/5 solution. Make the required dilution with the least possible exposure, and pour the solution into a *paraffined* bottle to which a calibrated 50 c.c. burette and soda-lime guard tubes have been attached (Fig. 60). The solution should now be most carefully standardized. One of the simplest methods of doing this, and one which should always be used in this instance, is the method of Dodge (1915) in which use is made of

the acid potassium phthalate purified as already described. Weigh out accurately on a chemical balance with standardized weights several portions of the salt of about 1.6 grams each. Dissolve in about 20 c.c. distilled water and add 4 drops phenolphthalein. Pass a stream of CO_2—free air through the solution and titrate with the alkali until a faint but distinct and permanent pink is developed. It is preferable to use a factor with the solution rather than attempt adjustment to an exact M/5 solution.

M/5 Hydrochloric Acid Solution.—Dilute a high grade of hydrochloric acid solution to about 20 per cent and distill. Dilute the distillate to approximately M/5 and standardize with the sodium hydroxide solution previously described.

The only solution which it is absolutely necessary to protect from the CO_2 of the atmosphere is the sodium hydroxide solution. Therefore all but this solution may be stored in ordinary bottles of resistant glass. The salt solutions, if adjusted to exactly M/5, may be measured from clean calibrated pipettes.

Table XLVI shows the proportions in which the stock solutions are to be mixed to make buffer solutions of varying hydrogen concentration.

TABLE XLVI

COMPOSITIONS OF MIXTURES GIVING P_H VALUES AT 20° C. AT INTERVALS OF 0.2

P_H		Composition	
	KCl – HCl Mixtures		
1.0	50 c.c. M/5 KCl	97.0 c.c. M/5 HCl	Dilute to 200 c.c.
1.2	50 c.c. M/5 KCl	64.5 c.c. M/5 HCl	Dilute to 200 c.c.
1.4	50 c.c. M/5 KCl	41.5 c.c. M/5 HCl	Dilute to 200 c.c.
1.6	50 c.c. M/5 KCl	26.3 c.c. M/5 HCl	Dilute to 200 c.c.
1.8	50 c.c. M/5 KCl	16.6 c.c. M/5 HCl	Dilute to 200 c.c.
2.0	50 c.c. M/5 KCl	10.6 c.c. M/5 HCl	Dilute to 200 c.c.
2.2	50 c.c. M/5 KCl	6.7 c.c. M/5 HCl	Dilute to 200 c.c.
	Phthalate – HCl Mixtures		
2.2	50 c.c. M/5 KHPhthalate	46.70 c.c. M/5 HCl	Dilute to 200 c.c.
2.4	50 c.c. M/5 KHPhthalate	39.60 c.c. M/5 HCl	Dilute to 200 c.c.
2.6	50 c.c. M/5 KHPhthalate	32.95 c.c. M/5 HCl	Dilute to 200 c.c.
2.8	50 c.c. M/5 KHPhthalate	26.42 c.c. M/5 HCl	Dilute to 200 c.c.
3.0	50 c.c. M/5 KHPhthalate	20.32 c.c. M/5 HCl	Dilute to 200 c.c.
3.2	50 c.c. M/5 KHPhthalate	14.70 c.c. M/5 HCl	Dilute to 200 c.c.
3.4	50 c.c. M/5 KHPhthalate	9.90 c.c. M/5 HCl	Dilute to 200 c.c.
3.6	50 c.c. M/5 KHPhthalate	5.97 c.c. M/5 HCl	Dilute to 200 c.c.
3.8	50 c.c. M/5 KHPhthalate	2.63 c.c. M/5 HCl	Dilute to 200 c.c.

TABLE XLVI—CONT'D

	Phthalate – NaOH Mixtures		
4.0	50 cc. M/5 KHPhthalate	0.40 c.c. M/5 NaOH	Dilute to 200 c.c.
4.2	50 cc. M/5 KHPhthalate	3.70 c.c. M/5 NaOH	Dilute to 200 c.c.
4.4	50 cc. M/5 KHPhthalate	7.50 c.c. M/5 NaOH	Dilute to 200 c.c.
4.6	50 cc. M/5 KHPhthalate	12.15 c.c. M/5 NaOH	Dilute to 200 c.c.
4.8	50 cc. M/5 KHPhthalate	17.70 c.c. M/5 NaOH	Dilute to 200 c.c.
5.0	50 cc. M/5 KHPhthalate	23.85 c.c. M/5 NaOH	Dilute to 200 c.c.
5.2	50 cc. M/5 KHPhthalate	29.95 c.c. M/5 NaOH	Dilute to 200 c.c.
5.4	50 cc. M/5 KHPhthalate	35.45 c.c. M/5 NaOH	Dilute to 200 c.c.
5.6	50 cc. M/5 KHPhthalate	39.85 c.c. M/5 NaOH	Dilute to 200 c.c.
5.8	50 cc. M/5 KHPhthalate	43.00 c.c. M/5 NaOH	Dilute to 200 c.c.
6.0	50 cc. M/5 KHPhthalate	45.45 c.c. M/5 NaOH	Dilute to 200 c.c.
6.2	50 cc. M/5 KHPhthalate	47.00 c.c. M/5 NaOH	Dilute to 200 c.c.
	KH_2PO_4 – NaOH Mixtures		
5.8	50 c.c. M/5 KH_2PO_4	3.72 c.c. M/5 NaOH	Dilute to 200 c.c.
6.0	50 c.c. M/5 KH_2PO_4	5.70 c.c. M/5 NaOH	Dilute to 200 c.c.
6.2	50 c.c. M/5 KH_2PO_4	8.60 c.c. M/5 NaOH	Dilute to 200 c.c.
6.4	50 c.c. M/5 KH_2PO_4	12.60 c.c. M/5 NaOH	Dilute to 200 c.c.
6.6	50 c.c. M/5 KH_2PO_4	17.80 c.c. M/5 NaOH	Dilute to 200 c.c.
6.8	50 c.c. M/5 KH_2PO_4	23.65 c.c. M/5 NaOH	Dilute to 200 c.c.
7.0	50 c.c. M/5 KH_2PO_4	29.63 c.c. M/5 NaOH	Dilute to 200 c.c.
7.2	50 c.c. M/5 KH_2PO_4	35.00 c.c. M/5 NaOH	Dilute to 200 c.c.
7.4	50 c.c. M/5 KH_2PO_4	39.50 c.c. M/5 NaOH	Dilute to 200 c.c.
7.6	50 c.c. M/5 KH_2PO_4	42.80 c.c. M/5 NaOH	Dilute to 200 c.c.
7.8	50 c.c. M/5 KH_2PO_4	45.20 c.c. M/5 NaOH	Dilute to 200 c.c.
8.0	50 c.c. M/5 KH_2PO_4	46.80 c.c. M/5 NaOH	Dilute to 200 c.c.
	Boric Acid, KCl – NaOH Mixtures		
7.8	50 c.c. M/5 H_3BO_3 M/5 KCl	2.61 c.c. M/5 NaOH	Dilute to 200 c.c.
8.0	50 c.c. M/5 H_3BO_3 M/5 KCl	3.97 c.c. M/5 NaOH	Dilute to 200 c.c.
8.2	50 c.c. M/5 H_3BO_3 M/5 KCl	5.90 c.c. M/5 NaOH	Dilute to 200 c.c.
8.4	50 c.c. M/5 H_3BO_3 M/5 KCl	8.50 c.c. M/5 NaOH	Dilute to 200 c.c.
8.6	50 c.c. M/5 H_3BO_3 M/5 KCl	12.00 c.c. M/5 NaOH	Dilute to 200 c.c.
8.8	50 c.c. M/5 H_3BO_3 M/5 KCl	16.30 c.c. M/5 NaOH	Dilute to 200 c.c.
9.0	50 c.c. M/5 H_3BO_3 M/5 KCl	21.30 c.c. M/5 NaOH	Dilute to 200 c.c.
9.2	50 c.c. M/5 H_3BO_3 M/5 KCl	26.70 c.c. M/5 NaOH	Dilute to 200 c.c.
9.4	50 c.c. M/5 H_3BO_3 M/5 KCl	32.00 c.c. M/5 NaOH	Dilute to 200 c.c.
9.6	50 c.c. M/5 H_3BO_3 M/5 KCl	36.85 c.c. M/5 NaOH	Dilute to 200 c.c.
9.8	50 c.c. M/5 H_3BO_3 M/5 KCl	40.80 c.c. M/5 NaOH	Dilute to 200 c.c.
10.0	50 c.c. M/5 H_3BO_3 M/5 KCl	43.90 c.c. M/5 NaOH	Dilute to 200 c.c.

The mixtures should be made up freshly once a week. They are conveniently kept in 200 c.c. bottles, each of which is provided with a 10 c.c. pipette. The bottles used for the alkaline borate mixtures should be paraffined.

2. **Indicators.**—Table XLVII of indicators shows the concentration of the solution used to test gross color changes and the range in which each indicator is useful.

TABLE XLVII

LIST OF INDICATORS

CHEMICAL NAME	COMMON NAME	CONCENTRATION	COLOR CHANGE	RANGE P_H
Thymol sulphone phthalein (acid range)	Thymol blue	0.04	Red-Yellow	1.2-2.8
Tetra bromo phenol sulphone phthalein	Brom phenol blue	0.04	Yellow-blue	3.0-4.6
Ortho carboxy benzene azo dimethyl aniline	Methyl red	0.02	Red-yellow	4.4-6.0
Di bromo ortho cresol sulphone phthalein	Brom cresol purple	0.04	Yellow-purple	5.2-6.8
Di bromo thymol sulphone phthalein	Brom thymol blue	0.04	Yellow-blue	6.0-7.6
Phenol sulphone phthalein	Phenol red	0.02	Yellow-red	6.8-8.4
Ortho cresol sulphone phthalein	Cresol red	0.02	Yellow-red	7.2-8.8
Thymol sulphone phthalein (see above)	Thymol blue	0.02	Yellow-blue	8.0-9.6
Ortho cresol phthalein	Cresol phthalein	0.02	Colorless-red	8.2-9.8

The indicators are supplied in dry form from which stock solutions are made as follows: 0.1 gram of dry powder is ground in an agate mortar with the following quantities of N/20 NaOH.

Indicator	N/20 NaOH per 0.1 gm.
Phenol red	5.7 c.c.
Brom phenol blue	3.0 c.c.
Cresol red	5.3 c.c.
Thymol blue	4.3 c.c.
Brom thymol blue	3.2 c.c.
Brom cresol purple	3.7 c.c.

When solution is complete dilute to 25 c.c.

This gives a 0.4 per cent solution. For use the stock solution of thymol blue, brom thymol blue, brom phenol blue and brom cresol purple are diluted with water to make 0.04 per cent solutions, those of phenol red and cresol red to make 0.02 per cent solutions.

Methyl red is made by dissolving 0.1 gram in 300 c.c. of alcohol and diluting to 500 c.c. Orthocresol phthalein is used in 0.02 per cent solution in 95 per cent alcohol.

3. **Comparison Color Standards.**—Select test tubes of the same bore. Fill into them 10 c.c. of the standard buffer solutions to make a set of standards whose P_H range from 1.2 to 9.6 with

increments of 0.2 P_H allowing extra tubes for the overlapping of indicators.

Add to each 5 drops of indicator solution as given below:

P_H of Buffer Solution	Indicator
1.2 – 2.8	Thymol blue
3.0 – 4.6	Brom phenol blue
4.4 – 6.0	Methyl red
5.2 – 6.8	Brom cresol purple
6.0 – 7.6	Brom thymol blue
7.0 – 8.4	Phenol red
7.8 – 8.6	Cresol red
8.8 – 9.6	Cresol phthalein

If corked and sealed with paraffin, these standards are fairly permanent except those containing methyl red as the indicator.

Procedure.—If the approximate P_H of the solution is not known, find the range within which it falls by adding to a portion

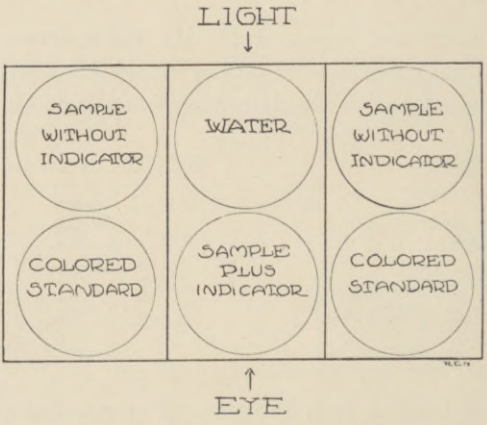


Fig. 61.—Arrangement of tubes in comparator in the determination of hydrogen-ion concentration.

the sulphonephthalein series of indicators, beginning with thymol blue.

Measure 10 c.c. of the solution to be examined into a test tube of the same bore as those containing the standard solutions. Add 5 drops of the indicator showing the sharpest color changes over the range within which the solution falls, as indicated by the preliminary test.

Compare this solution with those of the comparison color stand-

Table XLVIII

True H-Ion Concentration Corresponding to Logarithmic Figures

P_H	H-ION CONC. (gms. of hydrogen per li.)	P_H	H-ION CONC. (gms. of hydrogen per li.)	P_H	H-ION CONC. (gms. of hydrogen per li.)	P_H	H-ION CONC. (gms. of hydrogen per li.)	P_H	H-ION CONC. (gms. of hydrogen per li.)
1.0	1.0×10^{-1}	3.0	1.0×10^{-3}	5.0	1.0×10^{-5}	7.0	1.0×10^{-7}	9.0	1.0×10^{-9}
1.1	7.9×10^{-2}	3.1	7.9×10^{-4}	5.1	7.9×10^{-6}	7.1	7.9×10^{-8}	9.1	7.9×10^{-10}
1.2	6.3×10^{-2}	3.2	6.3×10^{-4}	5.2	6.3×10^{-6}	7.2	6.3×10^{-8}	9.2	6.3×10^{-10}
1.3	5.0×10^{-2}	3.3	5.0×10^{-4}	5.3	5.0×10^{-6}	7.3	5.0×10^{-8}	9.3	5.0×10^{-10}
1.4	4.0×10^{-2}	3.4	4.0×10^{-4}	5.4	4.0×10^{-6}	7.4	4.0×10^{-8}	9.4	4.0×10^{-10}
1.5	3.2×10^{-2}	3.5	3.2×10^{-4}	5.5	3.2×10^{-6}	7.5	3.2×10^{-8}	9.5	3.2×10^{-10}
1.6	2.5×10^{-2}	3.6	2.5×10^{-4}	5.6	2.5×10^{-6}	7.6	2.5×10^{-8}	9.6	2.5×10^{-10}
1.7	2.0×10^{-2}	3.7	2.0×10^{-4}	5.7	2.0×10^{-6}	7.7	2.0×10^{-8}	9.7	2.0×10^{-10}
1.8	1.6×10^{-2}	3.8	1.6×10^{-4}	5.8	1.6×10^{-6}	7.8	1.6×10^{-8}	9.8	1.6×10^{-10}
1.9	1.2×10^{-2}	3.9	1.2×10^{-4}	5.9	1.2×10^{-6}	7.9	1.2×10^{-8}	9.9	1.2×10^{-10}
2.0	1.0×10^{-2}	4.0	1.0×10^{-4}	6.0	1.0×10^{-6}	8.0	1.0×10^{-8}	10.0	1.0×10^{-10}
2.1	7.9×10^{-3}	4.1	7.9×10^{-5}	6.1	7.9×10^{-7}	8.1	7.9×10^{-9}	10.1	7.9×10^{-11}
2.2	6.3×10^{-3}	4.2	6.3×10^{-5}	6.2	6.3×10^{-7}	8.2	6.3×10^{-9}	10.2	6.3×10^{-11}
2.3	5.0×10^{-3}	4.3	5.0×10^{-5}	6.3	5.0×10^{-7}	8.3	5.0×10^{-9}	10.3	5.0×10^{-11}
2.4	4.0×10^{-3}	4.4	4.0×10^{-5}	6.4	4.0×10^{-7}	8.4	4.0×10^{-9}	10.4	4.0×10^{-11}
2.5	3.2×10^{-3}	4.5	3.2×10^{-5}	6.5	3.2×10^{-7}	8.5	3.2×10^{-9}	10.5	3.2×10^{-11}
2.6	2.5×10^{-3}	4.6	2.5×10^{-5}	6.6	2.5×10^{-7}	8.6	2.5×10^{-9}	10.6	2.5×10^{-11}
2.7	2.0×10^{-3}	4.7	2.0×10^{-5}	6.7	2.0×10^{-7}	8.7	2.0×10^{-9}	10.7	2.0×10^{-11}
2.8	1.6×10^{-3}	4.8	1.6×10^{-5}	6.8	1.6×10^{-7}	8.8	1.6×10^{-9}	10.8	1.6×10^{-11}
2.9	1.2×10^{-3}	4.9	1.2×10^{-5}	6.9	1.2×10^{-7}	8.9	1.2×10^{-9}	10.9	1.2×10^{-11}

ards containing the same indicator. The P_H of the unknown solution is the same as that of the standard buffer solution the color of which it most nearly matches.

If the unknown solution is colored, compare in a comparator holding six tubes (Fig. 60). Arrange the tubes as indicated in Fig. 61.

If the native color of the solution still interferes, the solution may be diluted up to 1.5 without appreciably changing the hydrogen-ion concentration.

Remarks.—Table XLVIII shows the true hydrogen-ion concentration corresponding to the logarithmic figures (1.0-10.9).

The hydrogen-ion concentration of the body fluids is given in Table XLIX. (Clark.)

TABLE XLIX

FLUID	P_H	FLUID	P_H
Blood	7.4	Muscle juice (fresh)	6.8
Urine	6.0	Muscle juice (autolyzed)	Variable
Saliva	6.9	Pancreas extract	5.6
Gastric juice (adult)	0.9-1.6	Peritoneal fluid	7.4
Gastric juice (infant)	5.0	Pericardial fluid	7.4
Pancreatic juice (dog)	8.3	Aqueous humor	7.1
Small intestinal contents	8.3	Vitreous humor	7.0
Small intestinal contents, (infant)	3.1	Cerebrospinal fluid	7.5-7.7
Bile from liver	7.8	Amniotic fluid	7.1
Bile from gall bladder	5.3-7.4	Milk (human)	7.0-7.2
Perspiration	4.5		
Tears	7.2		

CHAPTER XII

MISCELLANEOUS CHEMICAL PROCEDURES AND SOLUTIONS

The Use of the Colorimeter and Nephelometer

The Colorimeter.—The quantitative determination of substances which are present in only minute amounts but which form colored compounds is made with a colorimeter.

Three types of colorimeter are in common use: (1) plunger; (2) wedge and (3) dilution type. With the plunger type, (Fig. 62), the intensity of the color of either the standard or unknown is varied by changing the depth of solution through which the light passes with the aid of a plunger. The two halves of the field of view are illuminated by the light passing through the standard and unknown solutions simultaneously. With the wedge type, (Fig. 34) the standard solution is placed in a wedge and the unknown in a small cup with sides parallel to the wedge. The wedge is moved up and down until the intensity of the color is the same as the unknown. With the dilution type the standard and unknown are placed in small test tubes of equal diameter. The unknown is diluted until it has identically the same color as the standard.

Each type of instrument has its advantages and disadvantages. The wedge type is quite satisfactory where determinations need to be made quickly and only relatively accurate results are necessary. The wedge must be carefully calibrated, however, as described on page 98. For the most exact work an instrument of the plunger type such as the Duboscq (Fig. 62) or Bock-Benedict (Fig. 63) is necessary.

In using a colorimeter of the plunger type, the instrument and the mirror should be adjusted to the source of light, by placing a colored solution in both cups, setting them at the same point on the scale and noting the two halves of the field. If they are not alike, the zero point or the optics of the instrument must be

wrong. Suitable allowance must be made in the readings after the degree of variation has been calculated. After having determined the point at which the two halves of the field are alike, change the height of the plungers and then make a color comparison of the color standard against itself, readjusting the moved plunger until the fields look alike again. The error should not exceed 0.2 mm.

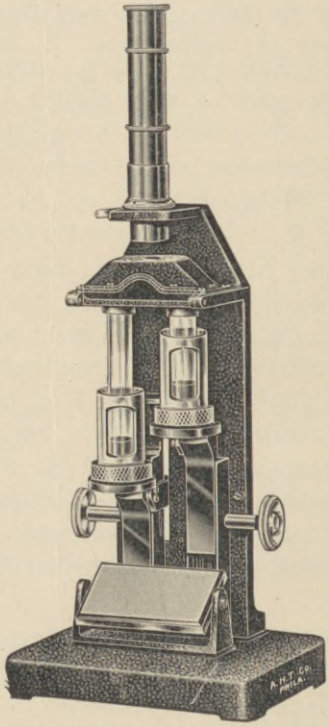


Fig. 62.—Duboscq colorimeter. (*Bausch and Lomb.*)

Having adjusted the instrument, the solutions are poured into the cups. The cup containing the standard solution is then lowered to a definite thickness, usually 20 mm. of the standard solution between the bottom of the cup and the end of the plunger. The cup containing the unknown solution is now moved until the two halves of the field are brought to the same intensity of color, after which the height at which the two liquid columns

display this equal absorptive power is read by means of the scale. Since the proportion of coloring matter in two solutions is inversely proportional to the heights of the two columns necessary to obtain the same intensity of illumination, if the standard tube is set at 20 mm. and the solution under examination is the same intensity of color at 10 mm. the latter is just twice the concentration of the standard. This is expressed by the formula:

$$\frac{\text{Color of Test Solution}}{\text{Color of Standard Solution}} = \frac{\text{Height of Standard Solution}}{\text{Height of solution to be tested}}$$

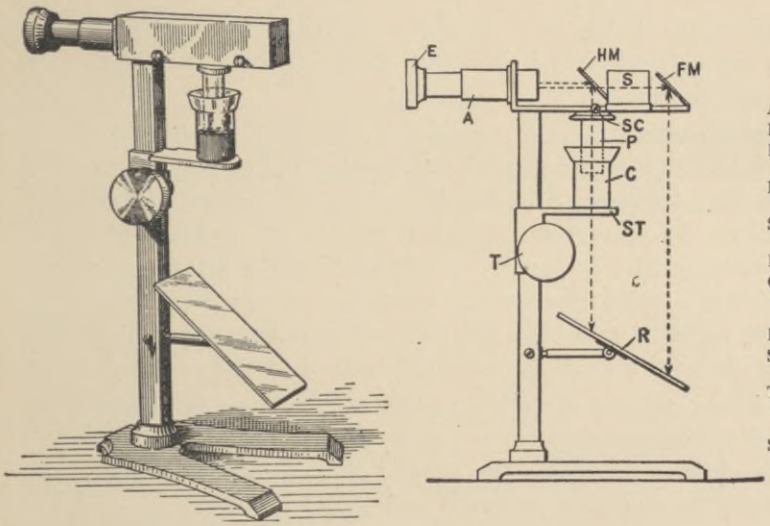


Fig. 63.—Bock-Benedict colorimeter.

If, therefore, the scale reading is 20 mm. for the standard and 15 mm. for the solution to be tested, the formula reads: $\frac{20}{15} = 1.33$. If for example the standard solution contains 4 mg. of coloring matter in 100 c.c., the solution under test will be found to contain $4 \times 1.33 = 5.32$ mg. in 100 c.c.

The color comparisons should be made rapidly as the eye fatigues quickly. The eye should be closed often while making the readings. The optical parts of the instrument must be kept free from dust, and cups, plungers and mirror must be kept clean.

Alkaline solutions such as those containing Nessler's reagent must not be spilled on the mirror. The spilling of solutions is usually due to filling the cups too full.

The focus should be adjusted with the draw tube so the line dividing the fields is distinct. The readings are best made in a dark-room, both eyes being kept open.

The more nearly the composition and concentration of the test solution equal that of the standard the more accurate will be the determination. Hence it is best to vary the strength of the test solution or standard until the two nearly match.

The tables given under the colorimetric determinations in blood and urine can be used with a Duboscq, Kober or Bock-Benedict, (20 mm. cell) instrument.

The Nephelometer.—Minute amounts of substances which do not form colored solutions may be determined in the nephelometer. This is an instrument for the determination of the quantity of a substance by measurement of the density of a precipitate which it produces with a reagent. It differs from the colorimeter in that the light which reaches the eye is not transmitted light but is light reflected from the particles of the suspension. The brightness of the two fields is compared instead of their color.

The Duboscq colorimeter with movable cups may be easily adapted for nephelometric purposes, as suggested by Bloor, (Jour. Biol. Chem., 1915, xxii, 145). The method of transformation may be seen from the diagrams (Figs. 64 and 65).

The brass plate carrying the colorimeter plungers is replaced by the plate *A*, with two slots in which are supported the nephelometer tubes *B*, with their flanges resting on the edges of the slots. The slots are so cut that the center lines of the tubes are exactly in line with the centers of the lower opening of the prism case *E*. If desired they may be countersunk to receive the flanges.

The colorimeter cups are replaced by the jackets *F* and are supported on them by the collars *D*. They move when the cup supports move. The mirror is turned to the horizontal position, so that it reflects no light; the light in the nephelometer comes from in front and not from below (Fig. 64).

The nephelometer tubes are small test tubes, 100 × 15 mm.,

preferably made from the same sample of colorless glass tubing so that they are of exactly the same bore. The flanges at the top should be well made so that the tubes rest firmly and evenly in the slots. The glass should be as free as possible from imperfections or striations. After the tubes are made and fitted into place the jackets are moved up on each tube by means of the

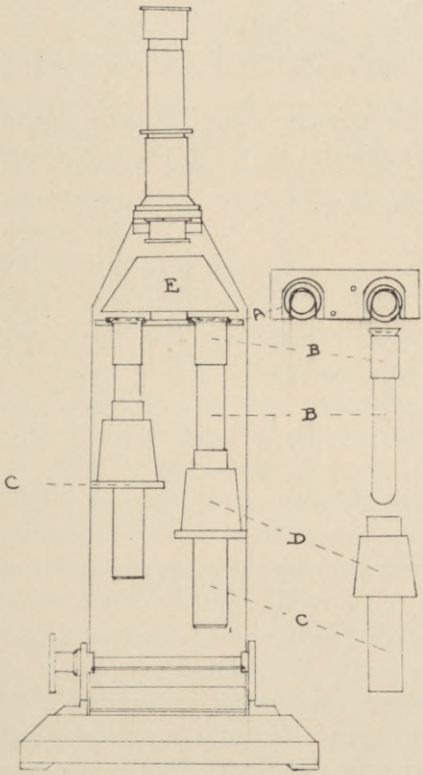


Fig. 64.—The Duboscq colorimeter converted into a nephelometer with the necessary extra parts. (*After Bloor.*)

rack and pinion, until the indicator on the scale is exactly at zero. Marks are made on each tube at the point reached by the top of the jacket and the portion above that point is made opaque by a ring of black paper or paint. Tubes and jackets are then marked "right" and "left" and always used on the same side.

Since it is rare to find two tubes, which when filled with the

same solution, give exactly the same readings, it is necessary to take this fact into account and correct accordingly.

The jackets *C* are made of tubing—metal or glass—a little larger than the tubes and about the same length, (they should just clear the mirror when it is turned horizontal), closed at the bottom and made light tight by black paint or paper. The collars *D*, supporting the jackets may be made of cork or more permanently of metal. A little cotton wool in the bottom of the jackets will prevent breakage if the tubes should fall into the jackets.

Artificial light is necessary and the lamp should be enclosed in a tight box, into one end of which the nephelometer fits snugly.

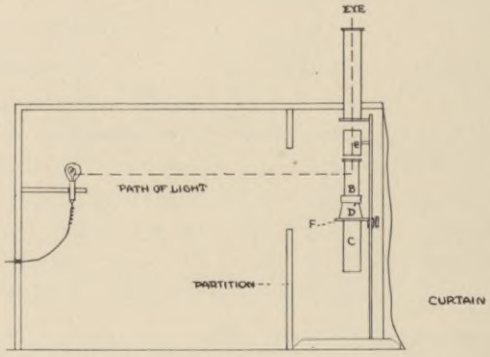


Fig. 65.—The nephelometer in position in its box, showing its relation to the source of light. (*After Bloor.*)

A partition extending part way up the box, as shown on the diagram (Fig. 65), serves the double purpose of shutting off the light from the lower part of the instrument and of providing a stop against which the instrument is pushed, so that its distance from the light is kept constant. The box is conveniently made without a bottom and the end closed with a dark curtain after the nephelometer is pushed into place. The inside of the box should be painted black. A dark room is desirable but not necessary, as the instrument may be used satisfactorily in a room darkened by a dark shade, or even in a dark corner of the laboratory.

The relations of the nephelometer and light source may be seen in the diagram (Fig. 65). The lamp used is an ordinary 50 Watt tungsten, supported by a bracket at 30 cm. from the nephelometer

and at the height of the nephelometer tubes. The extra parts necessary for the conversion of the colorimeter into a nephelometer may be obtained from the International Instrument Company of Cambridge, Massachusetts.

The above description applies only to the type of instrument with movable cups.

The total light reflected from a given depth of suspension depends not only on the number of reflecting particles but also on the condition of the precipitate, such as size and density. Hence it is very important that the condition of the particles in the standard and in the test solution be the same. It is thus necessary to make the physical and chemical conditions as regards salts, reaction, temperature and volume be as nearly alike in the two solutions as possible.

The readings obtained with the different suspensions in the nephelometer are not exactly proportional to the amount of substance present, and the difference between the observed and theoretical readings increases as the substance increases in amount. Kober has proposed a formula to take care of such corrections. But practically where the reading of the test solution does not differ from the standard by more than 25 per cent the correction falls within the limits of error of the determination and no correction need be made.

The nephelometer tubes should be so filled that the meniscus comes just above the bottom of the black collar of the tube *B*, (Fig. 64).

Standardization of Blood Chemical Determinations

The technic and reagents employed in blood chemical determination should be checked by making quantitative analyses on specimens of blood to which known amounts of the substance to be estimated have been added.

The procedure to be followed in the check determination is given below. The protocols show the amount to be added in each test, and the calculated amount per 100 c.c. of blood with which results obtained are to be compared. Blank determinations in which no blood is used should also be run with each new lot of chemicals.

Blood Urea Nitrogen.—Pipette 3 c.c. of whole blood into each of seven urea tubes. Add urea to each tube as indicated in the protocol below. Make a urea determination on each specimen in the usual way (page 133). Compare the results obtained with the calculated figures (Table L).

TABLE L

TUBE NO.	UREA ADDITION	CALCULATED UREA NITROGEN
1	0.0	X mg. per 100 c.c.
2	0.3 mg. urea = 1 c.c.S_1	X +4.67 " " " "
3	0.6 " " = 2 c.c.S_1	X +9.34 " " " "
4	0.9 " " = 3 c.c.S_1	X +13.00 " " " "
5	1.2 " " = 4 c.c.S_1	X +17.67 " " " "
6	1.5 " " = 5 c.c.S_1	X +22.34 " " " "
7	1.8 " " = 6 c.c.S_1	X +26.00 " " " "

Where S_1 = a solution containing 300 mg. of urea dissolved in 1000 c.c. water.

Total Non-protein Nitrogen.—Into each of seven Erlenmeyer flasks pipette 2 c.c. of oxalated blood. Add nitrogen and water as indicated in the protocol below. Finally add 2 c.c. each of 10 per cent sodium tungstate and ⅔ normal sulphuric acid. Determine the non-protein nitrogen on the filtrate in the usual way (page 131). Compare the results obtained with the calculated figures (Table LI).

TABLE LI

FLASK NO.	NITROGEN ADDITION	WATER	CALCULATED TOTAL NON-PROTEIN NITROGEN
1	0.0	14 c.c.	X mg. per 100 c.c. blood
2	0.2 mg. = 2 c.c.S_1	12 c.c.	X +10 " " " " "
3	0.4 mg. = 4 c.c.S_1	10 c.c.	X +20 " " " " "
4	0.6 mg. = 6 c.c.S_1	8 c.c.	X +30 " " " " "
5	0.8 mg. = 8 c.c.S_1	6 c.c.	X +40 " " " " "
6	1.0 mg. =10 c.c.S_1	4 c.c.	X +50 " " " " "
7	1.2 mg. =12 c.c.S_1	2 c.c.	X +60 " " " " "

Where S_1 = a solution containing 0.1 mg. nitrogen per c.c. (0.4716 gms. ammonium sulphate per liter).

Blood Sugar.—Pipette 2 c.c. of oxalated blood into each of nine Erlenmeyer flasks. Add glucose and water as indicated in the protocol below. Finally add 2 c.c. each of 10 per cent sodium tungstate and ⅔ normal sulphuric acid. Determine the sugar on

the filtrate in the usual way (page 141). Compare the results obtained with the calculated figures (Table LII).

TABLE LII

FLASK NO.	GLUCOSE ADDITION	WATER	CALCULATED SUGAR
1	0.0	14.0 c.c.	X mg. per 100 c.c.
2	0.5 mg. = 5.0 c.c.S_1	9.0 c.c.	X +25 " " " "
3	1.0 mg. =10.0 c.c.S_1	4.0 c.c.	X +50 " " " "
4	1.5 mg. = 7.5 c.c.S_2	6.5 c.c.	X +75 " " " "
5	2.0 mg. =10.0 c.c.S_2	4.0 c.c.	X +100 " " " "
6	2.5 mg. =12.5 c.c.S_2	1.5 c.c.	X +125 " " " "
7	3.0 mg. = 3.75 c.c.S_3	10.25 c.c.	X +150 " " " "
8	3.5 mg. = 4.375 c.c.S_3	9.625 c.c.	X +175 " " " "
9	4.0 mg. = 5.0 c.c.S_3	9.0 c.c.	X +200 " " " "

Where S_1 = standard containing 10 mg. glucose per 100 c.c.
S_2 = standard containing 20 mg. glucose per 100 c.c.
S_3 = standard containing 40 mg. glucose per 100 c.c.

Blood Uric Acid.—Pipette 2 c.c. of oxalated blood into each of nine Erlenmeyer flasks. Add uric acid and water as indicated in the protocol below. Finally add 2 c.c. each of 10 per cent sodium tungstate and ⅔ normal sulphuric acid. Determine the uric acid on the filtrate from each flask in the usual manner, (page 135.) Compare the results obtained with the calculated figures (Table LIII).

TABLE LIII

FLASK NO.	URIC ACID ADDITION	WATER	CALCULATED URIC ACID
1	0.0	14 c.c.	X mg. per 100 c.c. blood
2	0.01 mg. = 1 c.c. S_1	13 c.c.	X +0.50 " " " " "
3	0.02 " = 2 c.c. S_1	12 c.c.	X +1.00 " " " " "
4	0.03 " = 3 c.c. S_1	11 c.c.	X +1.50 " " " " "
5	0.04 " = 4 c.c. S_1	10 c.c.	X +2.00 " " " " "
6	0.05 " = 5 c.c. S_1	9 c.c.	X +2.50 " " " " "
7	0.06 " = 6 c.c. S_1	8 c.c.	X +3.00 " " " " "
8	0.07 " = 7 c.c. S_1	7 c.c.	X +3.50 " " " " "
9	0.08 " = 8 c.c. S_1	6 c.c.	X +4.00 " " " " "

Where S_1 = a solution containing 10 mg. uric acid (50 c.c. of the stock phosphate solution) in one liter of distilled water.

Blood Creatinine.—Into each of eight Erlenmeyer flasks pipette 2 c.c. of oxalated blood. Add creatinine and water as indicated in the protocol below. Finally add to each flask 2 c.c. each of 10 per cent sodium tungstate and ⅔ normal sulphuric acid. De-

termine the creatinine on the filtrate in the usual manner (page 138). Compare the results obtained with the calculated figures (Table LIV).

TABLE LIV

FLASK NO.	CREATININE ADDITION	WATER	CALCULATED CREATININE
1	0.0	14 c.c.	X mg. per 100 cc. blood
2	0.012 mg. = 2 c.c.S_1	12 c.c.	X +0.6 " " " " "
3	0.024 " = 4 c.c.S_1	10 c.c.	X +1.2 " " " " "
4	0.036 " = 6 c.c.S_1	8 c.c.	X +1.8 " " " " "
5	0.048 " = 8 c.c.S_1	6 c.c.	X +2.4 " " " " "
6	0.060 " =10 c.c.S_1	4 c.c.	X +3.6 " " " " "
7	0.072 " =12 c.c.S_1	2 c.c	X +3.0 " " " " "
8	0.084 " =14 c.c.S_1	0	X +4.2 " " " " "

Where S_1 = a solution containing 6 mg. creatinine in one liter of distilled water.

Blood Cholesterol.—Introduce into each of six beakers, 1 c.c. of blood plasma, 4 or 5 grams of plaster of Paris, and cholesterol as indicated in the protocol below. Determine the cholesterol in the usual way (page 148). Compare the results obtained with the calculated figures (Table LV).

TABLE LV

BEAKER NO.	CHOLESTEROL ADDITION	CALCULATED CHOLESTEROL
1	0.0	X mg. per 100 cc. plasma
2	0.2 mg. = 2 c.c. S_1	X +20 " " " " "
3	0.4 " = 4 c.c. S_1	X +40 " " " " "
4	0.6 " = 6 c.c. S_1	X +60 " " " " "
5	0.8 " = 8 c.c. S_1	X +80 " " " " "
6	1.0 " =10 c.c. S_1	X +100 " " " " "

Where S_1 = a solution containing 10 mg. of cholesterol in 100 c.c. chloroform.

Blood Chloride.—Pipette 4 c.c. of blood into each of five Erlenmeyer flasks. Add water and sodium chloride as indicated in the protocol below. To each flask then add 4 c.c. each of ten per cent sodium tungstate and ⅔ normal sulphuric acid. Shake and filter. To 20 c.c. of the filtrate of each flask add 10 c.c. of silver nitrate solution (page 146). Filter off the silver chloride and determine the excess of silver nitrate in the filtrate as described on page 145. Compare the results with the calculated figures (Table LVI).

TABLE LVI

FLASK NO.	SODIUM CHLORIDE ADDITION	WATER	CALCULATED SODIUM CHLORIDE
1	0.0	28 c.c.	X mg. per 100 cc.
2	1.0 mg. = 1 c.c. S_1	27 c.c.	X + 25 " " " "
3	3.0 " = 3.0 c.c. S_1	25 c.c.	X + 75 " " " "
4	5.0 " = 5.0 c.c. S_1	23 c.c.	X +125 " " " "
5	7.0 " = 7.0 c.c. S_1	21 c.c.	X +175 " " " "

Where S_1 = a solution containing 1 gram of sodium chloride per liter.

Preparation of Volumetric Solutions

A normal solution of an acid contains in a liter 1.008 grams of replaceable hydrogen, of an alkali 17.008 grams of hydroxyl. The amount of an acid required to make one liter of a normal solution is the molecular weight of the acid in grams divided by the number of replaceable hydrogen atoms which it contains. Similarly the molecular weight of a base divided by the number of hydroxyls which it contains is the amount necessary to make one liter of a normal solution.

Equal volumes of a normal solution exactly neutralize each other. If one known normal solution is available, others can be made indirectly by titration against the known solution. The normal solution which can be most easily prepared accurately is that containing acid potassium phthalate. This salt is a crystalline substance of high molecular weight and is easily procured in pure form. The alkalis can then be made by titration against the phthalate solution, and the other acids by titration against an alkali.

The preparation of normal hydrochloric acid by the distillation is also a simple and accurate procedure. This method may be employed if acid potassium phthalate is not available.

N/10 and N/100 solutions are made either by dilution of a normal solution or by direct titration.

1. **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

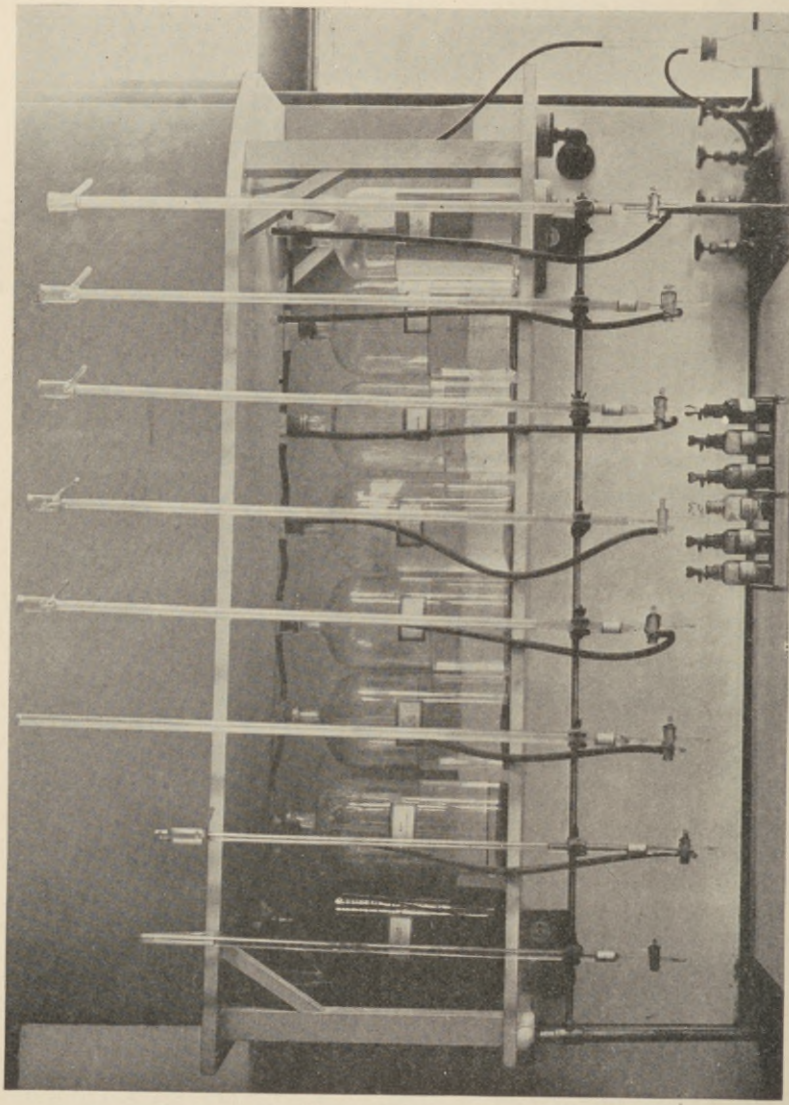


Fig. 66.—Burette arrangement. The reagent bottles are connected in series with the compressed air outlet. The burettes have three way stopcocks (Fig. 52) and fill automatically. Such an arrangement saves much time in making protein-free blood filtrates and in titrations and quantitative chemical procedures.

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 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

$$C = \frac{N.d}{n}$$

$$C = \frac{1900 \times .5}{49.5}$$

$$C = 19.2$$

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.

2. **N/1 Sulphuric Acid.**—A normal solution of sulphuric acid

may be made by the same procedure as that given for the N/10 acid. Dissolve the 5.3 grams of sodium carbonate in 100 c.c. of distilled water. Take 62.0 c.c. of chemically pure H_2SO_4 (sp.gr. 1.84), dilute with four or five volumes of water, cool and dilute to two liters.

Titrate and correct as indicated above.

3. **Normal and N/10 Hydrochloric Acid.**—(Hulett and Bonner, Jour. Amer. Chem. Soc., 1909, xxxi, 390.) 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 degrees of 1.096 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	Grams of solution to make 1 liter of N/1 HCl
770	20.218	18.04	180.4
760	20.242	18.02	180.2
750	20.266	18.00	180.0
740	20.290	17.97	179.7
730	20.314	17.95	179.5

4. **Normal and 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 N sodium hydroxide remove with a graduated pipet 57 c.c. of the clear solution and dilute to 1,000 c.c. Standardize against normal acid as described on the preceding page; for each liter of N/10 sodium hydroxide dilute 5.7 c.c. of the clear solution to 1,000 c.c. and standardize against N/10 acid.

5. **N/10 Iodine.**—Weigh out 12.685 grams of pure resublimed iodine into a small weighing bottle using a porcelain spatula. Dissolve 18 grams of pure KI in about 150 c.c. of water. Transfer

the iodine to a liter flask washing out the last traces with some of the KI solution, which is then poured into the flask. Stopper and shake occasionally until dissolved. If necessary a few more crystals of KI may be added to aid solution. Dilute to the mark and mix well. Keep in glass stoppered bottle in cool dark place. Standardize at once against N/10 sodium thiosulphate solution. Measure out accurately 25 c.c. of the iodine solution into an Erlenmeyer flask, run in sodium thiosulphate until the color is pale yellow, then add a few cubic centimeters of a 1 per cent solution of starch (preferably soluble starch) and titrate to disappearance of blue color. Care should be taken near the end point.

6. **N/10 Potassium Permanganate.**—Dissolve 3.162 grams of pure potassium permanganate in a liter of distilled water, allow to stand a few days, and filter through glass wool. Standardize against N/10 oxalic acid solution or against pure dry sodium or potassium oxalate. One c.c. of N/10 permanganate is equivalent to 7.0 mg. of sodium oxalate.

7. **N/10 Sodium Thiosulphate Solution.**—Weigh out 25 grams of ordinary c.p. sodium thiosulphate or 24.83 grams of the pure dry recrystallized salt. Dissolve in water and dilute to a liter. Boiled distilled water must be used. Keep in a bottle with a siphon arrangement and carrying a soda lime tube to exclude CO_2.

It is best standardized against acid potassium iodate $KH(IO_3)_2$. Weigh out accurately 0.3249 gram of acid potassium iodate. Dissolve in 50 c.c. of water, heating gently if necessary. Transfer the solution to a 100 c.c. flask, rinsing the beaker carefully and make to mark with water. This solution is exactly decinormal. Pipet out 25 c.c. into an Erlenmeyer flask, add 1 gram of potassium iodide dissolved in a little water, and a few cubic centimeters of dilute hydrochloric acid. Titrate immediately with the thiosulphate solution. When the solution becomes a pale yellow add a few cubic centimeters of one per cent solution of soluble starch and titrate to loss of blue color.

8. **Normal Acid Potassium Phthalate.**—This acid salt may be conveniently used as a starting point for the preparation of standard acids and alkalis. It can be obtained chemically pure, has no water of crystallization, has a high molecular weight, and is freely soluble in water.

The salt is dried at 110° to 115° C. to constant weight. A normal solution contains 204.14 grams in a liter.

Calibration of Volumetric Apparatus

(Medical War Manual No. 6)

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 of the temperature of calibration. Table LVII 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.

TABLE LVII

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 coordinate paper, which is hung by the burette for reference.

The accompanying figures (Table LVIII) for the first 10 c.c. of a burette serve as an example.

TABLE LVIII

BURETTE READING C.C.	WEIGHT OF WATER DELIVERED AT 22°C. GM.	VOLUME OF WATER DELIVERED (= wt. x 1.0033) C.C.	CORRECTION TO BURETTE C.C.
2	2.000	2.006	plus 0.01
4	4.002	4.008	" 0.01
6	6.009	6.017	" 0.02
8	8.020	8.050	" 0.05
10	10.020	10.050	" 0.05

The plus sign indicates that each correction is to be added to the observed reading in order to give the actual volume of liquid delivered. Were the volumes of water delivered at any points less than indicated by the burette readings the corresponding corrections would be indicated by minus signs.

Pipettes are calibrated by filling to the mark with distilled water and discharging into a weighing bottle. The water delivered should be weighed to within 1 part per 1000. If the mark is not accurate a correct one should be made with a wax pencil, subsequently etched in 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."

Use of Indicators

Indicators are chemical substances possessing an intense color which changes in the presence of acid and alkali. A small amount of the indicator will tinge a large quantity of fluid. The change in color of the indicator is due to the fact that its color is dependent upon the degree of its dissociation, which in turn is determined by the presence of free acid or free alkali.

Different indicators change color at greater or less hydrogen-ion concentration. The indicator selected for any titration should

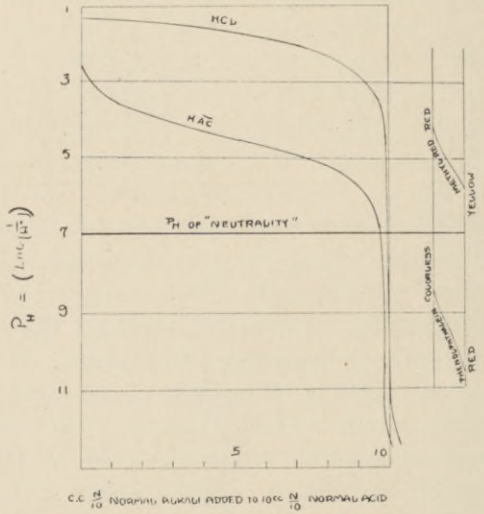


Fig. 67.—Titration curves of hydrochloric acid and acetic acids, showing the gradual color change of methyl red and phenolphthalein within their PH zones of transformation. Curves are approximate only. (*After Clark.*)

be one which gives a sharp color reaction which is sensitive to the form of acidity to be determined.

If we use Sorenson's expression P_H to indicate the logarithm of the reciprocal of the H-ion concentration, at the neutral point P_H is 7; values of P_H greater than 7 are on the alkaline side; values less than 7 are on the acid side of neutrality.

A general idea of the principles applied in the use of indicators for titration may be gained by the comparison of the titration of hydrochloric acid with sodium hydroxide and of acetic acid with sodium hydroxide (Clark). In Fig. 67 the curves show the P_H

(logarithm of the reciprocal of the hydrogen-ion concentration) plotted as ordinates as equal concentrations of hydrochloric and acetic acids respectively are neutralized with successive quantities of NaOH (abscisses).

At the right of these curves are indicated the conduct of methyl red and of phenolphthalein within the P_H zones of their color changes. It will be seen that in the titration of hydrochloric acid either indicator would show a sudden color change with the slightest addition of alkali when practically complete neutralization is approached. On the other hand in the acetic acid solution methyl red undergoes a slow color change in a P_H zone within which acetic acid is only partly neutralized. Only by the use of an indicator changing color at a much lower hydrogen-ion concentration (higher P_H) will the end point of this titration be reached. A similar set of curves might be drawn to show similar relations in the titration of alkalis with acids. It will be noted in the cases cited that phenolphthalein is serviceable in both cases.

It is simply the extension of such considerations which leads to the choice of phenolphthalein for the general titration of acids by bases. Similar considerations lead to the choice of methyl red in general titrations of alkalis with acids. In many cases, however, the choice must be determined by a close study of the acid or base and the special conditions employed.

Only indicators which change color well on the acid side at $P_H = 5$ or less, such as methyl red, can be used for titrating strong acids with weak bases, and for the titration of alkalis in the presence of carbonic acid. Likewise for the titration of strong bases with weak acids the indicator must change at $P_H = 8$ or more such as phenolphthalein. Almost any indicator can be used in the titration of strong bases such as NaOH against strong acids, as one drop of the solution will carry the hydrogen-ion concentration so far beyond neutrality that the turning point of any common indicator will be passed.

Phenolphthalein is unsatisfactory in the presence of ammonium salts so cannot be used as an indicator in ammonia titration. Indicators which change at $P_H = 3$ to 4 can be used for the titration of mineral acids in the presence of weak organic acids, as at

TABLE LIX

TABLE OF INDICATORS[1]

INDICATOR		COLOR		P_H AT WHICH COLOR CHANGES	FORM IN WHICH INDICATOR IS EMPLOYED	SPECIAL USE
CHEMICAL NAME	COMMON NAME	ACID	ALKALINE			
Dimethyl-amino-azo-benzene	Topfer's reagent	Red	Yellow	3-4	0.4 per cent in 95 per cent alcohol	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.
Diazo compound of benzidine and naphthionic acid	Congo red	Blue	Red	4-5	0.5 per cent sol. in 95 per cent alcohol	Titration of weak bases, (ammonia) with mineral acid.
Sodium-alizarin-sulphonate	Alizarin red	Yellow	Red	5-6	1.0 per cent solution of Na salt in 50 per cent alcohol	Titration of weak bases, (ammonia) with mineral acid.
Ortho carboxy-benzene-azo-dimethyl-aniline	Methyl red	Red	Yellow	4-6	0.02 per cent sol. in 60 per cent alcohol	Titration of bases with strong acid
	Litmus	Red	Blue	About 7	Paper	
Phenolphthalein		Colorless	Red	8-9	1.0 per cent solution in 95 per cent alcohol	Titration of organic acids with mineral alkali.

[1]Abbreviated from Medical War Manual, No. 6.

such an end point the weak organic acid exerts but little influence on the titration results.

Table LIX summarizes the important points about the indicators commonly employed in chemical titrations and shows the use for which each is best adapted. A special set of indicators is used in the determination of the hydrogen-ion concentration of biological fluids (see page 239).

Preparation of Dakin's Solution

1. **From Liquid Chlorine and Sodium Carbonate.**—Dakin's solution is to be made routinely from liquid chlorine and sodium carbonate.

Fig. 68.—Apparatus for making Dakin's solution from liquid chlorine. (Courtesy of Wallace and Tiernan Co.)

PROCEDURE.—Prepare a solution containing 18.0 grams anhydrous sodium carbonate (or 21.0 grams monohydrate sodium carbonate) per liter in distilled water. Run in chlorine through diffusor (Fig. 68) until a sufficient quantity has been introduced as indicated by the gauge. The gauge indicates the number of cubic centimeters of Dakin's solution made per minute.

TITRATION.—Measure 10 c.c. of Dakin's solution into beaker

containing 50 c.c. of water. Add 5 c.c. of 10 per cent potassium iodide solution and 2 c.c. of glacial acetic acid. Then run tenth normal sodium thiosulphate solution in the flask until the decolorization of the solution is complete.

The number of cubic centimeters of tenth normal thiosulphate required to decolorize the solution multiplied by the factor 0.0372 gives the percentage of sodium hypochlorite. The solution must titrate between 0.45 and 0.50 per cent sodium hypochloride.

Table LX shows the percentage of hypochlorite corresponding to the amount of thiosulphate used.

TABLE LX

C.c. N/10 sodium thiosulphate used	Per cent of sodium hypochlorite in solution
12.1	0.4507
12.2	0.4545
12.3	0.4582
12.4	0.4619
12.5	0.4656
12.6	0.4694
12.7	0.4731
12.8	0.4768
12.9	0.4805
13.0	0.4843
13.1	0.4880
13.2	0.4917
13.3	0.4954
13.4	0.4992

TESTS FOR ALKALINITY.—A. With powdered phenolphthalein. A few crystals of powdered phenolphthalein are dropped on the surface of about 5 c.c. of the solution, and the solution vigorously shaken. The solution must remain colorless. B. With alcoholic solution of phenolphthalein. About a half cubic centimeter of alcoholic solution of phenolphthalein is added to 5 c.c. of the Dakin's solution. A red color should develop, but quickly disappear.

If the solution does not titrate between 0.45 and 0.50 per cent sodium hypochlorite, or gives a reaction with powdered phenolphthalein, or fails to give a flash of color with alcoholic solution of phenolphthalein, it should be discarded. Store in dark bottles in a cool place.

Each time a portion is dispensed, it should be titrated, and the bottle labeled as follows:

DAKIN'S SOLUTION

Titration per cent sodium hypochlorite on

2. **From Chlorinated Lime, Sodium Bicarbonate and Sodium Carbonate.**—If liquid chlorine is not available, the Dakin's solution may be made by the following method.

TITRATION OF CHLORINATED LIME.—Weigh out 20 grams of an average sample of chlorinated lime; mix as completely as possible with one liter of tap water, and leave in contact for 6 hours, shaking from time to time.

Measure exactly 10 c.c. of the clear fluid; add 20 c.c. of a 10 per cent solution of potassium iodide, and 2 c.c. of acetic acid. Add a decinormal solution of sodium thiosulphate until decoloration is complete.

The number of cubic centimeters of thiosulphate solution required for complete decoloration, multiplied by 1.775 gives the weight of the active chlorine contained in 100 grams of the chlorinated lime.

The figure being known, it is applied to the accompanying

TABLE LXI

TITER OF CHLORINATED LIME	CHLORINATED LIME GM.	ANHYDROUS SODIUM CARBONATE GM.	SODIUM BICARBONATE GM.
20	230	115	96
21	220	110	92
22	210	105	88
23	200	100	84
24	192	96	80
25	184	92	76
26	177	89	72
27	170	85	70
28	164	82	68
29	159	80	66
30	154	77	64
31	148	74	62
32	144	72	60
33	140	70	59
34	135	68	57
35	132	66	55
36	128	64	53
37	124	62	52

table, which will give the quantities of chlorinated lime, of sodium carbonate, and of sodium bicarbonate, which are to be employed to prepare 10 liters of the solution.

To prepare 10 liters of the solution:

1. Weigh exactly the quantities of chlorinated lime, sodium carbonate and sodium bicarbonate, which have been determined in the course of the preceding trial.

2. Place in a 12 liter jar the chlorinated lime and 5 liters of ordinary tap water, agitate vigorously for a few minutes, and leave in contact for from 6 to 12 hours, overnight, for instance.

3. At the same time dissolve, cold, in the 5 other liters of water the sodium carbonate and the bicarbonate.

4. Pour all at once the solution of the sodium salts into the jar containing the maceration of chlorinated lime, agitate vigorously for a few moments, and leave it quiet to permit the calcium carbonate to settle as it forms. At the end of the half hour, siphon the liquid and filter it through double paper to obtain an entirely limpid product which must be protected from light.

Titrate and test for alkalinity as indicated above.

Preparation of Nessler's Solution (Folin Method)

This reagent is essentially a solution of the double iodide of mercury and potassium (HgI_2, 2KI) containing sodium or potassium hydrate. A stock solution of the double iodide is best prepared as follows:

Transfer 150 grams of potassium iodide and 110 grams of iodine to a 500 c.c. Florence flask; add 100 c.c. of water and an excess of metallic mercury, 140 to 150 grams. Shake the flask continuously and vigorously for seven to fifteen minutes, or until the dissolved iodine has nearly disappeared. The solution becomes quite hot. When the red iodine solution has begun to visibly pale, though still red, cool in running water and continue shaking the solution until the reddish color of the iodine has been replaced by the greenish color. Decant and wash the sediment. Dilute the solution and washings to a final volume of 2 liters. If the cooling was begun in time the resulting reagent is clear enough on immediate dilution with 10 per cent alkali and water.

Prepare the final Nessler solution as follows:

From completely saturated caustic soda solution containing 55 grams of NaOH per 100 c.c., decant the clear supernatant liquid, and dilute to a concentration of 10 per cent. Determine by titration that a 10 per cent solution has been obtained.

Introduce into a large bottle 3,500 c.c. of 10 per cent sodium hydroxide solution, add 750 c.c. of the double iodide solution and 750 c.c. of distilled water, giving 5 liters of Nessler's solution.

In the absence of modifying circumstances such as much acid or alkali, this reagent should be added in the proportion of 10 c.c. per 100 c.c. of volume to which the Nesslerized solution is to be diluted. As a general rule the volumetric flask should be at least two-thirds full before adding the Nessler reagent, otherwise, turbid mixtures may be obtained.

Preparation of Creatinin

(Benedict, S. R.: Jour. Biol. Chem., 1914, xviii, 182)

To 10 liters of undecomposed urine in a large precipitating jar add with stirring a hot solution of 180 grams of picric acid in 450 c.c. of boiling alcohol. Allow to stand overnight and syphon off the supernatant fluid. Pour the residue upon a large Buchner funnel, drain with suction, wash once or twice with cold saturated picric acid and suck dry. Treat the dry or nearly dry picrate in a large mortar or evaporating dish with enough concentrated HCl to form a moderately thin paste (about 60 c.c. of acid for each 100 grams of picrate) and stir the mixture thoroughly with the pestle for 3-5 minutes. Filter with suction on a hardened paper, and wash the residue twice with enough water to cover it, sucking as nearly dry as possible each time. Transfer the filtrate to a large flask and neutralize with an excess of solid magnesium oxide (the "heavy" variety is best). Add this oxide in small portions with cooling of the flask under running water between the additions. Neutralization of the acid will be indicated by a bright yellow color of the mixture, or litmus paper may be used to test it. Filter with suction. Wash the residue twice with water. Immediately add a few cubic centimeters of glacial acetic acid to the filtrate to make it strongly

acid. Pay no attention to any precipitate that may form, but dilute the solution with about 4 volumes of 95 per cent alcohol. After 15 minutes filter off the slight precipitate which forms. Treat the final filtrate with 30-40 c.c. of 30 per cent zinc chloride. Stir and let stand overnight in a cool place. Pour off the supernatant liquid and collect the creatinine zinc chloride on a Buchner funnel, wash once with water, then thoroughly with 50 per cent alcohol, finally with 95 per cent alcohol and dry. A nearly white, light crystalline powder should be obtained. The yield should be 90-95 per cent of the original creatinine (usually about 1.5-1.8 grams of creatinine zinc chloride per liter of urine).

Recrystallize the creatinine-zinc chloride by treating 10 grams with 100 c.c. of water and about 60 c.c. of normal sulphuric acid, heating the mixture until a clear solution is obtained. Add about 4 grams of purified animal charcoal, continue boiling for about a minute, filter with suction through a small Buchner funnel, pouring the filtrate back on the filter three or four times until it runs through perfectly colorless. Wash residue with hot water and transfer the total filtrate to a beaker and while hot treat with a little strong zinc chloride solution (3 c.c.) and with about 7 grams of potassium acetate dissolved in a little water. After ten minutes dilute with an equal volume of alcohol, and allow to stand in a cold place for some hours. Filter off the crystalline product. To remove the small amount of potassium sulphate which it contains stir up with twice its weight of water, filter, wash with a little water and then with alcohol. The preparation should be snow white. Yield, 85-90 per cent.

Place the finely powdered recrystallized creatinine zinc chloride in a dry flask and treat with seven times its weight (by volume) of concentrated aqueous ammonia. Warm slightly and agitate gently until a clear solution is obtained, care being taken to drive off no more ammonia during the warming than is necessary to obtain a clear solution. Stopper the flask, allow to cool, place in the ice box for an hour or more. Pure creatinine crystallizes out. It may be recrystallized from boiling alcohol or concentrated ammonia, but this is usually unnecessary. The product is perfectly pure and can be used as a standard in the quantitative determination of creatine and creatinine.

Method for Purification of Picric Acid

(Folin and Doisy: Jour. Biol. Chem., 1916-17, xxviii, 349)

Transfer about 600 gm. of wet picric acid, or about a pound of dry picric acid, to a large beaker (capacity not less than 4 liters). Pour on boiling water until the beaker is nearly full and add 200 c.c. of saturated (50 per cent) sodium hydroxide solution. Stir, and if necessary heat again until all the picric acid has dissolved, yielding a deep red picrate solution. To the hot solution add rather slowly, with stirring, 200 gm. of sodium chloride. Cool in running water to about 30° C., with occasional stirring. Filter on a large Buchner funnel and wash a few times with 5 per cent sodium chloride solution. Transfer the picrate to the large beaker, fill with boiling water, and when the picrate is dissolved add, with stirring, first 50 c.c. of 10 per cent sodium hydroxide solution, and then 100 gm. of sodium chloride. Cool to 30° C., with stirring, filter, and wash with sodium chloride solution, as before. Repeat the solution and precipitation of the sodium picrate twice more, but for the last washing of the last precipitated picrate use distilled water instead of sodium chloride solution.

Dissolve the purified picrate in the same large beaker, with boiling distilled water, and filter hot on a large folded filter, collecting the filtrate in a large flask. To the hot filtrate add 100 c.c. of concentrated sulphuric acid, previously diluted with about two volumes of water. The liberated picric acid begins to come out at once. Put a beaker over the mouth of the flask and cool under running tap water to about 30° C. Filter with suction as before and wash free from sulphates with distilled water.

Preparation of Solutions for Intravenous Use

1. **Physiological Salt Solution.**—Dissolve 25.5 grams of chemically pure sodium chloride in three liters of distilled water. Filter through paper.

Scrub thoroughly a dozen 12 ounce bottles. Rinse with tap water and then with distilled water. Measure 275 c.c. of the salt solution into each bottle. Plug the mouth with cotton and gauze. Autoclave for 15 minutes at 20 pounds pressure.

Label as follows:

Sterile Physiological (0.85%) Salt Solution for Intravenous Use.

Prepared ..... By .....

2. **Glucose Solution.**—

Weigh 750 grams of pure glucose and dissolve with the aid of heat in about 1500 c.c. of distilled water. Make up to three liters with distilled water. Filter through cotton to remove major part of flocculent material, then filter through paper. Thoroughly scrub bottles of 500 c.c. capacity. Rinse well with tap water and then with distilled water. Measure 275 c.c. of the 25 per cent glucose solution into each bottle. Plug the mouth with cotton and gauze. Autoclave for fifteen minutes at twenty pounds pressure.

Label as follows:

Sterile 25% Glucose Solution for Intravenous Use.

Prepared ..... By .....

3. **Sodium Bicarbonate.**—Dissolve 75 grams of chemically pure anhydrous sodium carbonate in three liters of freshly distilled water. Filter through paper. Add several drops of phenolphthalein solution.

Place in the flask a Folin absorption tube connected by rubber tubing to a calcium chloride drying tube filled with absorbent cotton. Stopper the flask with cotton. Place the flask with the entire apparatus in the autoclave and heat under 20 pounds pressure for 15 minutes. After cooling connect the drying tube with the carbon dioxide tank and bubble gas through until the solution becomes colorless. This converts the 2.5 per cent sodium carbonate solution into a 4 per cent sodium bicarbonate solution.

Distribute this sterile bicarbonate solution into sterile 500 c.c. bottles, putting 275 c.c. in each bottle. Replace the sterile stoppers and store in cool place.

Label as follows:

Sterile 4% Sodium Bicarbonate Solution for Intravenous Use.

Prepared ..... By .....

Preparation of Sodium Citrate Solution for Blood Transfusion

Dissolve 25 grams of chemically pure crystalline sodium citrate in one liter of freshly distilled water. This makes a 2.5 per cent solution. Filter through paper if not perfectly clear.

Scrub thoroughly ten 100 c.c. bottles. Rinse with tap water followed by distilled water. Fill with the citrate solution. Stopper with cotton, and autoclave for 15 minutes at 20 pounds pressure.

Label as follows:

Sterile 2.5% Sodium Citrate for Blood Transfusion.

Prepared ..... By .....

Preparation of Antiformin

1. **From Chlorinated Lime.**—

Sodium carbonate	600.0 grams
Chlorinated lime	400.0 grams
Distilled water.....	4,000.0 c.c.

Dissolve the sodium carbonate in 1,000 c.c. of distilled water. Triturate thoroughly the chlorinated lime in the remainder of the water. Filter. Mix the two and filter again.

To the solution so prepared add an equal volume of 15 per cent sodium hydroxide.

2. **From Liquid Chlorine.**—If liquid chlorine is available the antiformin solution may be made by running the chlorine gas into a solution containing 75 grams of sodium carbonate and 75 grams of sodium hydroxide per liter until the solution titrates about 2 per cent sodium hypochlorite.

Methyl Violet Shellac (for Marking Glassware)

Best white shellac.....	10 gr.
Alcohol, 95%	20-25 c.c.
Methyl-violet	0.1 gr.

Apply with a small brush or a tooth pick.

Bichromate Cleaner for Glassware

Potassium bichromate (powdered)	200 gr.
Water distilled up to	1500 c.c.
Sulphuric acid concentrated	500 c.c.

Stopcock Grease (Boothby)

Wash ordinary black rubber tubing in water to remove the talc. Cut in small pieces and add slowly to an equal quantity of lanolin which has been melted.

The rubber is allowed to melt until the resulting mixture is free from lumps.

Foam Killer

Twenty per cent solution of rosin in turpentine.

CHAPTER XIII

HISTOLOGICAL TECHNIC

Preparation of Permanent Sections for Histological Examination

Reagents.—

1. Delafield's Hematoxylin.—

Hematoxylin crystals	4 grams
Alcohol—95%	25 c.c.
Saturated (11%) aqueous solution of ammonia alum	400 c.c.

Add the hematoxylin dissolved in the alcohol to the alum solution, and expose the mixture in an unstoppered bottle to the light and air for two to three weeks, filter and add:

Glycerine	100 c.c.
Alcohol—95%	100 c.c.

Allow the solution to stand in the light until the color is sufficiently dark, then filter and keep in a tightly stoppered bottle. So long as it is good it has a purplish tinge.

2. Formaldehyde Fixing Solution.—

Formalin (commercial)	100 c.c.
Water to	1000 c.c.

3. Zenker's Fixing Solution.—

Potassium bichromate	2.5 grams
Mercury bichloride	5.0 grams
Water	100 c.c.

Five c.c. of glacial acetic acid is added to 100 c.c. of this stock solution just before use.

4. Ammonia Water.—Add 5 c.c. concentrated ammonia to 95 c.c. distilled water.

5. Acid Alcohol.—Add 1 c.c. concentrated hydrochloric acid to 100 c.c. 95 per cent alcohol.

6. Eosin.—Use saturated solution in 95 per cent alcohol.

7. Carbol—Xylol.—

Carbolic acid crystals (melted) .....1 part
Xylol .....3 parts

8. Mayer's Glycerine Albumen.—Mix equal parts white of egg and glycerine. Beat the mixture thoroughly, filter and add one per cent sodium salicylate as preservative.

Procedure.—(1) Fixation.—Specimens will be sent directly to the laboratory from the operating room. Enter at once in the Surgical Pathological Record Book, the patient's name, case number, name of the operator, the operation, and the nature of the specimen received.

If small the specimen is placed in 10 per cent formalin. Sections of characteristic portions of larger specimens are cut and placed in 10 per cent formalin. If the entire specimen is to be saved, it is to be placed in Kaiserling's solution.

When the specimen consists of uterus and adnexa, cut at least one block from uterus showing endometrium, one from each tube and one from each ovary. Two sections are to be cut from an appendix, one near the middle, and one from the distal end. One section is taken through the middle of each tonsil, cutting at right angles to the mucous membrane, and from upper to lower pole.

The specimen is allowed to remain in the formalin solution overnight.

(2) Embedding and Cutting.—After fixation, tissue blocks, not over 3 millimeters in thickness, are cut and embedded as follows:

1. Acetone I	2 hours
2. Acetone II	2 hours
3. Acetone III	Overnight
4. Chloroform I	1 hour
5. Chloroform II	1 hour
6. Saturated solution of paraffin in chloroform at room temperature	1 hour
7. Paraffin I in oven at 60° C.	1 hour
8. Paraffin II in oven at 60° C.	1 hour

Fill a small paper box with freshly melted paraffin and arrange the piece of tissue in the box with a hot needle. Place the surface of the block from which the bottom is to be cut, on the bottom of the box.

Cool as quickly as possible.

Remove paper and turn block of tissue and fasten to a small wood block. Number block with pencil.

Cut sections in microtome about ten microns thick and drop on surface of a pan of water at about 48° C.

Rub a small amount of Meyer's glycerine albumen on slides with the finger and push flat sections to two clean slides. Stand on end to drain, and then place in incubator overnight.

(3) STAINING.—Sections are stained as follows:

1. Xylol I	2 minutes
2. Xylol II	2 minutes
3. Absolute alcohol I	2 minutes
4. Absolute alcohol II	2 minutes
5. Alcohol 95%	2 minutes
6. Alcohol 70%	2 minutes
7. Hematoxylin (Delafield's)	5 minutes
8. Distilled water	2 minutes
9. Acid alcohol until brick red	
10. Ammonia water until blue	
11. Alcohol 70%	2 minutes
12. Sat. alcoholic solution of eosin	2 minutes
13. Alcohol 95% I	2 minutes
14. Alcohol 95% II	2 minutes
15. Carbol-Xylol	2 minutes
16. Carbol-Xylol	2 minutes
17. Xylol	2 minutes
18. Mount in Canada balsam	

Frozen Sections

Temporary sections can be quickly prepared for examination by the following technic (Wilson: Jour. Lab. and Clin. Med., 1915, i, 41.)

Reagents.—1. UNNA'S METHYLENE BLUE prepared by Terry's method as follows:

Place in a petri dish 0.5 gram of methylene blue, (Bausch and Lomb), 0.5 gram of potassium carbonate (Merck), and dissolve in 50 c.c. of distilled water. Place in incubator at 37° C.

Each day the water lost by evaporation is made up by the addition of distilled water.

Leave in incubator six days. The stain should ripen in this time.

Test on section of uterus. All the nuclei should be sharply stained and the smooth muscle should stain a sharp and beautiful purple.

2. Brun's Glucose Medium.—

Glucose	40 gr.
Glycerine	10 c.c.
Camphor	10 gr.
Water	140 c.c.

Mix and filter.

3. Dextrin Solution.—Dissolve dextrin in distilled water until the solution is the consistency of thin molasses. Add 0.5 per cent phenol.

Procedure.—1. Freeze bits of fresh tissue, not more than 2x10 mm. in the dextrin solution and cut sections 5 to 15 microns thick.

2. Remove sections from knife with tip of finger and allow them to thaw thereon.

3. Unroll sections with camel's hair brush or glass lifter in 1 per cent sodium chloride solution.

4. Stain 10 to 20 seconds in Unna's polychrome methylene blue.

5. Wash momentarily in 1 per cent sodium chloride solution.

6. Mount in Brun's glucose solution.

Permanent frozen sections may be made by the following technic:

Slices of the tissue (not over 2 or 3 mm. thick and 1 cm. square) are cut with a sharp scalpel and dropped into 10 per cent formalin heated to about 40° C. After about ten minutes the preparation is removed from the formalin and frozen, and the sections are placed in water and then on the slide. 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 piece of "Royal" blotting paper. 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 saturated alcoholic solution of eosin. A strength of 1 to 1000 is sufficient. After a few seconds it is rinsed in alcohol 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 with carbolxylol. 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, and cover pressed down upon it.

Mallory's Stain for Connective Tissue Fibers

1. Fix in Zenker's fluid.

2. Embed in celloidin or paraffin.

3. Stain sections in a 0.5 per cent solution of acid fuchsin five minutes or longer, depending on the freshness of the tissue.

4. Transfer directly to the following solution and stain from fifteen to twenty minutes or longer:

5.

Anilin blue soluble in water	0.5
Orange G	2.0
One per cent aqueous solution of phosphomolybdic acid	100.0

6. Wash and dehydrate in several changes of 95 per cent alcohol.

7. Clear in xylol.

8. Mount in balsam.

The fibrils and reticulum of connective tissue, amyloid, mucus, and certain other hyaline substances stain blue; nuclei, cytoplasm, fibroglia fibrils, axis cylinders, neuroglia fibers, and fibrin red; red corpuscles and myelin sheaths yellow; elastic fibers pale pink and yellow. The various structures do not stain with equal intensity, so that certain ones are brought out with great sharpness. This is particularly true of the connective tissue, and of fibrin and smooth and striated muscle fibers.

Technic for Staining Mitochondria (Bensley)

1. **Fixation.**—The fixing solution is freshly made just before use as follows: 2.5 per cent potassium bichromate, 8 c.c.; 4 per cent osmic acid, 2 c.c.; glacial acetic acid, one drop.

The tissue must be perfectly fresh and should be cut into pieces not larger than 1 cm. cube and placed in the fixing fluid 24 hours.

Wash in running water for 24 hours.

2. **Dehydration and Embedding.**—Fifty per cent alcohol 12 hours; 70 per cent and 95 per cent alcohol 24 hours each; absolute alcohol 6 to 12 hours; half absolute and xylol 6 hours; xylol I, 3 hours; xylol II, 3 hours; paraffin 60° C. 3 hours; cut sections not more than 4 microns thick.

3. **Staining.**—(1) Pass the sections, mounted on slides, down through toluol, absolute, 95, 70 and 50 per cent alcohol to distilled water.

(2) One per cent aqueous solution of potassium permanganate 1 minute.

(3) Five per cent aqueous solution of oxalic acid also 1 minute.

(4) Rinse in several changes of distilled water about a minute. Incomplete washing prevents the staining with fuchsin.

(5) Stain in Altman's anilin fuchsin, which is made up as follows: Make a saturated solution of anilin oil in distilled water by shaking the two together. Filter and add 30 grams of acid fuchsin to 100 c.c. of the filtrate. The stain should be ready to use in about 24 hours. It deteriorates in about a month. Stain for 5 minutes in the acid fuchsin solution which has previously been warmed to 60° C.

(6) Dry off most of the stain with a towel and rinse in distilled water so that the only stain remaining is in the sections. If a large amount of the free stain remains it will form a troublesome precipitate with the methyl green; on the other hand if too much of the stain is removed the coloration of the mitochondria will be impaired.

(7) Allow a little 1 per cent methyl green, added with a pipette, to flow over the sections, holding the slide over a piece of white paper so that the colors may be seen. Apply the methyl green for about 5 seconds at first and then modify the time to suit the needs of the tissue.

(8) Drain off the excess of stain and plunge the slide into 95 per cent alcohol for a second or two, then rinse in absolute for the same length of time, clear in toluol, and mount in balsam.

The mitochondria stain a bright crimson.

Stain for Tubercle Bacilli in Tissues

1. Delafield's hematoxylin, 20 to 30 minutes;
2. Water, 30 minutes;
3. Carbol fuchsin, 1 hour at 37° C. (incubator);
   (Place slide in petri dish and flood it; then cover to prevent evaporation.)
4. Decolorize in acid alcohol, 1 minute;

Hydrochloric acid	1 c.c.
95% alcohol	70 c.c.
Water	30 c.c.

5. Wash in 70 per cent alcohol, 2 to 3 minutes;
6. Wash in water;
7. Lithium carbonate solution until section takes blue color;

Sat. sol. lithium carbonate	1 part
Water	10 parts

8. Wash in water, 5 minutes;
9. 95% alcohol, 5 minutes;
10. Absolute alcohol, 5 minutes;
11. Xylol, 5 minutes;
12. Mount in gum dammar.

To obtain the best results the material should be fixed in 95 per cent alcohol, though formalin preparations occasionally stain well. After fixation in Zenker's solution, the bacteria do not stain satisfactorily.

Gram-Weigert Method for Demonstration of Gram-Positive Bacteria in Tissue

Carry section into water after removing paraffin as for eosin-hematoxylin stain.

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

Lithium carmine is made as follows:

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

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

Kaiserling's Method of Preserving Gross Specimens

1. Fixation for one to five days in:

Formaldehyde	200 c.c.
Water	1000 c.c.
Nitrate of potassium	15 grams
Acetate of potassium	30 grams

Change the position of the specimen frequently, using rubber gloves to protect the hands from the injurious effect of the formaldehyde. The time of fixation varies with the tissue or organ and size of the specimen.

2. Drain and place in 80 per cent alcohol one to six hours, and then in 95 per cent alcohol for one to two hours, to restore the color, which is somewhat affected in the fixing solution.

3. Preserve in:

Acetate of potassium	200 grams
Glycerine	400 c.c.
Water	2000 c.c.

Exposure to light gradually affects the colors. The process of fixation should be performed in the dark, and the specimens when preserved should be kept in the dark except when on exhibition.

If it seems desirable to cut a thin slice from the face of a specimen, this should not be done until the preparation has been in the preservative fluid two weeks. The specimen may then be placed in alcohol for one to two hours to brighten up the colors.

It is advisable to add camphor, thymol, carbolic acid (one

per cent), or some other preservative to the third solution to prevent the growth of molds.

Mixture for Sealing Museum Jars

Warm 200 grams of asphalt (Merck) and dissolve the melted asphalt in 200 c.c. of linseed oil and 400 c.c. turpentine.

Warm, and with a small brush apply the cement to the ground surface of the jar.

CHAPTER XIV

EXAMINATION OF MILK AND WATER

Bacteriological Examination of Water

Collect the sample of water in sterile container, with a sterile pipette. If the specimen is collected from a tap, allow the water to run several minutes before obtaining the specimen.

With a sterile pipette place 1 c.c. of the water in a petri dish. Ten cubic centimeters of melted agar at a temperature of 40° C. are then added to 1 c.c. of water in the petri dish. The medium and sample are thoroughly mixed and uniformly spread over the bottom of the dish by tilting or by rotation of the dish.

Incubate for twenty-four hours at 37° and count colonies.

In each of five Dunham fermentation tubes (Fig. 54) containing lactose broth, place 10 c.c. of water with sterile pipette and incubate at 37° C.

If 10 per cent of gas is formed within twenty-four hours, report as positive for B. coli group. If no gas is formed in twenty-four hours—or if the gas formed is less than 10 per cent, incubate for forty-eight hours. Subculture on Endo medium and identify organisms as belonging to B. coli group before reporting as positive. (Page 204.)

Examination of Milk and Cream

Collection of Sample.—Mix the milk or cream thoroughly in container by shaking or by pouring from one container to the other several times. Remove sample in a sterile bottle.

Bacterial Count.—With a sterile pipette withdraw 1 c.c. of milk from the sample bottle and introduce into a bottle containing 99 c.c. of sterile water, thus making a 1-100 dilution. Mark bottle 1/2.

One cubic centimeter of this dilution is transferred to a second bottle containing 99 c.c. of sterile water, giving a dilution of 1-10,000. Mark bottle 1/4.

Introduce 0.1 c.c. of the 1/2 dilution into a sterile petri dish, marking the plate 1/3. Put 1 c.c. of the 1/4 dilution into a second plate and mark 1/4.

Pour enough melted meat extract agar cooled to 45° C. into each plate to well cover the bottom. Mix the agar and sample well by rotating and tilting the dish.

Incubate 48 hours at 37° C. and count the colonies of bacteria. Each colony on the 1/3 plate represents 1000 bacteria per cubic centimeter and each on the 1/4 plate represents 10,000 bacteria per cubic centimeter.

Put 0.1 c.c. of the original milk or cream into a lactose fermentation tube (Fig. 54) labeling it 1/1; 1 c.c. of the 1/2 dilution

Fig. 69.—Babcock test bottle for determining the fat in milk.

into a second tube and marking it 1/2; 0.1 c.c. of the same dilution into a third, marking it 1/3; and 1 c.c. of the 1/4 dilution into a fourth tube, labeling it 1/4.

Incubate the tubes for 24 hours and examine for gas formation. Ten per cent or more gas in the fermentation tubes represents respectively 10, 100, 1000 and 10,000 of B. coli per cubic centimeter of milk. If desired, the organism causing the fermentation may be isolated in pure culture and further tests made to verify the assumption that it is the bacillus coli.

Determination of Butter Fat.—With a pipette deliver 17.6 c.c. of well mixed milk into a Babcock bottle (Fig. 69). Add 17.5 c.c. commercial sulphuric acid using the acid measure. Mix well.

Centrifuge at 1200 revolutions per minute for five minutes. Add hot water to neck of bottle. Centrifuge two minutes and add hot water to about the 7 per cent mark on graduated tube of the bottle and two drops of alkanet root in glymol to facilitate the reading. Centrifuge one minute. The per cent of butter fat is read off directly from the scale. In determining the butter fat in cream, mix the sample with equal parts of water and proceed as with milk except that 18 c.c. of the diluted cream is used instead of 17.6 c.c.

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