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LABORATORY MANUAL
OF
PHYSIOLOGICAL CHEMISTRY

INTRODUCTION TO
PHYSIOLOGICAL CHEMISTRY

By MEYER BODANSKY

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LABORATORY MANUAL
OF
PHYSIOLOGICAL CHEMISTRY

BY

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FOURTH EDITION

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AND
MARION FAY

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PREFACE TO THE FOURTH EDITION

In preparing the present edition the authors have been guided partly by the suggestions of teachers of the subject, many of whom have been consistent users of the manual in its previous editions. From the outset it has been our aim to provide some experiments that were essentially of descriptive value and others which would acquaint the student with the quantitative procedures commonly employed in the analysis of urine, blood, and other body fluids.

The subject affords unusual opportunities for a large variety of other experiments which either utilize some special technic, or which teach some fundamental principle in biochemistry. To present even a small proportion of the very large number of such special experiments would be impossible in a small manual of this type, and it is therefore deemed best to leave the choice of such experiments to the individual teacher who is in a position to be guided in his assignments by the equipment in his laboratory, as well as by the aptitudes and previous training of his students.

Most of the changes in the present revision have been made in an attempt to clarify directions and to render them more workable for the elementary student. However, specific instructions for calculating the results of quantitative analyses have been avoided as much as possible. While calculation from a simple arithmetical formula may save time, such a procedure has a serious disadvantage in that it leaves little to the imagination of the student. Quite often the purpose of an experiment and the principles involved are not fully realized by a student until he is obliged to reason through a series of calculations.

The authors appreciate the warm reception accorded the previous editions and will continue to welcome suggestions for future revisions. Acknowledgment is due especially to Dr. B. M. Hendrix, Professor of Biological Chemistry at the University of Texas, to Miss Virginia B. Duff for assistance in correcting the galley proof, and to Miss Elisabeth D. Runge, Librarian, for her assistance in checking the references.

M. BODANSKY
MARION FAY

May 30, 1938

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LABORATORY MANUAL OF PHYSIOLOGICAL CHEMISTRY

CHAPTER I

INTRODUCTION: THE APPLICATION OF CHEMICAL ANALYSIS TO PHYSIOLOGICAL CHEMISTRY

This manual contains descriptions of both qualitative and quantitative experiments. The former have a place in descriptive as well as in applied biochemistry. Thus the tests for the detection of sugar, protein, and bile pigments in urine, and of organic acids in gastric juice, may be cited as examples of qualitative procedures that are of clinical value. In the performance of tests of this type, the student who has had even elementary training in chemistry should have little, if any, difficulty. However, in the carrying out of lengthier, more exacting quantitative analyses, closer attention to details will be found essential.

The subject of quantitative analysis is of fundamental importance in all branches of chemistry. It is recommended that the student of physiological chemistry who has had no previous training along these lines should spare no pains in becoming familiar with the principles of quantitative analysis, and with the methods of calculation employed in chemistry. The importance of precision in measuring, weighing, and recording results cannot be emphasized too strongly. Moreover, the student should always seek to understand the chemical basis of the particular procedure in which he may be engaged.

The purpose of quantitative analysis is to determine the quantity of the constituents present in a given compound or mixture. There are several general methods of quantitative analysis. These will be briefly considered in the following paragraphs.

Gravimetric Analysis. Gravimetric analysis depends ordinarily upon the separation of the constituent to be determined in a form in which it can be weighed. For example, in the determination of sulfate in urine (page 176) this is converted into barium sulfate, which

is insoluble and may be filtered off, dried, and weighed. From the weight of the barium sulfate, the amount of sulfur that was present in the urine as sulfate may be calculated.

The Chemical Balance. The student should become familiar with the construction and use of the chemical balance. Consult instructor. For additional information refer to a standard textbook on quantitative analysis.

Volumetric Analysis. In volumetric analysis a reaction is allowed to proceed to completion between the constituent under analysis and some suitable reagent of accurately known concentration. As chemical compounds react in definite or stoichiometric proportions, the amount of substance under analysis may be calculated if the volume of the known or standard reagent is measured. For example, in the determination of phosphate in urine (page 172), a given volume of urine is titrated with a solution of uranium acetate of known concentration until all the phosphate is precipitated as uranium phosphate. Knowing the amount of phosphate that is equivalent to (i.e., precipitated by) 1 cc. of the uranium acetate solution, and measuring the number of cubic centimeters of this solution required to react with the phosphate contained in the volume of urine taken for analysis, one may calculate the amount of phosphate present in that volume and subsequently in the total specimen.

Volumetric Apparatus. Since precision is required in quantitative procedures, it is important that all apparatus intended for accurate measurements (volumetric flasks, burettes, pipettes, etc.), be accurately calibrated before using. Accurately calibrated apparatus, certified by the United States Bureau of Standards, may be purchased on the market, or apparatus may be sent to the Bureau for calibration. However, the calibration of volumetric apparatus, though requiring care and precision, is not difficult and may be carried out by the student himself.[1]

Colorimetric Methods of Analysis. Many substances of biochemical interest occur in amounts too small to be conveniently or accurately determined by the usual gravimetric or volumetric methods. The determination of creatinine in the urine is a case in point (page 162).

[1] Consult Reprint 92, Bureau of Standards. N. S. Osborne and B. H. Veazey, "The Testing of Glass Volumetric Apparatus," Washington, page 565 (1908). See also Treadwell and Hall, "Analytical Chemistry," Vol. II, pages 460–472, John Wiley & Sons, Inc., New York (eighth edition); J. P. Peters and D. D. Van Slyke, "Quantitative Clinical Chemistry," Vol. II, Methods, Williams & Wilkins, Baltimore, 1932.

In this determination, a convenient volume of urine is treated with a solution of picric acid and with alkali (or an alkaline picrate solution). The deep-orange color which develops within a few minutes depends upon the presence of creatinine, the intensity of the color being determined by the amount of this constituent contained in the urine. This color may be compared with that obtained when a solution containing a known amount of creatinine is treated in a similar manner. The colorimeter is an instrument by means of which quantitative comparisons of the intensity of the color may be made. From the reading obtained with this instrument, the amount of creatinine present in the urine may be calculated.

The Colorimeter. The colorimeter is an instrument designed primarily for the comparison of colors. It may be used in determining the amounts of certain substances that form colored compounds, for the colorimeter is based on the principle, that when light is transmitted through a colored medium, the amount of light absorbed is directly proportional to the concentration of the colored substance.

In the Duboscq [2] type of colorimeter, light from some even source of illumination is passed through prism systems on the two sides of the instrument. The substances that are to be tested are interposed in these two light-paths. Some of the light is absorbed in passing through the liquids, the amount of absorption depending on the depth of the column of solution. The two *beams* of light are now brought to a common axis by means of the rhombohedral prisms. Light from one cup illuminates one half of a circular field, and the light from the other cup illuminates the other half. The observing microscope is focused on the line of separation of the two halves. It is now possible to alter the depths of the two columns of liquid until the two halves of the field are identical in intensity. *The concentrations of the two solutions are*

[2] This type of colorimeter was devised by Jules Duboscq in 1854, and in its improved form is manufactured at present in France by the firm of Pellin, his successors. Modifications of the Duboscq colorimeter are manufactured in this country by a number of concerns, including Spencer, Klett, Leitz, and Bausch & Lomb. The description given here is based on a descriptive circular on colorimeters, issued by Bausch & Lomb, Rochester, N. Y.

J. H. Yoe's "Photometric Chemical Analysis," Vol. I; John Wiley & Sons, Inc., New York (1928), is concerned with the subject of colorimetry and includes an excellent discussion of the principles of various types of colorimeters.

Several types of photoelectric colorimeters have been devised. These differ somewhat in principle. Certain instruments, using the wavelengths of the characteristic absorption bands, measure the concentration of a substance in solution from the amount of light which the solution absorbs.

inversely proportional to the depths, which are read on the scales at the sides of the instrument. (In different models, the scales are differently placed.)

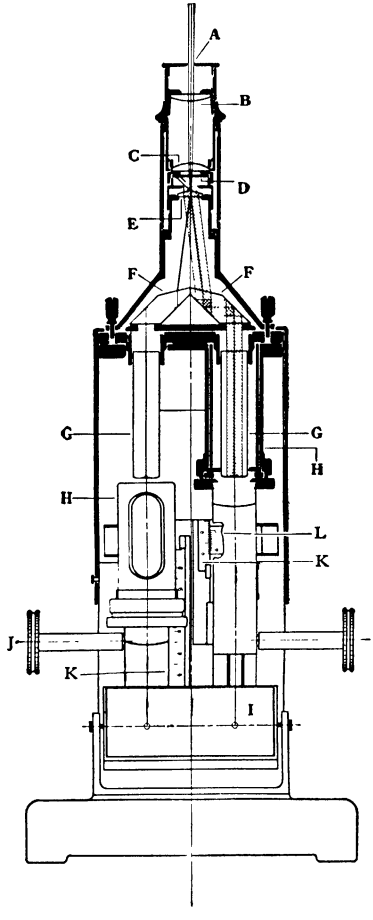


FIG. 1.—Diagram of Bausch & Lomb Duboseq Colorimeter; *A*, eyepiece diaphragm; *B*, eye lens; *C*, collective; *D*, cover glass; *E*, bi-prism; *F*, rhomboid prism; *G*, plungers; *H*, cups; *I*, mirror; *J*, pinion buttons; *K*, scales; *L*, verniers.

The Bausch & Lomb colorimeters are built on the same principle. A convenient form, known as the biological colorimeter, is illustrated in Fig. 1.

ACIDIMETRY AND ALKALIMETRY

Acidimetry and alkalimetry include the titration[3] of acids by means of bases and the titration of bases by means of acids. The point at which the reaction between an acid and a base is complete (stoichiometric point, or point of neutralization) may be determined by means of a suitable indicator.

Standard Acids and Bases. The strength of an unknown acid solution may be determined by titration with a basic solution of known strength; and, *vice versa*, the strength of an unknown basic solution may be ascertained by titration with a known acid solution. This general principle of titrating unknown solutions with known, or standard, solutions may be employed in reactions other than those involving the neutralization of an acid by a base (e.g., oxidation-reduction reactions).

Standard solutions may be prepared so that 1 liter of solution contains the molecular weight, or some fraction or multiple of the molecular weight, in grams of the solute. Thus, a molar solution of hydrochloric acid contains 36.47 g. of the acid per liter. A tenth-molar solution (0.1 *M*) of this acid contains 3.647 g. per liter. A molar solution of sodium hydroxide contains 40 g. per liter; and one of sulfuric acid, 98.09 g. per liter.

Standard solutions may also be prepared so that 1 liter will contain 1.008 g. of reactive or replaceable hydrogen, or its equivalent. Thus, in a solution containing 36.47 g. of hydrochloric acid, there is 1.008 g. of replaceable hydrogen. A solution such as this is said to be "normal" in strength.[4] Hence, a 0.2 *N* solution of hydrochloric acid may be prepared by dissolving 7.294 g. of hydrochloric acid in water and diluting to 1 liter. As regards solutions of hydrochloric acid, their normality is equivalent to their molarity. This is true also in the case of sodium hydroxide, since an atom of hydrogen is replaceable by an atom of sodium. On the other hand a molecule of sulfuric acid has two replaceable hydrogen atoms. Hence, a normal solution of this acid contains, in grams per liter, one-half the molecular weight, or 49.045 g. of sulfuric acid. Acetic acid, $CH_3 \cdot COOH$, contains only one

[3] In volumetric analysis, the strength of an unknown solution is determined by measuring it in terms of a solution of known concentration. The process is called titration.

[4] The term "normal" should not be confused with "normal saline," more properly designated as physiological saline. This is a solution of approximately 0.9 per cent sodium chloride and is isotonic with the blood.

replaceable hydrogen atom in the molecule, and hence requires one molecular weight, in grams, for each liter of normal solution.

Factors. It is usually convenient that solutions employed in volumetric analysis be made up to exact molarity or normality. However, this is not always obligatory, for precision is attainable with solutions that are actually of approximate molarity or normality, provided their exact strength is known. Thus, a solution may be 0.1025 normal. One cubic centimeter of this solution is equivalent to 1.025 cc. of a tenth-normal solution. Hence, all titrations made with this solution, if they are to be expressed in terms of a tenth-normal solution, should be multiplied by the *correction factor* 1.025.

Frequently, reagents are prepared in such concentration that 1 cc. is equivalent to a definite amount of the substance to be determined. Thus, the uranium acetate solution used in the titration of phosphate in urine (page 172) is made up so that 1 cc. precipitates an amount of phosphate equivalent to 5 mg. of P_2O_5. The selection of arbitrary factor values such as this has for its main purpose the simplification of calculations.

Experiment 1. Preparation of 0.5 *N* Oxalic Acid (200 cc.[5]). Weigh accurately a watch crystal or small crucible. Record the weight in your notebook, checking the weights twice. Now weigh in this container the exact amount of oxalic acid ($H_2C_2O_4 \cdot 2H_2O$) necessary to make 200 cc. of 0.5 *N* oxalic acid. Record the weight of container plus acid in your notebook and by subtraction show the weight of the acid. Have ready a clean 200-cc. volumetric flask into which a funnel with a short, wide stem has been inserted. Transfer the acid without loss into the funnel, using a camel's-hair brush, or by washing the crystals from the watch glass with a fine stream of distilled water from a wash bottle. Wash down the sides of the funnel and flask with about 100 cc. of distilled water and shake vigorously but carefully until the acid is dissolved. Dilute exactly to the mark, making the final adjustment by adding water, drop by drop, from a pipette or wash bottle; stopper the flask and invert 30 to 40 times to insure thorough mixing.[6] The solution may be transferred to a clean, *dry* bottle, labeled, and preserved for use in Experiment 2.

[5] Some other volume may be prepared, such as 100, 250, or 500 cc. The directions may also be modified with respect to the strength of the solution. Thus, 0.2 *N* or 0.1 *N* oxalic acid are equally satisfactory for use in the standardization of 0.1 *N* sodium hydroxide (Experiment 2).

[6] The oxalic acid solution may be standardized against standard solutions of either sodium hydroxide or potassium permanganate. The titration with potas-

Experiment 2. Preparation and Standardization of 0.1 *N* Sodium Hydroxide. Measure 6.5–7.0 cc. of a saturated solution of sodium hydroxide (approximately 60 per cent) into a liter volumetric flask.[7] Dilute to the mark with distilled water and mix thoroughly. This solution is stronger than 0.1 *N*. Determine the exact concentration as follows: Measure with an accurately calibrated pipette, into a clean Erlenmeyer flask or beaker, exactly 5 cc. of the 0.5 *N* oxalic acid prepared in Experiment 1.[8] Add 25 cc. of distilled water and 2 or 3 drops of phenolphthalein indicator. (Why is phenolphthalein used in this titration, and not methyl orange? See Experiment 4.) With a clean dry pipette remove 50 cc. (or 100 cc.) of the sodium hydroxide to a clean, dry beaker. Record the volume removed, so that the remaining volume may be known. Fill, or partly fill, a clean and dry burette with the newly prepared sodium hydroxide (if wet, the burette must first be rinsed with successive small portions of the sodium hydroxide), record the reading of the burette, and titrate the oxalic acid until a faint pink color forms that pervades the solution and does *not* fade within 1 minute. Record the reading of the burette and from the volume of sodium hydroxide required to neutralize the 5 cc. of 0.5 *N*

sium permanganate may be performed as follows: By means of an accurately calibrated pipette, measure exactly 5 cc. of the oxalic acid solution into a beaker or flask. Add 10 cc. of 1 : 4 sulfuric acid and dilute with hot water (70° C.) to a volume of about 200 cc. Run in the permanganate solution (0.1 *N*), with constant stirring, from a glass-stoppered burette. At first the solution may be colored red for several seconds; then it becomes colorless. After the reaction is once started, further additions of the permanganate are rapidly decolorized until an excess of permanganate is present. The permanent pink color is imparted to the solution by the permanganate as soon as all the oxalic acid is oxidized; this is taken as the end point. (For further details, see Treadwell and Hall.) The oxalic acid-permanganate titration is an example of an oxidation-reduction reaction.

Exactly 25 cc. (duplicates checking within ±0.1 cc.) of 0.1 *N* potassium permanganate solution should be used in titrating 5 cc. of 0.5 *N* oxalic acid.

[7] Sodium hydroxide sticks are rarely free from encrusted sodium carbonate. The latter is relatively insoluble in very concentrated solutions of sodium hydroxide. A saturated solution of sodium hydroxide should be prepared with freshly boiled water and allowed to stand for some days to permit the settling out of any carbonate that may have formed. However, if sodium hydroxide sticks that are free from any encrusted carbonate are available, these may be used in the preparation of standard solutions. In this case, dissolve approximately 4.5 g. of stick sodium hydroxide in water and dilute to 1 liter in a volumetric flask.

[8] If 0.1 *N* oxalic acid has been prepared (see footnote 5) use 25 cc. of it (measured with an accurately calibrated pipette) for the titration by the sodium hydroxide solution.

oxalic acid, calculate the strength of the sodium hydroxide solution. Calculate the amount of distilled water to add to the known volume of solution to yield 0.1 *N* sodium hydroxide. Mix thoroughly and titrate against duplicate portions of the oxalic acid as before; the results should not differ by more than 0.2 cc. From the average, calculate the normality, and if the alkali is now between 0.098 and 0.102 *N*, transfer to a clean dry bottle, label, and keep for future use. If the normality of the solution is not within this range, make stronger or weaker as necessary and restandardize.

Experiment 3a. Preparation and Standardization of 0.1 *N* Hydrochloric Acid. Concentrated hydrochloric acid is usually about 36 per cent, or approximately 10 *N*. To prepare 1 liter of 0.1 *N* hydrochloric acid, measure 10–11 cc. of concentrated hydrochloric acid (use small graduate for measuring the concentrated acid) into a 1-liter volumetric flask and dilute to the mark with distilled water. Mix thoroughly by inverting and shaking the flask. Determine the concentration of the acid solution by titrating 25 cc. with the 0.1 *N* sodium hydroxide prepared in Experiment 2, keeping accurate count of the acid removed from the flask. Use phenolphthalein as the indicator. Adjust the concentration of the acid solution until 25 cc. is exactly equivalent to 25 cc. of the alkali solution.[9] To obtain checks titrate at least in duplicate or triplicate. Repeat the titration by using (*a*) Congo red, and (*b*) methyl orange as the indicators.

Transfer the standard acid solution to a bottle, label, and keep for future use.

Experiment 3b. Preparation of Standard Hydrochloric Acid (Method of Hulett and Bonner[10]). Using an hydrometer, dilute concentrated hydrochloric acid (commercial acid will give satisfactory results) to a specific gravity of 1.1. Distill off three-fourths of the liquid and discard this portion of the distillate into the waste bottle provided for this purpose. Taking care not to carry it to dryness, continue the distillation, collecting the distillate in a clean, dry flask, or bottle (a glass-stoppered container is to be preferred).

Analysis has shown that the last fourth of the distillate is of very constant composition, the percentage of hydrochloric acid being determined by the barometric pressure.

[9] If the acid solution is nearly equivalent to the 0.1 *N* alkali, calculate the factor for the acid in terms of 0.1 *N*, label the solution accordingly, and employ the factor in subsequent calculations.

[10] *J. Am. Chem. Soc.*, **31**, 390 (1909); see also C. W. Foulk and M. Hollingsworth, *ibid.*, **45**, 1220 (1923).

Barometric Pressure, mm.	Per cent HCl	Grams of Constant-Boiling Distillate, containing 1 mole of HCl
770	20.218	180.390
760	20.242	180.170
750	20.266	179.960
740	20.290	179.745

As a change in barometric pressure of 10 mm. causes a change of only 1 part in 4,000, the barometric pressure need be considered for only very exact work.

Calculate the grams of the constant-boiling acid necessary to prepare 1 liter of 0.1 *N* HCl. Weigh out this amount of acid, first obtaining directions from the instructor for the proper handling of this material in the balance.[11] Transfer to a liter volumetric flask, dilute to the mark with distilled water, mix thoroughly and transfer to a clean, dry, glass-stoppered bottle for storage.

THE USE OF INDICATORS

The use of indicators in acid-base titrations depends upon the sharp color changes which they undergo with a small change in the concentration of hydrogen ions. Thus, phenolphthalein is colorless at *p*H 7.8 and faintly pink at *p*H 8.0 whereas methyl orange is pink at *p*H 4.2 and faintly yellow at *p*H 4.4.[12]

The proper choice of an indicator in the titration of acids and bases is of much importance, as is brought out in the experiment outlined below.

[11] Weights are more accurate than volume measurements, since the latter vary with temperature. If a balance is not available, calculate the volume of the constant-boiling acid required to prepare 1 liter of 0.1 *N* HCl. At 25° C., 164.42 cc. contains 1 mole of hydrochloric acid (1 cc. contains 6.08 millimoles). Measure the required volume carefully and dilute with distilled water to the mark in a liter volumetric flask.

In laboratories where it is impractical to perform Experiment 1, owing to a lack of sufficient analytical balances, the standard HCl may be prepared by this method and used in standardizing the sodium hydroxide solution in Experiment 2.

[12] The student is at this point urged to consult suitable references for adequate discussions of the theory of indicators, such as: W. M. Clark, "The Determination of Hydrogen Ions," Third Edition (1928), Williams & Wilkins Company, Baltimore; I. M. Kolthoff and N. H. Furman, "Indicators," John Wiley & Sons, Inc., New York (1926), Chapter III; I. M. Kolthoff and C. Rosenbloom, "Acid-Base Indicators," Macmillan Company, New York (1937), Chapter VII.

Experiment 4. Use of Indicators. The following solutions will be needed for this experiment:

0.2 *N* hydrochloric acid.
0.2 *N* acetic acid.
0.2 *N* sodium hydroxide.
0.2 *N* ammonium hydroxide.
0.2 *N* sodium carbonate.

4*a*. Fill a clean dry burette (or one rinsed well with the solution) with the sodium hydroxide. Pipette 10-cc. portions of the hydrochloric acid into each of two beakers, dilute with about 25 cc. of distilled water, add 2–4 drops of phenolphthalein, and titrate with the sodium hydroxide. Record the results. Repeat, using 3–4 drops of sodium alizarine sulfonate as the indicator. Record the results.

4*b*. Repeat 4*a*, using acetic in place of the hydrochloric acid. Record the results.

4*c*. Repeat 4*a* and 4*b*, using ammonium hydroxide in place of the sodium hydroxide. Record the results.

4*d*. Repeat 4*a* and 4*b* with sodium carbonate as the base. Record the results.

4*e*. Fill a burette with the hydrochloric acid solution. Pipette 10-cc. portions of sodium hydroxide into each of two beakers and add 1–2 drops of methyl orange, and about 25 cc. of distilled water. Titrate with the acid. Repeat this titration on ammonium hydroxide and on sodium carbonate, recording all results.

4*f*. Repeat 4*e*, using acetic acid in the burette. Record the results.

Why is the acid titrated into the base when methyl orange is used? Tabulate and explain all the results, formulating rules for your guidance in the choice of indicators.

Experiment 5. Buffer Action. Fill a burette with 0.1 *N* NaOH and another with 0.1 *N* HCl.

(*a*) Into each of two beakers place 10 cc. of 0.1 *N* NaCl. Add a drop of phenolphthalein to the contents of one beaker and determine the quantity of 0.1 *N* NaOH required to turn it alkaline.

Using methyl orange as the indicator titrate the contents of the other beaker with 0.1 *N* HCl. Record the quantity required to turn the solution acid.

Does NaCl exert any buffer action?

(*b*) Into each of two beakers place 10 cc. of 0.1 *M* sodium acetate. Perform the titrations with phenolphthalein and methyl orange as

indicators, as in (*a*). Record and explain the results. Write equations for the reactions involved.

(*c*) Into each of two beakers place 10 cc. of 0.1 *M* sodium acetate dissolved in 0.1 *M* acetic acid. Repeat the titrations as before. Record and explain the results.

(*d*) Repeat, using 10-cc. quantities of 0.1 *M* $NaHCO_3$.

(*e*) Repeat, using 10-cc. portions of 0.1 *M* Na_2HPO_4.

(*f*) Repeat, using 10-cc. portions of 0.1 *M* NaH_2PO_4.

Record and explain the results in (*d*), (*e*), and (*f*), and write the equations for the reactions involved.

Experiment 6. Colorimetric Determination of *p*H. On page 280 is given a list of indicators with their respective ranges of *p*H through which they exhibit definite gradations in color, with respect to both tint and intensity. Certain indicators which show the most delicate gradations in color are used in the colorimetric estimation of the *p*H of solutions.

Preliminary Experiments. (*a*) Add 2–3 drops of phenolsulfonphthalein (phenol red) to a very dilute acid solution (hydrochloric or sulfuric). Note the color. Add 2–3 drops of the indicator to a very dilute solution of sodium hydroxide. Note the color.

(*b*) Repeat, adding the phenol red indicator to buffer solutions of the following *p*H : 6.0, 7.0, 8.6. Note the colors.

(*c*) Repeat, using the following indicators and buffer solutions:

Thymol blue; buffer solutions of *p*H 1.0, 2.0, 2.8, 8.0, 9.6.
Brom phenol blue; solutions of *p*H 3.0, 4.6.
Brom thymol blue; solutions of *p*H 6.0, 7.6.

(*d*) In this experiment a series of solutions will be required having *p*H values ranging from *p*H 6.8 to *p*H 8.0 in intervals of 0.2 of a *p*H. For the preparation of these solutions, see pages 278 and 279.

Clean and dry seven test tubes; label them in the following order: 6.8, 7.0, 7.2 . . . to 8.0. Arrange them in a test-tube rack in ascending order and place in each tube 10 cc. of a solution, the *p*H of which corresponds to the label. Now add to the solution in each tube 5 drops of the phenolsulfonphthalein indicator. Mix. Note gradations in color in the series of tubes.

NOTE: The instructor will provide unknown solutions having *p*H values within the range of *p*H 6.8–8.0. If suitable standards and the necessary indicators are provided for *p*H values outside this range, additional practice may be given the student by providing several unknown solutions within a wider range of *p*H.

Experiment 7. Buffer Ratio and *p*H. From the burette (or pipette) run into a beaker 5 cc. of 0.05 *M* solution of Na_2HPO_4. In the same way measure into this 5 cc. of 0.05 *M* NaH_2PO_4. Titrate the mixture with 0.1 *N* NaOH, using phenolphthalein as the indicator. What does the titration value represent? From the total phosphate expressed as cubic centimeters of 0.1 *M* solution, subtract the titration value. What does this figure represent? (The determination should be done in duplicate.)

Calculate the *p*H of the solution, using for pK_1 the value of 6.8.[13]

In the same manner mix 5 cc. each of the 0.05 *M* phosphate solutions. Determine the *p*H colorimetrically as in Experiment 6. Record the result. How does it compare with the value calculated from the titration figure?

Obtain a phosphate solution of unknown *p*H from the instructor. Determine the *p*H colorimetrically and, after titration, by calculation, assuming that the total phosphate concentration is the same as in the first part of the experiment. Hand in a report, showing all calculations.

[13] The *p*H may be calculated from the formula:

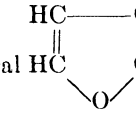
$$pH = pK_1 + \log \frac{[Na_2HPO_4]}{[NaH_2PO_4]}$$

See also M. Bodansky, "Introduction to Physiological Chemistry," 4th edition, 1938, pages 15–20 and 259–262.

CHAPTER II

CARBOHYDRATES

Experiment 1. The Molisch Reaction. To 2 cc. of 0.1 *M* solution of glucose, in a test tube, add 2 drops of Molisch's reagent [1] and mix. Now add about 3 cc. of concentrated sulfuric acid, pouring the acid carefully down the side of the tube so as to form a layer at the bottom of the tube. What appears at the junction of the two liquids? Repeat the test on 0.1 *M* solutions of sucrose, maltose, and arabinose, and on a 1 per cent starch solution.[2]

Molisch's reaction is a general test for carbohydrates. The concentrated acid acts on the sugar, yielding furfural  , hydroxy-methyl furfural, and other decomposition products. These form colored condensation compounds with the α-naphthol.

Is this test specific for carbohydrates alone?

Experiment 2. Reduction Tests. Alkaline solutions of copper, bismuth, silver, and other metallic salts are reduced by sugars having a free aldehyde group. This is the basis for Fehling's, Benedict's, Nylander's, and other reduction tests. Measure 2 cc. of a 1 per cent solution of copper sulfate and 2 cc. of 10 per cent sodium hydroxide into each of four test tubes. What is formed? Describe and explain. Write the equation explaining the reaction.

To tube 1, add drop by drop a solution of 0.1 *M* glucose, until the precipitate is dissolved. Explain. To tube 2, add in the same way a 30 per cent solution of sodium citrate and, to tube 3, a 30 per cent solution of sodium-potassium tartrate (Rochelle salts). What occurs? Why?

[1] A 5, 10, or 15 per cent solution of α-naphthol in 95 per cent alcohol may be used.

[2] Prepare the starch solution by heating about 80 cc. of distilled water to boiling, grinding to a paste 1 g. of starch in a mortar with about 10 cc. of cold water, and pouring the starch suspension into the boiling water with vigorous stirring. Cool and make up to 100 cc., mixing thoroughly.

Heat tube 4 to boiling. Describe what occurs. Now heat the other tubes to boiling. Explain the reaction in each tube. To tubes 3 and 4 add a few drops of the glucose solution. Explain the reactions involved and write the equations.

Experiment 3. Fehling's Test.[3] Equal amounts of the two solutions A and B (see footnote 3) are measured and mixed, and 5 cc. of the mixture taken for each test. The reagent should be heated before each test, to be sure that it is not reduced by heating alone. If no change occurs on heating, add to the warm reagent 8 drops of a glucose solution (0.1 *M* or some other suitable concentration), and boil. Describe the resulting phenomena and explain the part in the reaction taken by each ingredient of the reagent.

Experiment 4. Benedict's Test. This is a more delicate test than Fehling's. The reagent[4] has the practical advantage of keeping well in a single solution. It is not so easily reduced by urates or other constituents of the urine as is Fehling's reagent and is therefore more satisfactory in testing urine for sugar.

To 5 cc. of Benedict's solution, heated to boiling in a test tube, add 8 drops of the glucose solution (0.1 *M*). Boil for about 2 minutes. What occurs? Allow the tube to cool on standing and observe again. Repeat, using 0.1 *M* solutions of fructose, galactose, sucrose, maltose, lactose, and arabinose, and the 1 per cent starch solution. Do all carbohydrates reduce Benedict's reagent? Explain.[5]

Effect of Various Amounts of Sugar on the Test. Into each of eight test tubes, introduce 5 cc. of Benedict's reagent. Then carry out the test as outlined above upon solutions of glucose of the following concentrations: 0.05, 0.1, 0.2, 0.4, 0.6, 0.7, 0.8, 1.0, and 2 per cent. Observe carefully and note the differences in the appearance of the tubes. Allow the tubes to cool on standing and observe again. Could you tell the approximate amount of sugar present in a urine specimen from the appearance and amount of precipitate obtained in Benedict's qualitative test?

[3] Fehling's reagent consists of two solutions: Solution A is made by dissolving 69.38 g. of crystalline copper sulfate in distilled water and diluting to 1 liter; solution B is made by dissolving 250 g. of sodium hydroxide and 346 g. of sodium-potassium tartrate in water and diluting to 1 liter.

[4] Benedict's qualitative reagent is prepared by dissolving 173 g. of crystalline sodium citrate and 100 g. of anhydrous sodium carbonate in about 800 cc. of water. To the filtered solution is added 17.3 g. of copper sulfate dissolved in 100 cc. of water, and the whole is made up to 1 liter with distilled water.

[5] Benedict, S. R., *J. Biol. Chem.*, **5**, 485 (1908–09); *J. Am. Med. Assoc.*, **57**, 1193 (1911).

Experiment 5. Nylander's Test. Add 2 drops of Nylander's reagent[6] to 2 cc. of the 0.1 *M* glucose solution and heat in a boiling water bath for 5 minutes. Note the result. Repeat the test on other sugars.

Experiment 6. Barfoed's Test.[7] This test differs from Fehling's and Benedict's tests in that the reduction of the copper is brought about in an acid solution. To 5 cc. of Barfoed's reagent[8] in each of four test tubes add, respectively, 1 cc. of 0.1 *M* solutions of glucose, sucrose, maltose, and lactose. Place the tubes simultaneously in a boiling water bath and examine at intervals of 5 minutes, recording the results. Compare the time required for reduction to take place. Can Barfoed's test be used to differentiate between monosaccharides and disaccharides?

Experiment 7. Seliwanoff's (Resorcinol-Hydrochloric Acid) Reaction. To three 5-cc. portions of Seliwanoff's reagent[9] in three test

[6] Nylander's reagent is prepared by heating 2 g. of bismuth subnitrate and 4 g. of sodium-potassium tartrate in 100 cc. of 10 per cent potassium hydroxide. The solution is cooled and filtered.

Reaction: $Bi(OH)_2NO_3 + NaOH = Bi(OH)_3 + NaNO_3$.

$2Bi(OH)_3 +$ reducing sugar $= 2Bi + 3H_2O +$ oxidation products of sugar.

[7] The tests in Experiments 3, 4, 5, and 6 are only a few of those which have been proposed for the detection of reducing sugars. A convenient reagent to show the presence of sugar in urine has naturally been an important problem, and many tests, each with some advantage to recommend it, have been devised. Thus, Fehling's reagent may be made somewhat more sensitive by diluting. Benedict's solution, which is a modification of Fehling's reagent, was designed for the detection of relatively small amounts of sugar. Owing to its greater sensitivity Benedict's test has almost entirely replaced the older test of Fehling. Nylander's reagent will not yield a positive reaction with creatinine or uric acid. It does however reduce glycuronic acid.

[8] Barfoed's reagent is made by dissolving 13.3 g. of crystallized cupric acetate in 200 cc. of distilled water, filtering if necessary, and adding 5 cc. of 38 per cent acetic acid.

[9] Seliwanoff's reagent is made by dissolving 0.05 g. of resorcinol in 100 cc. of 12 per cent hydrochloric acid (1 part of concentrated hydrochloric acid diluted with 2 parts water).

For a modification of Seliwanoff's test and for other tests given by the keto-hexoses, consult C. A. Morrow and W. M. Sandstrom, "Biochemical Laboratory Methods," John Wiley & Sons, Inc. (1935), page 154.

The action of acid on fructose (as well as on other keto-hexoses) results in the formation of hydroxy-methyl-furfural, which in turn forms a condensation product with the resorcinol (1.3 dihydroxy-benzene). When allowed to stand, the condensation product separates out as a brownish-red precipitate which is soluble in alcohol, imparting to the solution an intensely red color. Aldo-hexoses, such as glucose, yield much smaller amounts of hydroxy-methyl-furfural; hence only a faint reaction is obtained even on prolonged heating.

tubes, add 1 cc. of fructose, glucose, and sucrose solutions, respectively. Place the tubes in boiling water and observe carefully for changes in color. Note the time necessary to bring about the changes in the tubes. Explain the results.

Experiment 8. The Action of Strong Bases on Carbohydrates. (*a*) Into five clean and properly labeled test tubes measure, respectively, 1 cc. of 1 per cent (or 0.05 *M*) solutions of the following carbohydrates: glucose, fructose, lactose, sucrose, starch. Next, add to each 1 cc. of 1 per cent sodium hydroxide solution and mix. Immerse all the tubes simultaneously in boiling water. Heat for 5 minutes, observing the odor and color from time to time.[10] Record the results.

(*b*) Now measure 1 cc. of Benedict's solution into each of the five tubes. Continue the heating for 3–5 minutes and note the results. What is the relation between the ease with which various carbohydrates are decomposed by alkali and their susceptibility to oxidation?

Experiment 9. The Action of Weakly Basic Solutions on Carbohydrates; Tautomeric Conversion of an Aldose into a Ketose and *vice versa*. Prepare four test tubes as follows, measuring the reagents carefully with suitably calibrated pipettes.

Tube 1a. 0.5 cc. of 0.1 *M* glucose and 0.5 cc. of saturated $Ba(OH)_2$.

Tube 1b. 0.5 cc. of 0.1 *M* glucose and 0.5 cc. of distilled water.

Tube 2a. 0.5 cc. of 0.01 *M* fructose and 0.5 cc. of saturated $Ba(OH)_2$.

Tube 2b. 0.5 cc. of 0.01 *M* fructose and 0.5 cc. of distilled water.

To each tube add toluene to form a layer approximately 3 mm. deep. The toluene is added to retard oxidation and bacterial decomposition. Stopper and set aside overnight.

After the tubes have stood overnight add to each tube 5 cc. of freshly prepared Seliwanoff's reagent (page 24) and immerse all the tubes in boiling water at the same time. Note carefully the rate of development of the color in each tube and its intensity, the latter being proportional to the amount of ketose present. Compare *tube 1a* with *1b*. Do your observations indicate that the presence of alkali has

[10] Sugars which contain a free aldehyde or ketone group form ionizable, unstable salts with bases. (See Bodansky's "Introduction to Physiological Chemistry," Fourth Edition, pages 44, 45.) Enols are first formed. In turn these decompose into a large variety of simpler compounds, many of which exhibit strong reducing properties. Certain of the fragments of the sugar molecule form a condensation product, brown in color, to which the term "humus" has been applied.

caused the partial transformation of glucose into fructose? Compare *2a* with *2b*. From the difference in the depth of the color in these tubes would you judge that some of the fructose may have disappeared? What is the mechanism of the reaction?

Experiment 10. Phloroglucinol Test (Tollens). To 2-cc. portions of 0.1 *M* solutions of arabinose, glucose, and galactose, add equal volumes of the phloroglucinol reagent,[11] and heat in a boiling water bath. Observe.

While the test may be used in differentiating between pentoses and hexoses, it is not altogether specific for the former. Galactose and glycuronic acid give the red color produced by the pentoses. The pentoses may be distinguished from galactose by extracting the colored compound in amyl alcohol and examining the absorption spectrum. The pentoses, treated in this way, yield an absorption band between the D and E lines. This band is not obtained with galactose. Since glycuronic acid yields the same band as the pentoses, other reactions must be resorted to in order to distinguish this acid from the five-carbon sugars.

Experiment 11. Orcinol Test (Bial). To 5 cc. of a solution of a pentose sugar, such as arabinose or xylose, add 3 cc. of a solution of orcinol[12] and heat in a boiling water bath. At the same time treat 5 cc. of a solution of glucose similarly. Observe the color changes in the two tubes and explain.

Experiment 12. Formation of Osazones. Weigh out 2 g. of phenylhydrazine-hydrochloride and 3 g. of sodium acetate and mix thoroughly in a mortar. Add about 0.5 g. of the mixture to each of eight test tubes, one of which contains 5 cc. of 1 per cent starch solution, while each of the others contains 5 cc. of a 0.1 *M* solution of one of the following: glucose, fructose, galactose, sucrose, lactose, maltose, and arabinose. Label carefully and place in a boiling water bath for 5 minutes. Remove the tubes, filter each solution while hot through a small, clean, dry filter into a clean test tube. Label the tubes con-

[11] *Phloroglucinol Reagent.* Mix equal volumes of distilled water and concentrated hydrochloric acid and saturate the solution with powdered phloroglucinol (1,3,5 trihydroxy-benzene).

[12] Dissolve 0.5 g. of orcinol (3,5 dihydroxy-toluene) in 250 cc. of 30 per cent hydrochloric acid to which 10–15 drops of 10 per cent ferric chloride have been added.

Bial's test is a modification of Tollens' original orcinol test, the addition of ferric chloride increasing somewhat the sensitivity of the reaction. This test depends, as does Tollens' phloroglucinol test, on the formation of a condensation product with furfural.

taining the filtrates and place them in the water bath, heating for half an hour. Remove the tubes, noting any precipitates that may form while the tubes are still hot. Allow to cool slowly. Examine the precipitate under the microscope and make drawings of the characteristic crystals. Do all carbohydrates form osazones? What sugars form the same osazone? Write the equations of the reactions involved in the formation of osazones.

Experiment 13. Reducing Power of the Disaccharides. From the results of the reduction tests performed in Experiment 4, what do you conclude about the reducing power of the disaccharides? Verify and explain these results.

Experiment 14. Hydrolysis of the Disaccharides. To 10-cc. portions of 5 per cent solutions of maltose, lactose, and sucrose, add 1 cc. of 20 per cent hydrochloric acid. Place the tubes in a boiling water bath for half an hour. At the end of this time, neutralize the solutions carefully. Test each, by Benedict's method, for the presence of reducing sugars. Using the remainder of each solution, prepare osazones (Experiment 12). Examine them microscopically. Explain the results.

NOTE: If practicable, determine the rotatory power of a solution of sucrose before and after acid hydrolysis. See Experiment 18. What is meant by inversion?

Experiment 15. Fermentation Test. Prepare a suspension of yeast by triturating a piece of yeast cake in about 10 cc. of water in a mortar. Add 1 cc. of this suspension to enough 1 per cent glucose solution to fill the long arm of the fermentation tube completely, and the short arm about halfway.[13] Place in the tube, making sure that there are no air bubbles at the top of the long arm. Repeat, using solutions of fructose, galactose, lactose, sucrose, maltose, and starch. Set the fermentation tubes aside in a warm place and observe after a few hours.

If gas forms in any of the tubes, test it by introducing 2–3 cc. of 10 per cent sodium hydroxide into the tube, filling the short arm with water, covering the opening with the thumb, and inverting the tube several times. Try to remove thumb. Explain. What is the gas, and how does it react with sodium hydroxide? Test further for the products of fermentation by warming some of the filtered alkaline solution with a dilute solution of iodine in potassium iodide. Note the odor of

[13] An Einhorn saccharimeter is recommended for this purpose. If not available a small test tube completely filled with the yeast-carbohydrate mixture may be inverted, without loss of liquid, in a larger test tube containing the same mixture. Fermentation is demonstrated by the accumulation of gas in the closed end of the smaller tube.

iodoform. For what substance is this a test? Is the test a specific one? Write the equations for the reactions involved.

Experiment 16. Mucic Acid Test. This test differentiates galactose and lactose from all other reducing sugars. To 10 cc. of a 10 per cent solution of lactose (or galactose) add 5–10 cc. of distilled water and 15 cc. of concentrated nitric acid. Evaporate the mixture to a small volume on a water bath in the hood. Cool, add 5 cc. of distilled water, and stir vigorously to start crystallization. Allow to stand overnight or longer. Examine the crystals of mucic acid under the microscope. Does sucrose or glucose when treated with nitric acid yield a product that is insoluble in water? Write the equation for the formation of mucic acid from galactose and lactose.

The mucic acid may be collected on a small filter paper, washed with a little distilled water and dried at 100° C. The melting-point of this material should be 212–215° C.

Experiment 17. Cole's Test for Lactose. This test is especially useful for distinguishing between lactose and glucose when either of these, or both, are present in urine. Advantage is taken of the greater adsorption of disaccharides by certain preparations of blood charcoal.

Treat 25 cc. of the solution to be tested (urine, a solution of glucose and lactose, lactose alone, or glucose alone) with 1 g. of Merck's medicinal blood charcoal. Shake, heat to boiling for a few seconds, cool thoroughly, then shake at intervals for 10 minutes. Filter (using a filter pump if available), retaining both the filtrate and the charcoal. Test the filtrate for glucose.

When the charcoal has completely drained, transfer it to a porcelain dish containing 10 cc. of water and 1 cc. of glacial acetic acid. Heat to boiling for 10 seconds and filter the hot solution through a small filter paper into a test tube containing a 1 : 2 mixture of phenylhydrazine-hydrochloride and sodium acetate, in sufficient amount to fill the rounded end of the tube. Mix by shaking and filter off the oily residue that may be present, collecting the filtrate in a test tube. Place the test tube in boiling water for 45 minutes, then allow at least an hour for cooling. Examine for the presence of the so-called "hedgehog" crystals of lactosazone.

Experiment 18. Optical Activity.[14] Optically active substances

[14] For a description of the polariscope and its uses, consult Browne's "A Handbook of Sugar Analysis," John Wiley & Sons, Inc., New York (1912), or Leach's "Food Inspection and Analysis," revised and enlarged by A. L. Winton, John Wiley & Sons, Inc. (1920). See also "Polarimetry, Bureau of Standards," Circ. 44, Second Edition (1918). Many of the standard textbooks of physics likewise contain good descriptions of polariscopes.

have the property of rotating the plane of polarized light. The specific rotation (or specific rotatory power) of such substances may be defined as the rotation in angular degrees produced under stated conditions by a length of 1 decimeter of a solution containing 1 g. of the substance in 1 cc. of the solution. When, as is usually the case, a concentration other than that demanded by the definition is employed, the specific rotation may be calculated by substituting the proper values in the following formula:

$$[\alpha]_D^{20} = \frac{a \times 100}{lc}$$

in which α = the specific rotation;
a = the observed rotation in angular degrees;
l = the length of the column of solution in decimeters;
c = the number of grams of the substance in 100 cc. of solution.[15]

The source of illumination must be taken into account. Sodium light (D line) is ordinarily employed, this being stated in the formula. The determination is usually made at 20° C., and this is likewise stated. The errors introduced by small differences in temperature are not very great, so that in ordinary work such differences are often disregarded.

Inasmuch as the specific rotations of the familiar sugars are known, the concentration of a solution of a given sugar may be calculated by applying the formula.[16]

$$c = \frac{a \times 100}{[\alpha] \times l}$$

Reading of the Zero Point. The zero point of the polariscope must first be determined. Set the scale near the zero mark and adjust the eyepiece until the field is clear and in focus. With distilled water in the polariscope tube, take a number of readings (about six), rotating the thumb screw until the field is uniformly illuminated. The read-

[15] The formula

$$[\alpha]_D^{20} = \frac{a \times 100}{lpd}$$

may be used for aqueous solutions, in which p is the number of grams of the substance in 100 g. of the solution, and d, the density.

[16] In the case of dextrose, the formula is:

$$\text{Per cent dextrose} = \frac{\text{Observed rotation} \times 100}{52.5 \times \text{length of tube in dcm.}}$$

ings should be made first from one side of the zero point and then from the other. An average of closely agreeing readings is taken. This figure represents the zero point of the instrument and should be used as a correction in the following determination.

Polarimetric Determination of Glucose. The polariscope tube should be clean and dry. If not dry, it may be rinsed several times with the solution to be used in the determination. Fill the tube with the sugar solution and tap gently with the finger so that no air bubbles remain trapped in the tube. Then slip the cover glass over the top and screw on the cap. Place the tube in the polariscope. As in the determination of the zero point, take a series of readings, noting whether the rotation is to the right or left (*plus* or *minus*). Take the average of the closely agreeing readings and correct for the zero point. Calculate the concentration of dextrose in the solution.

NOTES: Make polarimetric measurements of solutions of fructose and sucrose, of known as well as unknown concentration. In preparing solutions of glucose and fructose for polarimetry, sufficient time should be allowed for equilibrium to be reached. This may be hastened by the addition of one or more drops of dilute ammonium hydroxide.

Experiment 19. Mutarotation. Prepare a 4 per cent solution of *d*-glucose, without heating, and immediately take a reading in the polariscope. Make two or three additional readings at 20-minute intervals. Allow the solution to stand overnight so that equilibrium will be reached, and take another reading. Equilibrium may be attained much more rapidly by adding 1–2 drops of dilute ammonium hydroxide to the solution in the polariscope tube. From the results obtained, calculate both the initial and final specific rotations.

POLYSACCHARIDES

STARCH

Prepare 100 cc. of 1 per cent starch solution as directed on page 20.

Experiment 20. Iodine Test. To 5 cc. of the starch solution add 1 or 2 drops of dilute iodine solution.[17] Observe the color. Heat the solution and note the change. Allow the solution to cool, and observe.

Experiment 21. Reduction. Does starch reduce Benedict's reagent?

[17] The iodine solution may be prepared by dissolving 0.3 g. of iodine and 1.5 g. of potassium iodide in 100 cc. of distilled water. If desired, this solution may be diluted with distilled water.

Experiment 22. Precipitation with Alcohol. To 5 cc. of the starch solution add an equal volume of 95 per cent alcohol and shake. Then, after it has been allowed to stand for some time, filter off the precipitate and test the filtrate with iodine. (If precipitation of the starch does not take place in a short time, add a drop of saturated sodium chloride solution.)

Experiment 23. Precipitation wtih Ammonium Sulfate. To 10 cc. of the starch solution add an equal volume of saturated ammonium sulfate solution, and mix thoroughly. Explain the result.

Experiment 24. Products of Starch Hydrolysis. To 50 cc. of the starch solution in a flask, add 1 cc. of concentrated hydrochloric acid. Heat to boiling. At 2-minute intervals remove a drop of this solution with a stirring rod and test with iodine on a test tablet. Note the time required to bring about the first color change, and continue the testing, noting all changes until the reaction becomes colorless (that is, to the point where the iodine color remains unchanged upon adding a drop of the solution). What are the products of starch hydrolysis? How do these products react with iodine? Neutralize a portion of the solution, after hydrolysis, with sodium carbonate, and test for the presence of reducing sugars. Using the rest of the solution, prepare and identify the osazone.

Experiment 25. Dialysis. Prepare two small celloidin sacs,[18] or use small cellophane bags. Transfer to one 5 cc. of 1 per cent glucose and to the other 5 cc. of the starch solution. Carefully rinse the outside and suspend each in a separate small beaker containing distilled water. After 15 minutes and again after an hour test the dialysates for the presence of glucose in one, starch in the other. Explain the results.

Dextrin

Prepare about 50 cc. of a 2 per cent solution of dextrin, heating and filtering, if necessary, to obtain a clear solution.

Experiment 26. Reduction. Test the dextrin solution with Benedict's reagent. Commercial dextrin may contain appreciable amounts of reducing sugars.

[18] *Preparation of a Celloidin Dialyzer.* Fill a large test tube, that has been thoroughly cleaned and dried, with a solution of celloidin. After a few minutes, pour the celloidin back into the original container. Clamp the tube in an inverted position and allow it to drain. When the odor of ether has disappeared, fill the tube with lukewarm water and carefully loosen and withdraw the membrane. Larger celloidin sacs may be made by using larger test tubes, Erlenmeyer or other flasks.

Experiment 27. Iodine Test. Test the solution with iodine. If starch is present in the sample of dextrin, the blue color due to the starch may mask the characteristic purple-red or red-brown color of the dextrin-iodine reaction. Try to remove the starch by adding 10 cc. of saturated ammonium sulfate solution to 10 cc. of the dextrin solution, allowing the mixture to stand for 15–20 minutes and filtering off any precipitate that forms. The filtrate may now give the characteristic color reaction for dextrin with iodine. What is the effect of heat on the color?

Experiment 28. Precipitation with Alcohol. To 5 cc. of the dextrin solution add an equal volume of alcohol and allow to stand until precipitation is complete. Filter off the precipitate and wash it two or three times with alcohol. Does this precipitate give a positive reduction test?

Glycogen

Experiment 29. Preparation of Glycogen.[19] Take four fresh oysters (or scallops if they can be obtained fresh), cut into small pieces, and drop into vigorously boiling water (about 200 cc.). Continue boiling for several minutes. Remove the tissue from the water; grind it fine with sand in a mortar, then replace it in the same water and continue the boiling for some minutes. Make faintly acid with acetic acid to coagulate the proteins. Filter while hot. Note the appearance of the filtrate.

Use this solution as directed in Experiments 30 and 31. To the remaining solution add an equal volume of 95 per cent alcohol and set aside until the next day. Filter off the precipitate and redissolve in a little water. Test with iodine.

What are the characteristic properties of glycogen?

Experiment 30. Iodine Test. Add dilute iodine solution, drop by drop, to 5 cc. of the glycogen solution. Compare the color obtained with that found in testing starch and dextrin. Glycogen gives a wine-

[19] A demonstration of the glycogen-storing action of the liver may be performed in the following manner. Three rabbits are used. To one has been fed a stock diet of grain; to the second has been given, in addition to this diet, a dose of glucose by stomach tube a few hours previous to the experiment; the third animal has been fasted for 24 hours. The animals are stunned by a blow on the head; the livers are promptly excised and the gall-bladders removed. The livers are then treated separately, as outlined in Experiment 29. The filtrates are poured into twice their volumes of 95 per cent alcohol in tall cylinders and allowed to stand overnight. The amount of precipitate in each cylinder will show strikingly the effect of food upon the glycogen content of the liver.

red color with iodine. If there is difficulty in obtaining this color, add a drop of 10 per cent sodium chloride and more of the iodine. What is the effect of heat on the color?

Experiment 31. Hydrolysis of Glycogen. Test the glycogen solution with Benedict's reagent in the usual way and note whether any reduction occurs. Treat 25 cc. of the glycogen solution with 2 cc. of concentrated hydrochloric acid and heat on the water bath for about 15 minutes. Neutralize and again test with Benedict's reagent. Explain the results.

NOTES: How do cellulose, inulin, and gum arabic differ in reaction and composition from the polysaccharides which you have studied in the preceding experiments?

Make a chart showing the reactions of the monosaccharides, disaccharides, and polysaccharides which you have studied with the various reagents which you have used for carbohydrate tests.

QUANTITATIVE ESTIMATION OF REDUCING SUGAR

Experiment 32. Benedict's Method for the Quantitative Determination of Sugar.[20] This method is based on the principle that a given amount of glucose reduces a definite amount of copper. The copper is reduced to and precipitated as white cuprous sulfocyanate. This white compound serves as a good background against which the end point of the titration (i.e., the disappearance of the last trace of blue color) may be detected.

This is a suitable method for the determination of sugar in urine and is a routine procedure in many clinical laboratories. The method should be used for sugar concentrations ranging between 0.2 to 1 per cent. Diabetic urines usually require a ten-fold dilution.

Procedure. Ten cc. of the sugar solution (or urine) are delivered by pipette into a 100-cc. volumetric flask, diluted to the mark with distilled water, and mixed thoroughly. (If the sugar concentration is low, no dilution is necessary.)

Twenty-five cc. of Benedict's *quantitative* reagent[21] are measured

[20] Benedict, S. R., *J. Am. Med. Assoc.*, **57**, 1193 (1911).

[21] Benedict's quantitative reagent contains the following ingredients:

Copper sulfate (pure crystalline)	18 g.
Sodium or potassium citrate	200 g.
Potassium sulfocyanate	125 g.
Sodium carbonate (anhydrous)	100 g.
Potassium ferrocyanide (5 per cent solution)	5 cc.
Distilled water to 1 liter.	

All the dry ingredients, except the copper sulfate, are dissolved with the aid of

into a 250-cc. Erlenmeyer flask and crystalline sodium carbonate (10–20 gm., or one-half this amount if the anhydrous salt is used) is added, together with a small quantity of talcum to prevent bumping. This mixture is heated to boiling over a free flame until the carbonate has entirely dissolved, care being taken to keep the volume constant, if necessary, by the addition of small amounts of distilled water. The diluted solution (or urine) is now run in from a 50-cc. burette, keeping the reagent boiling rather vigorously throughout the titration. At first about 0.5 cc. is added at a time; when the chalk-white precipitate forms and the blue color of the mixture begins to lessen perceptibly, the titration is continued more slowly, adding a drop at a time. Disappearance of the last trace of blue color marks the end point of the titration. Instead of a white end point, urine may give a grayish-green tinge, due to the urinary pigments.

Duplicate determinations should agree within 0.2 cc.

Calculation. Since 50 mg. of glucose reduces exactly 25 cc. of the reagent, the titration figure represents the amount of solution containing 50 mg. of glucose. Calculate the percentage of sugar in the original sample.

Caution. Chloroform interferes with the determination. If it has been used as a preservative for urine, it should be removed by boiling a sample for a few minutes and diluting to the original volume.

heat in enough distilled water to make about 800 cc. This solution is then filtered. The copper sulfate is weighed accurately on the analytical balance, dissolved in about 100 cc. of water, and poured slowly, with stirring, into the other liquid. The 5 cc. of the 5 per cent potassium ferrocyanide is then added, the solution allowed to cool and diluted to 1 liter. Exactly 50 mg. of glucose reduces 25 cc. of this reagent.

CHAPTER III

FATS AND RELATED COMPOUNDS

Experiment 1. Solubility. To a small piece of solid fat (mutton or beef tallow, or lard) in a test tube, add 2–3 cc. of ether. (**Do not work with ether near a flame.**) Shake well. Does the fat dissolve? Repeat, using acetone, hot and cold alcohol, chloroform, and water. If you are uncertain as to the solubility of the fat in any of these reagents, test some of the liquid after shaking with the fat, by pouring a few drops on a piece of paper. When the liquid evaporates, a greasy spot will remain on the paper if any of the fat has been dissolved. Record your results.

NOTE: Ether, acetone, and alcohol are inflammable.

Experiment 2. Emulsification. (*a*) To about 5 cc. of water in a test tube add a few drops of 0.5 per cent sodium carbonate solution and a drop of oil (olive or cottonseed). Shake and note the result. Place a drop of the emulsion on a slide and examine under the microscope. (*b*) Repeat, omitting the carbonate solution. (*c*) Repeat (*a*), using a drop of rancid oil. (*d*) Repeat with a drop of oil and 5 cc. of 1 per cent albumin solution. Explain the results. Examine a drop of milk under the microscope. Is it similar to the emulsions you have just prepared?

Experiment 3. Acrolein Test. To about 1 g. of solid potassium-acid-sulfate contained in a test tube or crucible, add 1–2 drops of glycerol and heat gently over a low flame, under the hood. By a slight wave of the hand, enough of the fumes issuing from the tube may be directed towards the observer to be smelled. Do not inhale. Acrolein (acrylic aldehyde) is formed by the dehydration of glycerol. Write the equation for the reaction.

Repeat the test, using a small amount of fat. Note the odor. Why may this be used as a test for fats?

Experiment 4. (*a*) **Saponification of a Fat.** To 10 g. of a fat (such as beef tallow), contained in a flask, add 100 cc. of a saturated alcoholic solution of sodium hydroxide. Cover with a funnel and heat on the water bath for about an hour. Transfer the contents of the flask to a casserole and continue heating on the water bath until most

of the alcohol has evaporated. Then add 50 cc. of alcohol, stir, and evaporate again.

Dissolve the residue in about 200 cc. of hot water.

(*b*) To 25 cc. of the solution add solid sodium chloride, with stirring, until a coagulum forms. What is this coagulum?

(*c*) To 5 cc. of the solution add several drops of 10 per cent calcium chloride, and shake. Observe and explain the result. What is the cause of "hardness" of water? Repeat, using several drops of a 1 per cent solution of magnesium chloride. Explain.

What are the products of the alkaline hydrolysis of a fat? Write the equation for the reaction of tripalmitin with sodium hydroxide.

(*d*) To the remainder of the solution add 4–5 drops of methyl orange, and acidify with sulfuric acid. Allow to cool. What is the cake that forms at the top? Filter it off and save the filtrate for use in part (*e*). Wash the material on the filter paper with three or four 50-cc. portions of hot water. Add the washings to the filtrate. Remove the residue from the filter and apply the acrolein test to a small piece. Explain. To the remainder add enough 95 per cent alcohol to dissolve, heating gently on the water bath. Filter, and allow the filtrate to cool slowly. Examine under the microscope the crystals that form. Explain.

(*e*) Evaporate the filtrate, reserved for this part of the experiment, on the water bath, and apply the acrolein test to some of the residue. Explain.

Experiment 5. Determination of the Saponification Number. The saponification number is defined as the number of milligrams of potassium hydroxide required to saponify one gram of fat.

Method. Observing the precautions outlined in the footnote,[1]

[1] The flask should be cleaned thoroughly by washing with soap and water, and rinsing with water and alcohol. In weighing a solid fat, the following procedure is recommended. Melt the fat so that a truly uniform sample may be obtained. Small flat-bottomed glass cylinders, about 10 mm. in diameter and about 15 mm. high, are convenient for the weighing of the sample; or small glass dishes may be made by cutting off the closed ends of Pyrex test tubes. Such a cylinder or dish is weighed, the melted fat placed in it, and the whole reweighed. It is best to use 1.5–2 g. of the fat. The container and fat are then transferred to the flask, care being taken that no loss occurs and that no fat is spilled on the neck of the flask. If an oil is used in the determination, weigh out 5–10 g. of the oil in a small beaker, together with a small pipette or medicine dropper. After the weight has been recorded, the required amount of the oil may be transferred to the flask by means of the dropper. Care must be taken not to get any of the oil on the neck of the flask. The dropper is then replaced in the beaker, the whole reweighed, and the weight of the oil taken for the analysis determined by difference.

transfer to an Erlenmeyer flask a weighed amount of fat (between 1 and 2 g.), and add, by means of a carefully cleaned pipette, 25 cc. of an alcoholic 0.5 *N* solution of potassium hydroxide.[2] At the same time, from the same pipette, measure another 25-cc. portion of the alcoholic potassium hydroxide solution into a second flask for a blank determination.

NOTE: The student should perform both the determination and the blank in duplicate.

The flasks are connected with reflux condensers and boiled gently (preferably over an asbestos pad) for at least 30 minutes. When saponification is complete cool the flasks, add to each 3 cc. of 1 per cent phenolphthalein,[3] and titrate the excess of alkali with 0.2 *N* hydrochloric acid. If much alcohol has evaporated, add enough to restore to approximately the original volume.

From the titration figures obtained calculate the amount of potassium hydroxide required to saponify the fat taken in each experiment and the saponification number of the fat. Explain why the saponification number may serve as a measure of the mean molecular weight of the fatty acids constituting the fat.

Experiment 6. Iodine Number. Hanus' Modification of the Hübl Method.[4] The iodine number is defined as the number of grams of iodine that are absorbed by 100 g. of fat. It is a measure, therefore, of the unsaturation of a fat, and is a valuable means of identification.

[2] Dissolve 30 g. of C.P. potassium hydroxide (free from carbonate) in 1 liter of 95 per cent alcohol, which has been purified by standing over potassium hydroxide for several days and by subsequent distilling. For very accurate work, the alcohol may be purified with silver oxide (Dunlap, *J. Am. Chem. Soc.*, **28**, 395 (1906). Standardize the alcoholic potassium hydroxide solution with a standard acid solution using phenolphthalein as the indicator. Potassium hydroxide is preferable to sodium hydroxide as the potassium soaps are more soluble in alcohol.

[3] With dark, resinous oils that do not lose their color during saponification, phenolphthalein alone will not give a satisfactory end-point. Three cubic centimeters of 1 per cent phenolphthalein, together with 3 cc. of a cold-saturated alcoholic solution of Alkali-blue B (Coleman-Bell), has been found satisfactory.

[4] Several methods for the determination of the iodine number are in use. For descriptions of these methods consult Leach's "Food Inspection and Analysis," John Wiley & Sons, Inc., New York, (1920).

Free iodine is not readily absorbed by fat; hence more active solutions are used, containing an unstable compound of iodine. In Hanus' solution, the active agent is iodine monobromide; in Wijs' solution it is iodine monochloride. The halogen addition products are therefore not necessarily iodo derivatives exclusively. Thus in oleic acid, the dihalogen compound formed with Hanus' solution contains iodine and bromine; the compound formed with Wijs' solution contains iodine and chlorine [$CH_3(CH_2)_7CHI \cdot CHCl(CH_2)_7COOH$].

Method. Weigh out about 0.25 g. of oil, or 0.5 g. of solid fat (as described in the preceding experiment) and transfer to a glass-stoppered bottle of about 300 cc. capacity. Add 10 cc. of chloroform. When the fat has dissolved, add 30 cc. of Hanus' solution,[5] by pipette, delivering the liquid so that none of it touches the neck of the bottle. Insert the stopper and shake gently. Allow to stand in the dark for 30 minutes. Carry out a blank determination at the same time and in exactly the same way except that the fat is omitted.

The determination should, of course, be done in duplicate.

After half an hour, remove the stopper carefully and add 10 cc. of a 15 per cent solution of potassium iodide,[6] pouring it over the end of the stopper into the bottle, and 100 cc. of water. Titrate immediately with the standard solution of sodium thiosulfate,[7] which is run in rapidly until the solution is pale yellow in color. Then add 2 cc. of a freshly prepared starch paste (0.5 per cent) and continue to titrate until the blue color disappears. Toward the end of the titration, it is advisable to stopper the bottle and shake well its contents between

[5] *Hanus' Solution.* Dissolve 13.2 g. of iodine in a liter of glacial acetic acid (99.5 per cent). The acetic acid should be pure, giving no green color on warming on the water bath with potassium bichromate and sulfuric acid. Solution of the iodine may be brought about by warming gently on the water bath and adding the acetic acid in small amounts. Cool. Add enough bromine to double the halogen content, as shown by titration (3 cc. is usually sufficient).

[6] The potassium iodide is effective in removing the iodine from the chloroform layer. It also has another function in the titration. Since IBr is present, the reaction

$$KI + IBr = KBr + I_2$$

takes place, thus freeing the iodine for the titration reaction.

[7] *Standard Sodium Thiosulfate Solution.* Dissolve 24.8 g. of C.P. recrystallized sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) per liter of distilled water. This makes an approximately 0.1 *N* solution. It is standardized in the following manner.

Dissolve 3.8633 g. of pure potassium bichromate in a liter of distilled water. One cubic centimeter of this solution is equivalent to 0.01 g. of iodine. For very accurate work the bichromate solution should be standardized. See Treadwell and Hall, "Quantitative Analysis," Eighth Edition, 586 (1935). Measure 20 cc. of the bichromate solution into a flask, add an equal volume of water, 10 cc. of 15 per cent potassium iodide solution, and 5 cc. of concentrated hydrochloric acid. Run in the thiosulfate solution from a burette until the red color of the free iodine changes to a yellow: then add 2 cc. 0 5 per cent starch solution (freshly made), and titrate until the blue color disappears. Calculate the strength of the thiosulfate solution.

Equation:

$$K_2Cr_2O_7 + 14HCl + 6KI = 2CrCl_3 + 8KCl + 7H_2O + 3I_2$$

$$6Na_2S_2O_3 + 3I_2 = 6NaI + 3Na_2S_4O_6$$

additions of thiosulfate. The blank should be titrated in the same manner.

Calculate the iodine number of the fat.

Experiment 7. Extraction of Lipids from Brain Tissue. Chopped brain (hog, sheep, or cattle) is dried overnight at 100° C., or preferably in a vacuum drying oven at a lower temperature, or it may be mixed with three times its weight of plaster of Paris, ground well, and allowed to stand for 24 hours.

Cholesterol. Grind 10 g. of the dried brain tissue in a mortar, transfer to a flask, and extract three times with cold acetone, using 20–30 cc. for each extraction. Decant the acetone through a filter. Save the residue for subsequent work.

Combine the acetone extracts and evaporate on a steam bath. Avoid flames. Dissolve the dried residue in a small amount of boiling 95 per cent alcohol; filter hot. Set aside to cool. If crystals do not form, dissolve the amorphous residue in the smallest possible amount of hot acetone, filter, and allow to cool. When crystals of cholesterol have separated out, dissolve some in chloroform and test as directed in Experiments 8 and 9. The cholesterol may be further purified by recrystallization from absolute alcohol. Dry the crystals at 100° C. Examine microscopically.

Phospholipids. Treat the brain residue from the acetone extraction three times with cold ether, using about 50 to 60 cc. in all. Save the residue for the extraction of the cerebrosides. Evaporate the combined extracts to about 20 cc. and add 3 to 4 volumes of acetone. The precipitate consists chiefly of lecithin and kephalin. Filter, wash the precipitate with a little acetone, and allow to dry.

Incinerate a small amount of the phospholipid in a porcelain crucible. Cool and extract the residue with hot water (5–10 cc.). Filter and add to the filtrate 3 cc. of ammonium molybdate solution (5 per cent) and 5 drops of nitric acid. Heat to boiling, then allow to stand. Note the yellow crystalline precipitate of ammonium phosphomolybdate.

On another small amount perform the acrolein test (page 46).

Cerebrosides. Extract the brain tissue residue obtained from the phospholipid extraction three times with boiling alcohol. Evaporate the combined extracts to about 30 or 40 cc., cover, and allow to cool. The cerebrosides, or glycolipids, separate out. Filter.

Dissolve some of the precipitate in hot water. Cool. Apply the Molisch test to 3 or 4 cc. of the solution. Explain.

Test some of the solution for the presence of reducing sugar (Bene-

dict's test). To the remainder of the solution in an Erlenmeyer flask add hydrochloric acid (5 drops of concentrated acid for each 5 cc. of solution), cover with a watch glass, and heat on the water bath for 1–2 hours. Cool, neutralize, and test for the presence of reducing sugar. Explain.

Experiment 8. Salkowski's Test for Cholesterol. Transfer to a *dry* test tube, 2 or 3 cc. of the cholesterol solution prepared in Experiment 7. Add 2–3 cc. of concentrated sulfuric acid to form a layer at the bottom. Note the color of the ring produced. Agitate gently and note the colors in the two layers. Repeat, using a chloroform solution of pure cholesterol.

Experiment 9. Liebermann-Burchard Reaction. To another portion of the chloroform solution in a *dry* test tube, add 10 drops of pure acetic anhydride, mix, add 3 drops of concentrated sulfuric acid. Mix again and allow to stand. Note the changes in color and the final development of a blue or greenish-blue color.

Repeat the test, using a chloroform solution of pure cholesterol.

Experiment 10. Rosenheim's Test for Ergosterol. To 3 cc. of a saturated solution of trichloracetic acid (1 part water, 9 parts acid) add a few drops of a chloroform solution of ergosterol. Note the color changes and the final development of a relatively stable blue color. Repeat, using a few drops of a solution of cholesterol in chloroform.

NOTE TO STUDENT: Prepare a table to include the solubilities, properties, characteristic reactions and hydrolysis products of the fats and fat-like substances which you have studied.

CHAPTER IV

PROTEINS

Experiment 1. Test for Nitrogen. Mix thoroughly 2–3 g. of soda lime (sodium hydroxide and calcium oxide) with a small amount (about 0.5 g.) of dried egg albumin or casein. Place the mixture in a hard-glass tube and heat strongly under the hood. Note the odor. Test the fumes by holding a moistened piece of red litmus paper over the test tube. What has been formed during the fusion? [1]

Experiment 2. Test for Phosphorus. Working under the hood, melt a mixture of 1 g. of potassium carbonate and 1 g. of potassium nitrate in a porcelain crucible; carefully add about 0.5 g. of casein and continue heating until effervescence ceases. Cool, add 10 cc. of distilled water, and heat to boiling. Filter, add 1 cc. of concentrated nitric acid followed by 2–3 cc. of 5 per cent ammonium molybdate solution. Heat to boiling. If the precipitate does not form immediately, allow to stand. What is the precipitate? What is the chemistry of this test? [2]

Experiment 3. Test for Unoxidized Sulfur. To 1–2 g. of dry protein in a flask, add about 10 cc. of 20 per cent sodium hydroxide. Put a watch glass over the flask and boil vigorously for a time. Cool. Acidify with hydrochloric acid and heat again to boiling, placing a piece of filter paper, moistened in lead acetate solution, over the mouth of the flask. What is formed on the paper? Explain the chemistry of this test and write the equation.

Experiment 4. Ninhydrin Reaction. To 4 cc. of a protein (or amino acid) solution, neutral in reaction, add 1 cc. of 0.1 per cent ninhydrin (triketo-hydrindene hydrate).[3] Mix, boil for 1 minute, and

[1] A more satisfactory test for the detection of nitrogen in organic nitrogenous substances consists in fusing the material in question with either metallic sodium or potassium. The corresponding cyanide is formed, which, when treated with a freshly prepared solution of ferrous sulfate (and a small amount of potassium fluoride), and acidified after 5–10 minutes with 30 per cent nitric acid or with 5 per cent sulfuric acid yields ferric ferrocyanide (Prussian blue).

[2] See Treadwell and Hall, "Analytical Chemistry," Vol. I, "Qualitative Analysis," Ninth Edition, 400 (1937).

[3] The ninhydrin reagent should not be more than 2 or 3 days old.

set aside to cool. A pink color changing to purple and blue develops. Run a control test with distilled water.

This reaction depends on the presence of free carboxyl and α-amino groups and is therefore given by proteins, peptones, peptides, and some of the amino acids. However, the reaction is not specific, being obtained also in the presence of ammonium salts and certain amines and amides.

Experiment 5. Biuret Reaction. The biuret reaction is a general test for proteins, depending upon certain groupings in the protein molecule.[4] The color obtained is due essentially to the formation of a copper-protein compound in an alkaline solution. The substance biuret, which gives this test, may be prepared by heating urea.

5*a*. In each of two dry test tubes, place small equal amounts of urea. Heat one until the molten material solidifies. What fumes are given off? Dissolve the white residue in 5 cc. of water. Filter. Then perform the biuret test by adding to the filtrate an equal volume of 20 per cent sodium hydroxide and mixing. Now add, drop by drop, a very dilute solution of copper sulfate (the color of this should be *faintly* blue). Observe the characteristic color of the reaction. Write the equation for the formation of biuret.

Dissolve the untreated urea in the second tube in 5 cc. of water and perform the biuret test. Explain.

5*b*. Perform the biuret test on 2 cc. of a 1 per cent albumen (egg-white) solution.[5]

5*c*. To 2 cc. of egg-white solution, add an equal volume of a saturated solution of ammonium sulfate. Perform the biuret test on this mixture. What difference do you observe in the color as compared with that obtained in Experiment 5*b*. Now add an excess of 20 per cent

[4] The nature of the compounds formed in the biuret reaction is unknown, but they are evidently the result of the interaction of some group in the protein molecule with $Cu(OH)_2$ in alkaline solution. It is generally assumed that the reaction depends on the presence of two acid amide, or so-called peptide, groups, $—CONH_2$, or one such group together with some other complex containing the NH_2 radical.

[5] The white of eggs contains about 11 per cent protein and may be diluted with distilled water to a 1 per cent solution. On adding the water, the ovoglobulin and ovomucin will flock out. These proteins should be removed by filtering or straining.

Albumin is defined as a simple protein, soluble in water and coagulable by heat. Albumen is the old name for albumin, but is still used to denote the white of eggs. Synonymous with albumen is the term "egg albumin." (See Dorland's "Medical Dictionary.")

sodium hydroxide to the mixture. What gas is evolved? What color change occurs? What precaution is necessary in performing the biuret test in the presence of ammonium salts? Large amounts of magnesium likewise interfere with this test, owing to precipitation of magnesium hydroxide.[6]

Experiment 6. Millon's Reaction.[7] To 3 cc. of the egg-white solution add a few drops of Millon's reagent. A white precipitate forms. Heat gently (60–70° C. is the best temperature) and observe closely the behavior of the precipitate, its color and that of the solution. Repeat, using a small bit of solid boiled egg-white suspended in 3 cc. of water.

Inorganic salts, such as sodium chloride, which precipitate the mercury present in the reagent, interfere with the reaction.

Millon's reaction is due to the presence of the monohydroxy-benzene nucleus. Hence, it is given by phenol, salicylic acid, vanillin, etc. Confirm this by testing solutions of phenol and salicylic acid with Millon's reagent. Compare these with the protein tests.

Repeat the test, using 3 cc. of a 2 per cent solution of gelatin. From the results of these experiments, what do you conclude regarding the tyrosine content of egg albumin and gelatin?

Experiment 7. Xanthoproteic Reaction. To 3 cc. of the egg-white solution add 1 cc. of strong nitric acid in a test tube. Note the precipitate. Heat gently and note the color. Cool. Make alkaline with sodium or ammonium hydroxide, and note the change in color. Repeat the test, using a gelatin solution. The reaction is due to the presence of a substituted benzene nucleus in the protein molecule, and the yellow color is the result of the formation of nitrobenzene derivatives. What amino acids must be present to give a positive xanthoproteic test? Phenylalanine reacts slowly and with difficulty.

Experiment 8. The Glyoxylic Acid Reaction. (Adamkiewicz or Hopkins-Cole Test.[8]) To 2 cc. of the protein solution add a few drops

[6] Tests similar to the biuret reaction may be performed with nickel and cobalt, in place of copper, characteristic colors being given with these metals. See J. W. Pickering, *J. Physiol.*, **14**, 347 (1893).

[7] *Millon's Reagent.* Treat 1 part by weight of mercury with 2 parts by weight of nitric acid of sp. gr. 1.42, and warm gently until solution is complete. Dilute the resulting solution with 2 volumes of distilled water and allow to stand for several hours. Decant the supernatant liquid from the crystalline precipitate. The reagent contains mercurous and mercuric nitrates, together with an excess of nitric acid and a little nitrous acid.

[8] The original Adamkiewicz test is performed by adding glacial acetic acid to the protein, following this by the addition of sulfuric acid. Hopkins and Cole

of the glyoxylic acid reagent. Mix. Add 3–5 cc. of concentrated sulfuric acid, pouring the acid carefully down the side of the tube so that it will form a layer under the lighter fluid. A purple ring forms at the junction of the two fluids. Shake the tube gently from side to side. The color will spread throughout the mixture.

Repeat the test, using 2 cc. of a 2 per cent solution of gelatin.

The color produced in this test is due to the formation of a condensation product of the glyoxylic acid (CHO COOH) with the indole group of tryptophane. The test, therefore, depends upon the presence of this amino acid.

Nitrates, chlorates, nitrites, or an excess of chlorides, when present, interfere with the Hopkins-Cole reaction.

Experiment 9. Acree-Rosenheim Formaldehyde Reaction. In principle this test is similar to that of the Hopkins-Cole test, depending also on the formation of a condensation product of tryptophane and an aldehyde. To 2 cc. of the protein solution add 3 drops of dilute formaldehyde (1 : 5000). Mix and stratify above concentrated sulfuric acid. Note the color of the ring after standing for 5 minutes. Compare the results obtained with solutions of egg albumin, casein, and gelatin.

Experiment 10. Ehrlich's Diazo Reaction. Phenols and imidazoles react with diazo-benzene-sulfonic acid to form condensation products. A positive test therefore indicates the presence of either tyrosine or histidine. Mix 1 cc. of a solution of sulfanilic acid (0.5 per cent sulfanilic acid in 2 per cent hydrochloric acid) and an equal volume of 0.5 per cent sodium nitrite and allow to stand for 1 minute. Add 1 cc. of a protein solution, then make alkaline with 10 per cent sodium carbonate. Note the color. Run a control, using water in place of the protein solution. Repeat the test, using very dilute solutions of tyrosine and histidine.

Experiment 11. Molisch Test. This is essentially a test for car-

found that glacial acetic acid may contain glyoxylic acid as an impurity and that this substance is responsible for the test. They therefore employed glyoxylic acid which they prepared by treating oxalic acid with sodium amalgam (*J. Physiol.*, **27**, 418 (1902)). The preparation of the glyoxylic acid reagent has been modified by Benedict (*J. Biol. Chem.*, **6**, 51 (1909)). Benedict's method is as follows: Place 10 g. of magnesium in a flask and cover the metal with distilled water. Add slowly, with gentle shaking, 250 cc. of a cold, saturated oxalic acid solution, cooling the flask in running water. Filter off the insoluble magnesium oxalate, acidify the filtrate with acetic acid, and dilute to a liter with distilled water. The solution contains only the magnesium salt of glyoxylic acid.

bohydrates, but is given by a variety of proteins in which a sugar group is present in the molecule.

To 3 cc. of the egg-white solution add a few drops of the α-naphthol (Molisch) reagent. Mix and run in an equal quantity of sulfuric acid, pouring it down the side of the tube to form a layer under the lighter liquid. The characteristic violet ring should be formed in the presence of a glycoprotein.

To 3 cc. of the egg-white solution add an equal quantity of concentrated sulfuric acid, as above. Does a purple ring appear? If so, why? If the colored ring does not appear, agitate very gently. Set aside and observe from time to time. If done carefully a positive test can be obtained. What is the group in the protein molecule that can take the place of the α-naphthol?

PRECIPITATION REACTIONS

Proteins are readily brought out of solution by various physical and chemical agents. One variety of reagent, the neutral salts, may be used in high concentration to precipitate (salt out) a protein. Upon removal of the salt the protein may be recovered unchanged in properties. Other precipitants, such as the strong acids, have a more permanent action and "denature" the protein while precipitating it. Heat will denature certain proteins, that is, change the solubilities and chemical characteristics; precipitation occurs if the *p*H of the solution is near enough to the isoelectric point. Certain proteins are precipitated by alcohol. If the alcohol is removed immediately, the solubility of the protein appears to be unchanged. However, if after precipitation, the protein remains in contact with the alcohol for some time, it undergoes denaturation. This process is thought by some to be due to the removal of water either from around or from within the protein molecule. Other precipitating agents like the salts of heavy metals (mercury, lead, zinc, copper, iron) precipitate proteins from neutral or alkaline solution, a chemical reaction taking place between the protein as anion and the heavy metal as cation. Proteins also react with anions in acid solution as in the precipitation by ferrocyanic, picric, and tannic acids. These and other reagents have long been used in the precipitation of alkaloids in toxicological analysis; hence their designation as "alkaloidal reagents."

Some confusion exists as to the differentiation between the terms precipitation, coagulation, and denaturation. It may be tentatively suggested that precipitation may occur without denaturation and de-

naturation without precipitation; but that coagulation involves both denaturation and precipitation.

Experiment 12. Coagulation by Heat. Heat gently 3 to 4 cc. of egg-white solution in a test tube. Observe the amount of coagulation. Add 2 to 3 drops of 2 per cent acetic acid and observe the effect.

In a slightly modified form this test is employed in the examination of urine for the presence of protein (page 138). Repeat, using (*a*) a solution of gelatin, (*b*) a solution of casein.

Experiment 13. Influence of Acids, Bases, and Salts on Heat Coagulation. Prepare and label five test tubes. Measure 5 cc. of filtered egg-white solution into each tube. To tube 1 add 0.5 cc. of 0.1 *N* acetic acid; to tube 2, 0.5 cc. of 0.1 *N* HCl; to tube 3, 0.5 cc. of 0.1 *N* NaOH; to tube 4, 1 drop of 10 per cent NaCl (or $CaCl_2$). Keep tube 5 as a control. Place the tubes in a small beaker of water, suspended by means of cork wedges in a larger beaker of water. Heat very slowly, and by means of a thermometer placed in one of the tubes, determine the coagulation temperature of each solution. Record the results.

Neutralize the contents of tube 3. Note the coagulum. What change has taken place in the protein?

Experiment 14. Importance of Water in Heat Coagulation. Place a small amount of dried, powdered egg albumin into each of two test tubes. Add 5 cc. of distilled water to one and shake gently until the protein dissolves. Immerse both tubes in boiling water. After coagulation is complete in the first tube, remove both tubes from the water and cool. Now add 5 cc. of the 1 per cent NaCl to the dried albumin and agitate at intervals for a few minutes. Filter the contents of each tube. Test the solubility of the residues on each filter paper, if present. Apply the biuret test to equal portions of each filtrate. To other portions add a drop of dilute acetic acid and heat to boiling. From these observations would you conclude that water is necessary for heat coagulation?

Experiment 15. Strong Mineral Acids. To 3 cc. of the egg-white solution in a test tube, add strong nitric acid, pouring it down the side of the tube to form a layer under the protein solution. Observe the precipitate which forms at the junction of the two liquids. This is the well-known Heller's ring test and is employed clinically for the detection of albumin in urine.

Repeat, using concentrated hydrochloric acid.

Experiment 16. Precipitation with Alcohol. To 5 cc. of 5 per cent egg-albumin solution add 2 volumes of 95 per cent alcohol. Filter

off half the precipitate immediately. Test its solubility by shaking in water, filtering, and performing a biuret test on the filtrate. Allow the remaining portion to stand for half an hour. Filter, and test the filtrate for the presence of protein. Explain. Does alcohol completely precipitate protein? Is the second half of the precipitate soluble in water? What is the effect of alcohol on protein and how efficient is it as a precipitating agent?

Prepare three test tubes each containing 5 cc. of 95 per cent alcohol. Acidify the alcohol in one tube by adding a drop of 0.1 *N* HCl or H_2SO_4. To the second tube add a drop of 0.1 *N* NaOH. The alcohol in the third tube remains neutral. Add to each tube a few drops of the egg-albumin solution. Compare the results.

Experiment 17. Salting Out of Proteins. To 10 cc. of egg-white solution in a small beaker, add solid ammonium sulfate to saturation. Filter off the precipitate that forms. Test the filtrate and precipitate for the presence of protein.

Repeat, adding solid sodium chloride to saturation. Does a precipitate form? Add a few drops of dilute acetic acid. Filter, and test the precipitate and filtrate as before.

The use of neutral salts is a very convenient method of precipitation, since the protein may be recovered, unchanged, after dialysis.[9]

Experiment 18. Salts of Heavy Metals. To 5 cc. of the protein solution add very slowly, a drop at a time, a 1 per cent solution of ferric chloride. Note the effect and then add an excess of ferric chloride.

Repeat, using mercuric chloride, zinc sulfate, copper sulfate, and lead acetate. Explain the results of this experiment.

Experiment 19. Alkaloidal Reagents. The "alkaloidal" reagents are so called because of their property to precipitate alkaloids. Proteins as well are precipitated by a number of these reagents, among which may be included ferrocyanic, tannic, picric, phosphotungstic, phosphomolybdic, sulfosalicylic, dinitrosalicylic, metaphosphoric, and trichloracetic acids and potassium-mercuric iodide.

Ferrocyanic Acid. Acidify 5 cc. of egg-white solution with acetic acid and add a few drops of 5 per cent potassium ferrocyanide solution. Note the precipitate.

Picric Acid, Trichloracetic Acid, etc. To 5 cc. of the egg-white solution add a few drops of a saturated picric acid solution.

Repeat, adding to different portions of the egg-white solution a few

[9] For a discussion of the mechanism of precipitation of protein by neutral salts, see Loeb's "Proteins and the Theory of Colloidal Behavior," McGraw-Hill Book Co. (1924), page 95 *et seq.*

drops of 10 per cent trichloracetic acid, 20 per cent sulfosalicylic acid, and a freshly prepared 25 per cent solution of metaphosphoric acid.

Acidify three 5-cc. portions of the egg-white solution with dilute hydrochloric acid. Add to tube 1, a few drops of a freshly prepared solution of tannic acid, to tube 2, a few drops of 10 per cent sodium tungstate, and to tube 3, a few drops of 2 per cent phosphotungstic acid.

Experiment 20. Determination of the Isoelectric Point of Casein.[10] In a 50-cc. measuring flask place 0.3 g. of pure casein (according to Hammarsten). Add about 25 cc. of distilled water, previously warmed to about 40° C., and exactly 5 cc. of *N* sodium hydroxide. Agitate till the casein dissolves, taking care to prevent frothing. Rapidly add 5 cc. of *N* acetic acid, mix, cool, and make up to 50 cc. with distilled water. A fairly opalescent solution of casein in 0.1 *N* sodium acetate is thus obtained.

Make up the following series, using clean, dry test tubes:

Tube No.	1	2	3	4	5	6	7	8	9
Cc. casein in 0.1 *N* sodium acetate	1	1	1	1	1	1	1	1	1
Cc. distilled water	8 38	7 75	8 75	8.5	8	7	5	1	7 4
Cc. 0.01 *N* acetic acid	0 62	1 25							
Cc. 0.1 *N* acetic acid			0 25	0 5	1	2	4	8	
Cc. *N* acetic acid									1 6

Place the casein solution in the tubes first, then the water, and mix. Now add the acetic acid to the first tube and shake immediately. Then add the acid to the second tube and shake this, and so on. Examine the tubes on mixing, after 10 minutes, and after 20 minutes. Record your observations in tabular form, using these symbols:

O = no change. + = opalescence. × = precipitate

In which tube is precipitation greatest?

The concentration of hydrogen ions can be calculated approximately from the following formula, no allowance being made for the acidity of the casein.

[10] After S. W. Cole, "Practical Physiological Chemistry," W. Heffer & Sons, Ltd., Cambridge, Seventh Edition (1926), page 78. Published with the permission of Professor Cole and his publishers.

$$[H^+] = \frac{K \text{ (acetic acid in mols. per liter)}}{a \text{ (sodium acetate mols. per liter)}};$$

$[H^+]$ = Hydrogen ions in grams per liter;

K = Dissociation constant of acetic acid, 1.85×10^{-5}

a = Degree of dissociation of sodium acetate:

0.87 for 0.01 N,
0.79 for 0.1 N.

Thus in tube 1,

$$[H^+] = \frac{1.85 \times 10^{-5} \times 0.62 \times 10^{-3}}{0.87 \times 10^{-2}} = 1.32 \times 10^{-6}.$$

Below are the $[H^+]$ and pH of the various tubes.

Tube No.	$[H^+]$	pH
1	$1\ 32 \times 10^{-6}$	5 88
2	$2\ 66 \times 10^{-6}$	5 57
3	$5\ 32 \times 10^{-6}$	5 27
4	$1\ 06 \times 10^{-5}$	4 97
5	$2\ 13 \times 10^{-5}$	4 67
6	$4\ 25 \times 10^{-5}$	4 37
7	$8\ 52 \times 10^{-5}$	4 07
8	$1\ 70 \times 10^{-4}$	3 77
9	$3\ 40 \times 10^{-4}$	3 47

Still finer adjustments of the reaction can be obtained by suitably varying the concentration of acetic acid. The $[H^+]$ can be calculated from the formula.

Experiment 21. Extraction of Edestin, a Globulin, from Hemp Seed. Heat about 300 cc. of 5 per cent sodium chloride solution to 60° C. Have ready in a clean and dry mortar about 20 g. of ground hemp seed. Pour a portion of the warmed salt solution on the hemp seed and triturate thoroughly. Pour off the more fluid portion of the mixture into a 600-cc. beaker and, with another portion of the warm salt solution, treat the residue as before. With the last portion of the salt solution, wash the hemp seed into the beaker containing the washings. Warm on the water bath to 60° C. (*No higher! Why?*) and maintain the mixture at this temperature for about an hour, stirring frequently. Strain rapidly through cheesecloth wet with warm 5 per cent sodium chloride solution onto a filter previously moistened with warm 5 per cent sodium chloride solution. If the funnel and paper become cold, the globulin will precipitate and clog the pores of the

filter paper. For this reason it is very desirable to use a water-jacketed funnel, maintained at 60–65° C. The beaker in which the filtrate is received should be placed in a vessel of warm water to prevent rapid cooling. Slow cooling will increase the size of the crystals formed. After 24 hours filter off the precipitate. (If only a small amount is formed, try diluting the filtrate with distilled water to bring down the globulin remaining in solution.) Wash the precipitate with cold 5 per cent sodium chloride solution. Examine some of the material under the microscope and sketch the characteristic forms of crystals.

Test the solubility of edestin in water, in cold and warm 5 and 10 per cent sodium chloride, in dilute hydrochloric acid, and in dilute sodium hydroxide. Is edestin heat-coagulable? Try the biuret and Millon's reactions on portions of this material and explain the results.

DERIVED PROTEINS

Experiment 22. Acid and Alkali Metaproteins. Measure into each of two beakers 25 cc. of egg-white solution. To one add 10 cc. of 0.1 *N* sodium hydroxide and to the other the same amount of 0.1 *N* hydrochloric acid. Heat on the water bath for a half hour, covering the beaker with a watch glass. Remove the beakers and cool. To the acid solution add 0.1 *N* sodium hydroxide until neutral; to the alkaline solution add 0.1 *N* hydrochloric acid in the same way. At the neutral point a precipitate will form. What is the precipitate in each beaker? Filter off the precipitates and test them in the following manner, using comparable portions of the two.

Suspend a small portion of the precipitate in a few cubic centimeters of 0.1 *N* hydrochloric acid. Is it soluble? Heat. Does the metaprotein coagulate in acid? Repeat, using 0.1 *N* sodium hydroxide. Repeat, using water. Perform the biuret test on some of the metaprotein solution. Perform Millon's test on some of the precipitated metaprotein. Test a portion of the precipitate for unoxidized sulfur (page 58). Compare the properties of acid and alkali metaprotein.

Experiment 23. Proteoses and Peptones. (*a*) *Separation of Proteoses from Peptones.*[11] The proteoses may be more or less com-

[11] A mixture of proteoses and peptones may be prepared by hydrolyzing protein with mineral acids, or by digesting with pepsin in a hydrochloric acid solution. It is, however, more convenient to use commercial preparations in these experiments. Witte's peptone consists of a mixture of proteoses and peptones, the former predominating, and is prepared commercially by digesting fibrin with pepsin in hydrochloric acid. A solution may be prepared as follows: Add 5 g. of Witte's peptone to 100 cc. of distilled water and warm gently to dissolve

pletely separated from the peptones by saturation with ammonium sulfate.[12]

Saturate about 25 cc. of a solution of Witte's peptone with finely pulverized ammonium sulfate, stirring all the time. Filter off the proteoses that precipitate.

Test the filtrate by means of the biuret reaction. (It will be necessary to use a large excess of sodium hydroxide to counteract the effect of the ammonium sulfate present. Why?) Observe the color of the biuret reaction when applied to peptone and contrast it with the color given by a protein.

(*b*) *Tests for Proteoses.* Test a portion of the proteose precipitate by means of Millon's reaction.

Dissolve the remainder in a small quantity of distilled water and apply the following tests:

(1) Test by means of the biuret reaction, noting the color carefully and contrasting it with the color given by protein and peptone.

(2) Acidify with acetic acid and add a few drops of potassium ferrocyanide solution.

(3) To 5 cc. of the solution add 15 cc. of 95 per cent alcohol. Does precipitation occur?

(4) Slightly acidify with acetic acid and heat. Does coagulation occur?

(5) To a few cubic centimeters of the proteose solution add a few drops of lead acetate solution.

(6) Repeat, adding a few drops of phosphotungstic acid solution.

(7) Repeat, adding a few drops of picric acid solution.

it. Acidify faintly with acetic acid and filter. The filtrate contains proteoses and peptones.

Armour's peptone (obtainable from Armour & Co., Chicago, Ill.) and Bacto-Peptone (obtainable from the Digestive Ferments Co., Detroit, Mich.) consist mainly of peptones and are suitable for use in the peptone experiments.

[12] The proteoses precipitated by saturation with ammonium sulfate consists of a mixture of the so-called primary proteoses (proto- and hetero-proteoses) and secondary proteoses. The proteoses may be precipitated in two fractions as follows: Half saturate a solution of Witte's peptone by adding an equal volume of saturated ammonium sulfate solution. Stir the mixture rapidly with a rubber-tipped stirring rod. The primary proteoses separate out, collecting largely as a gummy mass around the stirring rod. Filter. The filtrate contains secondary proteoses and peptones. Saturate the filtrate with pulverized ammonium sulfate and stir. The secondary proteoses separate out.

Test each of the proteose fractions as in part (*b*).

Compare the relative complexity of proteins, primary proteoses, secondary proteoses, and peptones.

(8) Repeat, adding a few drops of trichloracetic acid solution.

(*c*) *Tests for Peptones.* Using a 3–4 per cent solution of Armour's peptone (or Bacto-Peptone), repeat the tests given in part (*b*).

Prepare a table to include protein (egg albumin), metaproteins, proteoses, and peptones, summarizing the results of the various tests that have been given: biuret reaction, precipitation with phosphotungstic acid, coagulation by heat, etc.

Experiment 24. Isolation of *l*-Cystine (Folin's Method).[13] (*a*) Heat 300 cc. of concentrated hydrochloric acid on a sand bath in the hood. Introduce 150 g. of washed wool or hair into the flask, about 50 g. at a time, shaking after each addition and allowing it to dissolve. Fit the flask with a reflux condenser and heat gently on the sand bath for 5–6 hours, or until the biuret test is negative. Remove from the bath, and to the hot mixture add solid sodium acetate (300–400 g.) until no free mineral acid can be detected in the mixture with Congo-red paper. Allow the mixture to stand for 3–5 days. The longer it stands, up to 3 weeks, the more cystine is obtained. Filter on a Büchner funnel and wash with cold water. Save the mother liquor for (*b*). Dissolve the precipitate in 3 per cent hydrochloric acid, add about 20 g. of a good grade of bone-black (which has been freed from calcium phosphate), or the vegetable decolorizing carbon Norit, and boil for 10 minutes. Filter. Heat the filtrate to boiling and neutralize the boiling solution by adding slowly a hot, concentrated, filtered solution of sodium acetate. Test with Congo-red paper at short intervals to avoid an excess of sodium acetate. The precipitate formed consists of cystine, and should be very white and pure. If it is not white, redissolve in water and hydrochloric acid and purify as before.

Filter off the crystals and wash with cold water. Examine under the microscope and sketch. Dry, and apply the test for cystine (Experiment 25).

(*b*) Decolorize the original mother liquor with bone-black, and allow to stand in a cold place. Tyrosine crystals should separate out. Filter off, examine under the microscope, and test for tyrosine.

Experiment 25. Sullivan's Naphthoquinone Reaction for Cysteine and Cystine. *Cysteine.* To 5 cc. of a solution of cysteine in 0.1 *N* HCl,[14] add 1 cc. of 1 per cent NaCN in 0.8 *N* NaOH. **Exercise great**

[13] Folin, O., *J. Biol. Chem.*, **8**, 9 (1910); see also Folin's "A Laboratory Manual of Biological Chemistry," D. Appleton & Co., Fourth Edition (1926), page 115.

For an alternative method, consult Morrow and Sandstrom, "Biochemical Laboratory Methods," John Wiley & Sons, Inc. (1935), page 76.

[14] The concentration of cysteine (and cystine) should not be more than 0.01 per cent.

caution as NaCN and HCN are very poisonous. Mix and add 1 cc. of a freshly prepared aqueous solution of 1,2-naphthoquinone-4-sodium sulfonate, followed, after mixing, by 5 cc. of a 10 per cent solution of sodium sulfite (anhydrous) in 0.5 *N* NaOH. Mix and wait 30 minutes. A reddish brown color appears. Then add 1 cc. of a 2 per cent solution of sodium hyposulfite ($Na_2S_2O_4$) in 0.5 *N* NaOH. The test is positive if the red color persists; negative if it is discharged.

Cystine. To 5 cc. of a solution of cystine in 0.1 *N* HCl [14] add 1–2 cc. of a freshly prepared 5 per cent aqueous solution of NaCN, and mix. The cystine is thus reduced to cysteine. Then add 1 cc. of a freshly prepared 0.5 per cent solution of 1,2-naphthoquionone-4-sodium sulfonate, sodium sulfite, etc., as given above in the test for cysteine.

Experiment 26. Preparation of *l*-Tyrosine from Silk Waste.[15] Place 200 g. of silk waste in a 3-liter round-bottomed Pyrex flask and add 600 cc. of hydrochloric acid of sp. gr. 1.115. Hydrolyze by boiling gently under a reflux condenser on a sand bath, heated on an electric hot plate, for 12 hours. After completion of the hydrolysis, filter off the acid-insoluble humin and wash with a small quantity of hot distilled water. In order to drive off the hydrochloric acid as completely as possible, concentrate the combined filtrate and wash water under diminished pressure in a 1000-cc. Claisen distilling flask in a boiling water bath, until a thick paste forms. Take up the residue in 200 cc. of distilled water and again evaporate to a thick paste. Repeat this operation three or four times. After the last concentration, take up the residue with 600 cc. of distilled water; add 20 g. of the vegetable decolorizing carbon, Norit; heat the mixture to boiling for about 10 minutes, and filter. The filtrate should be clear and amber-colored; otherwise, repeat the treatment with 10-g. portions of the decolorizing carbon. Neutralize the filtrate with 20 per cent sodium hydroxide solution. A heavy white precipitate, which is tyrosine, separates. Place the mixture in a refrigerator for 2 or 3 days, filter on a Büchner funnel, and wash with a small quantity of ice-cold distilled water. Concentrate the filtrate under diminished pressure to at least one-half of its volume, and cool as before to obtain a second crop of crystals. The mother liquor from tyrosine contains glycine and alanine.

To purify the tyrosine, unite the two crops of crystals; place in 3–4 liters of distilled water; boil until dissolved, and then filter. Allow the filtrate to stand overnight in a refrigerator, again filter and wash.

[15] After C. A. Morrow and W. M. Sandstrom, "Biochemical Laboratory Methods," page 77. The silk waste can be supplied by Cheney Brothers, South Manchester, Connecticut.

Evaporate this filtrate to one-half its volume to obtain another crop of crystals. The yield of *l*-tyrosine is 8–10 per cent of the weight of the silk used. Examine the crystals under the microscope. Test for tyrosine, using very small quantities.

Experiment 27. Tests for Tyrosine. (*Denigè-Mörner Test.*) To a small quantity of tyrosine add 2–3 cc. of the sulfuric acid-formaldehyde solution [16] and heat to boiling. Observe the development of a green color.

Folin-Denis Test. To 1–2 cc. of a dilute solution of tyrosine add an equal volume of the Folin-Denis phenol reagent [17] and 5–10 cc. of a saturated solution of sodium carbonate. In the presence of even minute traces of tyrosine a blue color develops.

Apply the Millon reaction to a small amount of tyrosine.[18]

[16] Add 1 cc. of 40 per cent formaldehyde to 100 cc. of 50 per cent sulfuric acid, and mix.

[17] *Phenol Reagent.* Transfer to a 2-liter flask 750 cc. of distilled water, 100 g. of sodium tungstate, 20 g. of phosphomolybdic acid, 50 cc. of 85 per cent phosphoric acid (H_3PO_4) and 100 cc. of concentrated hydrochloric acid. Boil with a reflux condenser for 2 hours, cool, dilute to 1 liter with distilled water and filter if necessary. This reagent, which is usually deep straw-yellow in color, should not turn more than slightly blue when a sample (5 cc.) is rendered alkaline with sodium carbonate.

[18] A recently described color reaction for tyrosine utilizes α-nitroso-β-naphthol. Add 1 drop of a 1 per cent solution in alcohol of this reagent to 1 cc. of a solution of tyrosine (or protein). Heat to boiling, then add 1–2 drops of concentrated nitric acid. Note the development of a pink to deep purple color.

CHAPTER V

PART I—MILK

Experiment 1. The Proteins of Milk. To 50 cc. of fresh, skimmed milk in a beaker, add dilute hydrochloric acid, drop by drop, avoiding an excess. The precipitate is casein, the chief protein of milk. Allow the precipitate to settle, and filter. Wash the precipitate with alcohol and then with small amounts of ether. Test the solubility of the casein in acid and alkali. Is it heat-coagulable when in solution? Test a small amount of the casein for the presence of phosphorus.

Boil the filtrate from the casein in a casserole until it is concentrated to about one-third the original volume. Note the formation of a coagulum. This consists of lactalbumin and lactoglobulin. Filter. Test the coagulum for protein. Use the filtrate for the following experiment.

Experiment 2. The Carbohydrate of Milk. Evaporate the last filtrate from the preceding experiment until it becomes thick and syrupy. Allow to stand in a cool place overnight. Lactose will separate out. Establish the presence of this sugar by appropriate tests.

Experiment 3. Babcock's Method for the Determination of Fat in Milk.[1] By means of a specially graduated pipette, measure 17.6 cc. of milk into a Babcock bottle. Add 17.5 cc. of commercial sulfuric acid (sp. gr. 1.82), mix, and when the curd is dissolved whirl the test bottle in the Babcock centrifuge for 5 minutes at the proper speed. (The correct speed varies from 700 revolutions for a 24-in. wheel to 1000 for one of 10-in. diameter.) Remove, add boiling hot water up to the neck of the bottle, replace in the centrifuge, and whirl for 1 minute. Remove the bottle and again add boiling hot water so as to bring the fat within the scale of the neck of the bottle. After whirling for one more minute, read the length of the column, taking care to make the reading at a temperature of about 60° C. The reading gives the percentage of fat in the milk.

The following is a simplified procedure for determining the fat content of milk. Measure 5 cc. of well-mixed milk into a special Bab-

[1] For details of this and other methods, consult A. E. Leach, "Food Inspection and Analysis," revised and enlarged by A. L. Winton, John Wiley & Sons, Inc., New York, Fourth Edition (1920).

cock tube. Add sufficient sulfuric acid (sp. gr. 1.82) to fill the body of the tube. After mixing, fill the neck of the tube with a solution containing equal volumes of concentrated hydrochloric acid and amyl alcohol. Centrifuge for 1–2 minutes. Read off the percentage of fat by means of the graduations in the neck of the tube.

Experiment 4. Determination of Protein in Milk (Kjeldahl's Method for Nitrogen). The milk should be thoroughly mixed before sampling. Measure 5 cc. of the milk into a Kjeldahl flask (a flat-bottomed Pyrex flask of 700-cc. capacity may be used for this purpose). Add 20 cc. (*from a graduate*) of concentrated sulfuric acid, 5–10 g. of sodium sulfate, and a small crystal of copper sulfate. The sodium sulfate is added for the purpose of raising the boiling-point of the mixture. The copper sulfate acts as a catalyst. The presence of these substances accelerates the digestion (and oxidation) of organic material by sulfuric acid.[2] Heat the flask in the hood, *gradually* at first, increasing the flame somewhat after several minutes and continuing the heating until digestion is complete, about half an hour after the solution in the flask has become perfectly clear and colorless (or bluish or greenish, because of the presence of the copper sulfate). During digestion rotate the flask carefully to wash down any particles which may have lodged on the sides. Cool and dilute carefully with 300 cc. of distilled water. Cool again.

In the meantime, set up a condenser.[3] A glass delivery tube dips into a receiver (a milk bottle, flask, or beaker may be used for this purpose), containing 50 cc. of tenth-normal acid and 3–4 drops of an indicator.[4] Measure the acid by means of a pipette.

NOTE: Beginners should consult the instructor for the proper method of adding the alkali. Add to the cooled contents of the flask

[2] It is necessary to run a blank on the reagents in order to determine whether they contain nitrogen and, if so, the amount. Set up a flask containing the same quantities of the various reagents as in the determination and carry through the entire procedure. The amount of nitrogen thus found should be subtracted from the determination value in the calculation. As with other quantitative procedures, the determination should be done in duplicate and the results should check within 1 per cent of each other.

[3] NOTE TO THE STUDENT: The condenser and other apparatus used in the distillation should be absolutely clean, this being insured by distilling water (distilled) through the condenser before using it in the analysis.

[4] The titration is essentially between ammonium hydroxide and acid (sulfuric or hydrochloric). Alizarin red, Congo red, or a mixture of methyl red and methylene blue may be used. The mixed indicator may be prepared as follows: Mix 1 part of 0.5 per cent methylene blue in 95 per cent ethyl alcohol with 30 parts of 0.5 per cent alcoholic solution of methyl red.

a few drops of an indicator. Cautiously tilting the flask at an angle, pour down the side, so as to make a layer at the bottom of the liquid, 60–80 cc. of a saturated solution of sodium hydroxide (60 per cent). Set upright without agitating. Add a pinch of talc which will help to prevent foaming during the distillation.

Connect the flask with the condenser, taking care not to shake the contents before the connections are secure. By a gentle rotary motion mix thoroughly the contents of the flask, making sure that the layer of alkali is distributed throughout the liquid. At this point the mixture should be strongly alkaline as shown by the indicator. Without unnecessary delay place a lighted Bunsen burner beneath the flask and bring rapidly to a boil. Continue the boiling until 150–200 cc. of liquid have distilled over, or until bumping begins, owing to concentration of the contents. At this point adjust the end of the delivery tube to a height just above the acid in the receiver and allow distillation to proceed for several minutes. With the tube well out of the liquid extinguish the flame. Wash off the end of the condenser with a small amount of distilled water, adding washings to distillate. Titrate the residual acid with standard alkali. The difference between the residual acid and the original 50 cc. 0.1 *N* acid represents the acid neutralized by the ammonia derived from the nitrogen of the milk. One cubic centimeter of 0.1 *N* acid is equivalent to 0.0014 g. nitrogen. To calculate the amount of protein the result for total nitrogen is usually multiplied by the factor 6.38. (Explain.[5]) Calculate the protein content of (*a*) 100 cc. of milk, (*b*) a quart of milk.

PART II—BONE AND CONNECTIVE TISSUE

Experiment 5. Bone. The inorganic constituents of bone include calcium phosphate, calcium carbonate, magnesium phosphate, calcium fluoride, and iron. The inorganic matrix consists of collagen (bone collagen is usually called ossein), a mucoid, osseomucoid, and an albuminoid.

(*a*) In a beaker, cover a small piece of bone (rib bone or chicken

[5] This calculation does not take into account the non-protein nitrogen, representing such constituents as amino acids. Theoretically, the calculations for total protein should be based on the total nitrogen minus the non-protein nitrogen, but this is rarely done in practice.

The student may obtain practice with the Kjeldahl method, employing definite quantities of such nitrogenous organic substances as urea and uric acid. These should be of a high degree of purity.

bone) with 10 per cent hydrochloric acid. Let stand at room temperature for 3–4 days. Note effervescence. To what is this due?

When the bone has become flexible and translucent, remove it from the acid, wash it with water, and place it in a second beaker or in a small casserole. Cover with about 50 cc. of water and boil until the organic matrix of the bone goes into solution. On cooling a gel is formed. Explain.

(*b*) *Inorganic Constituents.* Filter the acid solution and add ammonium hydroxide to the filtrate until the reaction becomes alkaline. Then acidify with acetic acid. The precipitate formed at first dissolves on the addition of the acetic acid, leaving a residue of ferric phosphate. Filter, saving the filtrate. Dissolve the ferric phosphate with a little dilute hydrochloric acid. Filter and test portions of the filtrate for iron (1) with potassium ferrocyanide, (2) with ammonium thiocyanate. Test another portion for phosphate with ammonium molybdate and nitric acid.

Perform the following tests on the filtrate from the ferric phosphate:

Test a small portion of the filtrate for phosphate, using ammonium molybdate and nitric acid. Repeat the test, using uranium acetate.

To the remainder of the filtrate add a solution of ammonium oxalate (5 per cent). What is the precipitate? Filter. (If the filtrate is not clear, return it to the filter paper a second time or until it is clear.) To the clear filtrate add a few drops of the ammonium oxalate solution. If no precipitate is formed, add ammonia until the reaction is alkaline. Let stand. A crystalline precipitate of ammonium magnesium phosphate separates out.

Make an outline of the various steps in this procedure, explaining the chemistry involved and writing the equations for the reactions.

Experiment 6. Preparation of Elastin from Yellow Elastic Connective Tissue (*After G. W. Vandegrift and W. J. Gies*).[6] Cut into small pieces 10 g. or more of ox ligament (ligamentum nuchae) and place in 5 per cent sodium chloride solution for 3–4 days, adding thymol as a preservative and shaking at regular intervals. In this way the albumin and globulin are extracted. The salt solution is poured off and the material boiled vigorously in water, with repeated renewals, in order to convert the collagen into gelatin. When all the collagen has been hydrolyzed, repeated boiling with water will no

[6] *Am. J. Physiol.*, **5**, 287 (1901). For an alternate method see A. N. Richards and W. J. Gies, *Am. J. Physiol.*, **7**, 93 (1902). Consult these papers for a detailed discussion of the chemistry of yellow elastic connective tissue.

longer yield gelatin, as may be determined by testing a small portion of the solution in a test tube with tannic acid. When only a faint turbidity is obtained with tannic acid, filter off the undissolved residue and wash free from traces of dissolved protein and chloride. Dry at 110° C. Grind to a powder in a mortar.

Test portions of the powdered material (*a*) for protein, (*b*) for the presence of unoxidized sulfur. (*c*) Determine the solubility of elastin in dilute acid and alkali.

Experiment 7. Preparation of Tendomucoid and Gelatin from White Fibrous Connective Tissue[7] (*After W. D. Cutter and W. J. Gies*).[8] (*a*) *Tendomucoid.* Remove extraneous material, such as fascia, from a piece of tendon (Achilles tendon of the ox). Cut the tendon (about 20 g.) in small pieces and wash these in cold water. Transfer the washed material to a flask, add about 200 cc. of half-saturated calcium hydroxide (lime water) and let stand for 24 hours, shaking at frequent intervals. Filter off the residue, which consists largely of collagen, and save for part (*b*). Add dilute hydrochloric acid to the filtrate in order to precipitate the mucoid. Filter and wash the precipitate, first in dilute hydrochloric acid to remove adherent protein impurities, and then in water until free from acid.

Test portions of the tendomucoid for protein and for the presence of sulfur. Test the solubility of the tendomucoid in sodium chloride, acid, and alkali.

Treat a portion of the tendomucoid with 25 cc. of water to which 0.5 cc. of concentrated hydrochloric acid has been added. Boil until the solution has become dark brown. Neutralize and test the solution for the presence of reducing sugar (Benedict's test). Explain the result of this experiment.

(*b*) *Formation of Gelatin from Collagen.* After the extraction of the tendon with calcium hydroxide in part (*a*), the remaining material consists mainly of collagen. Wash the pieces with water to remove

[7] Gelatin may also be prepared from cartilage and bone. The matrix of cartilage consists principally of inorganic salts, chondromucoid, chondroitin-sulfuric acid, an albuminoid, chondroalbuminoid, and collagen. When cartilage is boiled in water for several hours, the collagen is hydrolyzed to gelatin. Nasal septa, thyroidal, cricoidal, tracheal, and aretynoidal cartilages of cattle are the more available forms of cartilage. For the methods of preparation of chondroitin sulfuric acid, consult P. A. Levene, "Hexosamines and Mucoproteins," Longmans, Green & Co., London (1925), pages 113–114.

[8] *Am. J. Physiol.*, **6**, 155 (1901–2). See also the paper of L. Buerger and W. J Gies, *Am. J. Physiol.*, **6**, 219 (1901–2) for a description of the chemical constituents of tendon tissue.

the lime water, transfer to a casserole or beaker, add 150 cc. of water, and boil for 3–4 hours, adding water at intervals to replace that lost by evaporation. Filter while hot, concentrate the filtrate by evaporation to about one-third the original volume, and allow to cool.

Apply the various protein reactions to pieces of the gelatin or to a solution of the same.

CHAPTER VI

DIGESTION

The activity of enzymes is inhibited by a large variety of impurities. Hence it is important that glassware and other apparatus used in the following experiments should be very clean. The glassware may be treated with cleaning mixture, rinsed with water, then with strong nitric acid, again thoroughly rinsed with water, washed with soap and hot water, and finally rinsed with hot distilled water. It may then be allowed to drain until dry. If the necessary precautions are taken at the beginning, much time and effort will be saved, and successful experiments made possible.

SALIVA

Experiment 1. (a) Collection of the Saliva. Rinse out the mouth with distilled water. Then chew a piece of paraffin to stimulate the flow of saliva. Collect the saliva in a funnel fitted with an ashless filter paper. The filtered saliva is used in the following experiments. If the experiments should require more than one day, fresh saliva should be collected each day.

(b) Reaction. Test the reaction of fresh saliva to litmus, phenolphthalein, and Congo red. What is the approximate *p*H?

Dilute 2 cc. of saliva [1] with 3 cc. of water. (The water should be neutral in reaction. Recently boiled and cooled distilled water is to be preferred.) Add 2 drops of phenol red and compare with color standards containing this indicator and prepared from standard buffer solutions of known *p*H (*p*H 6.8 to 8.4). If the saliva is more acid than *p*H 6.8, treat another portion of saliva as before, using bromthymol-blue as the indicator, and compare with a set of color standards ranging in *p*H from 6.0 to 7.0.

(c) Mucin. To 15 cc. of saliva in a test tube add several drops of dilute acetic acid, a drop at a time. A stringy precipitate of mucin is formed. If the precipitate does not settle out, try stirring gently so that the stringy mass collects on the end of the rod and can be re-

[1] For the determination of *p*H, the saliva is preferably collected in a test tube containing neutral paraffin oil. The layer of oil diminishes the loss of CO_2.

moved for testing. Or allow to stand overnight for more complete precipitation. Filter, saving the filtrate for (*d*). Test a portion of the precipitate for solubility in 10 per cent acid and 10 per cent alkali. Test another portion with Millon's reagent.

(d) Inorganic Constituents. Test the filtrate obtained in (*c*) for the presence of chlorides, phosphates, sulfates, nitrites, and calcium.[2]

Experiment 2. The Digestion of Starch by Ptyalin. Measure 20 cc. of a solution of soluble starch (1 per cent) into a small beaker which is placed in a water bath maintained at 40° C. When the solution reaches this temperature, add 10 drops of filtered saliva and stir thoroughly. At 1-minute intervals remove a drop of the digest and add it to a drop of a very dilute solution of iodine in a depression of a test tablet. (The stock iodine solution should be diluted with distilled water to a pale straw-yellow color.) At the same time remove 1 cc. of the solution and test for reducing sugar by means of Benedict's reagent. (A series of test tubes, each containing 5 cc. of previously heated Benedict's reagent, should be held in readiness for this purpose. After addition of a 1-cc. portion of the digest, the reduction test may await completion until after the iodine tests have been completed.) Observe the various color changes obtained with iodine as the digestion progresses. Note the time required for (1) the disappearance of the opalescence of the solution, (2) the appearance of reducing sugar, and (3) the disappearance of the "iodine" reaction.

The achromic point is that stage in the digestion of starch at which the last trace of erythro-dextrin (gives a red color with iodine) is converted to achroo-dextrin (gives no color with iodine). If the achromic point is reached in less than 4 minutes, repeat the experiment, using less saliva. On the other hand, if the digestion of starch appears to be very slow, use more than 10 drops of saliva.

Compare the results of this experiment with those of Experiment 24, page 38. Are the end products the same?

Experiment 3. The Effect of Temperature on Salivary Digestion. To each of four test tubes, add 5 cc. of a soluble starch solution. Place one tube in a boiling water bath, one in a bath at 40–45° C., one at room temperature, and the last in a mixture of ice and salt. Allow the contents of the tubes to reach the surrounding temperature, and then

[2] Use the standard qualitative tests. If necessary, review the procedures in a textbook on qualitative analysis. To test for nitrites add 2 drops of 10 per cent H_2SO_4 to 1 cc. of saliva. Mix and add 2 drops of 10 per cent KI and a drop of starch solution. In the presence of nitrous acid, iodine is liberated and turns the starch blue.

add 1 cc. of undiluted saliva to each. Follow the progress of digestion in each tube by testing with iodine. In which tube is digestion most rapid? Is the enzyme destroyed by heat? By cold? Demonstrate this by placing tubes 1 and 4 in the water bath at 40° C. and observing the effect on digestion. What is the optimum temperature for salivary digestion?

Experiment 4. The Effect of Acids and Alkalies on Salivary Digestion. To each of a series of seven test tubes add 4 cc. of a 1 per cent solution of soluble starch. To tube 1, add 1 cc. of 0.5 *N* hydrochloric acid; to tube 2, 1 cc. of 0.05 *N* hydrochloric acid; to tube 3, 1 cc. of 0.005 *N* hydrochloric acid; to tube 4, 1 cc. of distilled water; to tube 5, 1 cc. of 0.005 *N* sodium hydroxide; to tube 6, 1 cc. of 0.05 *N* sodium hydroxide; and to tube 7, 1 cc. of 0.5 *N* sodium hydroxide. Place the tubes in a water bath and bring to a temperature of 40° C. Now add to each tube 5 drops of filtered saliva. Mix well. Test the contents of tube 4 with iodine from time to time, and, when the achromic point is reached, remove all the tubes from the bath, neutralize their contents rapidly, and test each with iodine and with Benedict's reagent. Tabulate the results.

Experiment 5. The Effect of Electrolytes on Salivary Digestion. Place 10 cc. (or more) of saliva in a dialysis bag[3] and suspend in a large beaker of distilled water. Change the water at frequent intervals after testing it for the presence of chloride. When the chloride test becomes negative, proceed as follows:

Measure 2 cc. of a 1 per cent starch solution into two test tubes. To one add 2 cc. of distilled water; to the other 2 cc. of a 0.5 per cent solution of sodium chloride. Immerse the tubes in a water bath at 40° C. To each tube add 1 cc. of the dialyzed saliva and follow the course of digestion by means of the iodine test. Explain the results.

Experiment 6. Is Enzyme Action Influenced by the Presence of Antiseptics? To each of five test tubes containing 5 cc. of starch solution, add one of the following: 2 drops of toluene, 2 drops of chloroform, 2 drops of a 1 per cent solution of mercuric chloride, 2 drops of a 2 per cent solution of phenol, 0.5 g. of sodium fluoride. Prepare a sixth test tube to contain only the starch for use as a control. Immerse the tubes in a water bath, and, when their contents have reached 40° C., add to each of the six tubes 1 cc. of dilute saliva (1 part of saliva diluted with 4 parts of water). Follow the

[3] A celloidin sac, a parchment-paper bag, or cellophane sausage casing may be used.

progress of digestion by means of the iodine test. Tabulate the results and explain them.

Experiment 7. The Salivary Glands as a Path of Excretion: Potassium Iodide. Place 0.2 g. of potassium iodide in a small gelatin capsule and swallow it, rinsing out the mouth immediately afterward with distilled water. At intervals of several minutes, test the saliva for iodides, noting the time expiring between the ingestion of the capsule and the appearance of iodides in the saliva.

To test the saliva for iodides place about 1 cc. of saliva in a test tube, acidify with dilute sulfuric acid, and add a few drops of sodium nitrite solution. Then add a drop or two of starch paste. A blue color appearing at this point shows the presence of iodides in the saliva. Upon what reactions does this test depend? Write the equations. Does the result of this experiment depend upon the rate of emptying of the stomach?

Experiment 8. The Excretion of Thiocyanate in the Saliva. Place 1 cc. of saliva in a small porcelain dish and add 2–3 drops of a dilute solution of ferric chloride. Acidify with a drop of dilute hydrochloric acid and note the formation of the red ferric thiocyanate. If the color is due to ferric phosphate, it will not disappear on adding a few drops of a solution of mercuric chloride; if, on the other hand, thiocyanate is responsible for the color, the addition of the mercuric chloride solution will render the solution colorless. Why?

GASTRIC DIGESTION

Experiment 9. Extraction of the Gastric Enzymes.[4] Turn a pig's stomach inside out, wash with water, and dissect or strip off the pinkish mucous membrane. Grind in a meat chopper. Place two-thirds of the ground tissue in a flask and mix with 200 cc. of 0.1 *N* hydrochloric acid. Add toluene. Set away in an incubator at 40° C. until the following day, filter, and save the filtrate for use in the following experiments.

Place the remaining one-third of the gastric mucosa in a flask, add 50 cc. of glycerol, stir well, and allow to stand at room temperature for at least 24 hours. The filtered glycerol extract is suitable for use

[4] When it is not feasible to prepare tissue extracts, commercial preparations of pepsin may be used with equal success. A 2 per cent solution of pepsin in 0.1 *N* hydrochloric acid should be satisfactory for most purposes. A 2 per cent solution in water should also be prepared for use where a neutral extract is indicated.

in the following experiments where a neutral pepsin preparation is called for.

Experiment 10. Optimum reaction of Peptic Digestion. Measure 4 cc. of the neutral pepsin solution into each of three test tubes. Add to (1), 1 cc. of 0.5 *N* HCl; to (2), 1 cc. of 0.5 *N* Na_2CO_3; to (3), 1 cc. of water. Incubate at 40° C., and when the contents of the tubes have reached this temperature, add a tiny piece of protein,[5] just large enough to be visible, to each tube. In which tube does digestion proceed most rapidly?

Experiment 11. Is Hydrochloric Acid Essential for Peptic Activity? Prepare a series of test tubes as follows:

1. 5 cc. of pepsin-hydrochloric acid solution.
2. 5 cc. of 0.1 *N* hydrochloric acid.
3. 5 cc. of neutral pepsin solution.
4. 2.5 cc. of neutral pepsin solution and 2.5 cc. of 0.2 *N* hydrochloric acid.
5. 2.5 cc. of neutral pepsin solution and 2.5 cc. of 0.01 *N* hydrochloric acid.
6. 2.5 cc. of neutral pepsin solution and 2.5 cc. of 0.2 *N* sulfuric acid.
7. 2.5 cc. of neutral pepsin solution and 2.5 cc. of 0.2 *N* lactic acid.
8. 2.5 cc. of neutral pepsin solution and 2.5 cc. of 0.2 *N* acetic acid.

Place all the tubes, after numbering them, in a bath at 40° C. When the contents of the tubes have reached this temperature, introduce into each tube a small piece of fibrin or coagulated egg white. Observe the progress of digestion and record the results. Does hydrochloric acid alone digest protein? Does pepsin without acid digest protein? Does pepsin act in an alkaline solution? What is the effect of acids other than hydrochloric acid on the activity of pepsin?

Experiment 12. Gastric Rennin. Prepare a series of test tubes as follows:

(*a*) 5 cc. of fresh milk to which 0.1 *N* hydrochloric acid is added, a drop at a time, until a precipitate results.

(*b*) 5 cc. of fresh milk.

[5] Fibrin, coagulated egg white, or a suspension of coagulated egg albumin may be used in this and later experiments. The suspension may be prepared by pouring filtered and diluted egg white into boiling water, faintly acidified with acetic acid, stirring vigorously so that the particles remain finely divided. Substitute 5 cc. of this suspension for the piece of fibrin or cube of coagulated egg albumin.

(*c*) 5 cc. of fresh milk.
(*d*) 5 cc. of fresh milk + 5 drops of 0.1 *N* hydrochloric acid.
(*e*) 5 cc. of fresh milk + 10 drops of 0.1 *N* sodium carbonate.
(*f*) 5 cc. of fresh milk + 10 drops of a saturated solution of ammonium oxalate.

Place all the tubes in a water bath or incubator at 40° C., and then add to each of tubes (*b*), (*d*), (*e*), and (*f*) 5 drops of a neutralized commercial rennin solution. To the contents of tube (*c*) add 5 drops of previously boiled rennin solution. Allow the tubes to remain in the water bath or incubator, and observe at the end of 10 and 15 minutes. Explain the results. How does ammonium oxalate prevent the coagulation of milk by rennin? What is the difference between the precipitates in tubes (*a*) and (*b*)? How does the reaction influence the activity of rennin?

To the contents of tube (*f*) add a 10 per cent solution of calcium chloride, a drop at a time. Note what happens and explain the result.

Is rennin present in the gastric mucosa in an active or an inactive form? Verify your answer by experimenting with the glycerol extract prepared in Experiment 9.

Experiment 13. The Products of Peptic Digestion. Combine the acid and glycerol extracts of the gastric mucosa prepared in Experiment 9 (or use commercial pepsin) in a flask, and add finely ground coagulated egg white or hashed, lean meat. Add 0.1 *N* hydrochloric acid to the mixture and shake well. Test for the presence of *free* hydrochloric acid by means of Töpfer's reagent (see Experiment 14). Add more acid if the test is negative. Continue shaking the mixture at intervals and add more acid if necessary. Incubate at 40° C. for two or three days, testing from time to time for the presence of free acid and adding more if required. Unless free hydrochloric acid is present, bacterial action will set in and the digest will putrefy. As an additional precaution, the digest may be preserved with toluene.

After digestion has proceeded sufficiently, filter off the undigested residue.

To 5 cc. of the filtrate, made slightly alkaline with sodium carbonate and then acidified with acetic acid, add a few drops of bromine water. A pinkish-lavender color indicates the presence of free tryptophane. Does gastric digestion proceed to the free amino-acid stage?

Neutralize the remaining filtrate with sodium hydroxide. What precipitates at the neutral point? Filter off any precipitate that may have formed, and identify it by appropriate tests.

To the filtrate, add solid ammonium sulfate to saturation. What precipitates? Identify the precipitate and the filtrate from this precipitate by appropriate tests.

Experiment 14. Free and "Combined" Hydrochloric Acid. Treat 5 g. of finely ground, dried egg albumin with 100 cc. of 0.1 *N* hydrochloric acid, until most of the protein has dissolved. Filter through a coarse filter. Titrate 10-cc. portions of the filtrate with 0.1 *N* sodium hydroxide, using phenolphthalein as the indicator. Calculate the total acidity in terms of cubic centimeters of 0.1 *N* acid per 100 cc. of the solution.

Repeat the titration, using dimethylamino-azo-benzene (Töpfer's reagent) as the indicator. Calculate the free hydrochloric acid present in 100 cc. of the mixture.

The combined hydrochloric acid, which represents the acid in combination with protein, is calculated by subtracting the value for free acidity from the value for total acidity.

What is the range of phenolphthalein; of Töpfer's reagent? Explain the use of these indicators in these two titrations.

Experiment 15. Analysis of Gastric Contents.[6] (*a*) *Free Hydrochloric Acid.* Titrate a 10-cc. specimen of strained gastric contents with 0.1 *N* sodium hydroxide, using dimethylamino-azo-benzene as the indicator. Calculate the amount of free hydrochloric acid in per cent

[6] Artificial gastric contents for use in this experiment may be prepared by dissolving dried egg albumin in hydrochloric acid. To this may be added various abnormal constituents, such as blood, lactic acid, and bile, if tests for these are to be made. Peptone heated with acid until the titration values reach the desired figures is also a convenient method of preparation.

If practicable gastric juice may be obtained from students who volunteer as subjects for fractional gastric analysis. Breakfast is omitted if the laboratory period is in the morning. However, if the laboratory period is in the afternoon, the noon-day meal is to be omitted. In clinical work, it is conventional to make the test in the morning.

The subject swallows a Rehfuss tube (or a duodenal tube of a similar type), and the stomach contents are removed by means of a large syringe. The volume is measured, the acidity (free and total) determined on a portion strained through cheesecloth, and a microscopic examination made of the sediment. Tests should also be made for the presence of blood, bile, and lactic acid.

The tube is removed and the test meal taken. This may consist of 200–250 cc. of weak tea, or water, and a roll, or 4–5 crackers. After half an hour the stomach tube is again introduced and a specimen (10–15 cc. if possible) removed for analysis. The tube is left in position, a little air being blown back from the syringe through the tube so that no fluid remains to affect the next sampling. Specimens are withdrawn at intervals of 15 minutes and labeled accordingly. The free and total acidity of each specimen is determined by titration.

HCl and in terms of cubic centimeters of 0.1 *N* acid in 100 cc. of the gastric contents.

(*b*) *Total Acidity; "Combined" Hydrochloric Acid.* Titrate 10 cc. of the strained gastric contents, using phenolphthalein as the indicator. Calculate the total acidity as before in per cent HCl and in cubic centimeters of 0.1 *N* acid in 100 cc. From this value subtract the result obtained in the titration with Töpfer's reagent in (*a*). The difference represents the "combined" acidity, i.e., the acid present in combination with protein, and organic acids.

If the amount of gastric juice is insufficient for the analyses as outlined, smaller amounts may be used (as little as 1 cc.) and more dilute alkali (0.01 *N*) employed in the titrations. Another alternative is to titrate 10 cc. of the gastric contents with Töpfer's reagent to its end point, recording the result, then adding phenolphthalein to the same specimen and continuing the titration to its end point. The first titration represents the free acid; the total alkali used in reaching the phenolphthalein end point represents the total acidity.[7]

What are the normal concentrations of total and free acidity and what changes occur pathologically? What other constituents are found in the gastric juice, normally and pathologically? [8]

[7] **Determination of Titratable Acidity (Method of Helmer and Fouts).** Gastric acidity may be determined by titration to a *p*H of 7.0, using phenol red as the indicator. Transfer to a 60-cc. porcelain dish 1 cc. of gastric contents, dilute with 10 cc. of distilled water, and add 2 drops of phenol red (0.1 g. of indicator + 5.7 cc. of 0.05 *N* NaOH + water to 100 cc.). Measure into a second dish 5–6 drops of a *p*H 7 phosphate buffer solution (61.1 cc. of *M*/15 Na_2HPO_4 + 38.9 cc. of *M*/15 KH_2PO_4), dilute with 10 cc. of distilled water, and add 2 drops of the phenol red indicator. Titrate the contents of the first dish with 0.01 *N* NaOH until the color matches that of the control solution. From the result thus obtained calculate the titratable acidity in terms of the customary clinical units (cubic centimeters of 0.1 *N* NaOH required to neutralize 100 cc. of gastric contents).

As pointed out by Helmer and Fouts (*Am. J. Clin. Pathol.*, **7**, *Technical Supplement*, **1**, 43 (1937), the titration to *p*H 7.0 yields information that is of greater physiological significance than that obtained by titrating to the dimethylamino-azo-benzene end-point (*p*H 2.8). Gastric juice with a *p*H of 7, or below, contains pepsin and rennin, whereas, when the *p*H is above 7, no pepsin or rennin is present.

[8] At this stage the student should refer to other sources for comprehensive descriptions of the methods employed clinically in the collection and analysis of gastric contents, and should familiarize himself with the clinical interpretation of the results of various analytical procedures. Read, for example, J. C. Todd and A. H. Sanford's "Clinical Diagnosis by Laboratory Methods," W. B. Saunders Company.

Lactic Acid. Specimens of gastric juice with a low free acidity should be tested for lactic acid. Dilute a few drops of ferric chloride solution with distilled water in a test tube until the color is but faintly yellow. Divide this solution into two portions, and to one portion add 1 cc. of the gastric juice. Compare the color produced with that of the untreated dilute ferric chloride solution. Lactic acid produces a distinct canary-yellow color.

A somewhat more satisfactory procedure is the *Strauss Test.* Shake 5 cc. of the strained gastric contents with 20 cc. of ether in a separatory funnel. After the two layers have separated, run out all but the top 5 cc. of the ether. To this add 20 cc. of water and 2 drops of ferric chloride solution (5 or 10 per cent) and shake. Depending on the content of lactic acid, a greenish-yellow to a much more intense yellow-green color will develop in the aqueous layer.

Uffelman's Reaction, though not specific, is also useful. Add dilute ferric chloride solution to a 1 per cent solution of phenol until an amethyst-blue color has developed. To 5 cc. of this reagent add an equal volume of strained gastric juice (or better, an aqueous solution of the residue obtained on evaporating the ether extract of strained gastric juice). A greenish-yellow or canary-yellow color will develop in the presence of lactic acid.

Run control tests with (*a*) a very dilute solution of lactic acid, (*b*) dilute hydrochloric acid, (*c*) dilute tartaric acid, (*d*) water. Tabulate the results.

Blood. Employ the tests given on page 202. *Bile.* Employ the tests given on page 142.

Experiment 16. Determination of the Hydrogen-ion Concentration of Gastric Contents. (*Colorimetric Method of Shohl and King.*) [9] Prepare Clark and Lubs' standards for *p*H 1.4, 1.6, 1.8, 2.0, 2.4, and 3.0.[10] (For very accurate work, standards for every 0.1 *p*H should be prepared.) The gastric contents, removed 1 hour after an Ewald test meal, are filtered or centrifuged. To 2 cc. of the contents in a test tube, 11 mm. in diameter, add 2 drops of a 0.2 per cent solution of thymolsulfonphthalein in 95 per cent alcohol. (The standards should be prepared in the same way, i.e., 2 cc. of the solution with 2 drops of

[9] Shohl, A. T., and King, J. H., *Johns Hopkins Bull.*, **31**, 158 (1920). The *p*H of gastric juice may be determined colorimetrically, using as little as 1 drop of material, according to a method described by J. H. Brown, *J. Lab. and Clin. Med.*, **9**, 239 (1924). See also Helmer and Fouts.[7]

[10] For the preparation of suitable buffer mixtures for the range, *p*H 1.0–2.2, see page 278.

the indicator in a tube of similar size.) Compare with the standards and record the result.

PANCREATIC AND INTESTINAL DIGESTION

Experiment 17. Extraction of Pancreatic Enzymes.[11] Grind a pig's or sheep's pancreas in a meat grinder after removing the fat. Place the ground pancreatic tissue in a flask and add 100 cc. of water and 50 cc. of 95 per cent alcohol. Shake well and allow to stand for 24 hours or somewhat longer. Strain the alcoholic extract through muslin or cheesecloth. Test the reaction of the extract. If not neutral, neutralize, being careful not to go beyond the neutral point.

Experiment 18. Pancreatic Amylase. Measure 125 cc. of starch solution [12] into a 250-cc. Erlenmeyer flask and place in the water bath or incubator at 40° C. When the contents of the flask have reached this temperature, add 25 cc. of pancreatin solution [13] and 5 cc. of toluene, to act as a preservative against bacterial action. Mix and return to the water bath or incubator, shaking the contents from time to time.

NOTE: After pancreatin is added, the starch mixture should have a reaction of about *p*H 7.0, which is the optimum for the activity of pancreatic amylase.

At the end of half an hour, after the toluene has been allowed to rise to the surface, remove 50 cc. of the digest by means of a pipette, boil to arrest digestion, transfer quantitatively to a 100-cc. volumetric flask, cool to room temperature, and dilute to the mark. Determine the amount of reducing sugar present by means of Benedict's quantitative procedure or some other suitable method.

At the end of 2 hours, remove 25 cc. of the digest by pipette, boil, dilute to 100 cc., as above, and again determine the sugar content.

[11] Instead of the extract described in this experiment, commercial preparations of trypsin and pancreatin may be employed in the experiments on pancreatic digestion.

[12] *Starch Solution.* The following directions give the quantities for eight experiments. Weigh out 24 g. of soluble starch and in a mortar stir to a paste with about 250 cc. of water. Test the reaction, and if necessary, make neutral to litmus with 0.1 *N* sodium carbonate. Pour the suspension into about 400 cc. of boiling water and stir vigorously. Cool, and to the mixture add 3.6 g. of C.P. sodium chloride and 0.24 g. of disodium phosphate, stirring to dissolve the salts. Transfer to a liter volumetric flask and dilute to the mark with distilled water.

[13] The alcoholic extract of the pancreas, prepared in Experiment 18, may be used, or a suspension of commercial pancreatin (50 mg. of pancreatin in 100 cc. of water).

Allow the digestion to proceed 24 hours and again remove 25 cc. of the digest. As before, determine the sugar content.

From the data obtained in this way, plot a curve in which the time in hours is represented as the abscissae, and the amount of reducing sugar, in milligrams of glucose per 5 cc. of digest, is represented as the ordinates.[14]

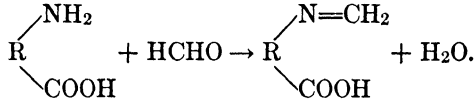
Experiment 19. The Hydrolysis of Fat by the Pancreatic Lipase, Steapsin. Prepare an emulsion of olive oil by adding to 25 cc. of the oil 2 drops of a 1 per cent alcoholic solution of phenolphthalein, and titrating to a very faint pink with 0.01 *N* sodium hydroxide, *shaking vigorously* after each addition of alkali. Place 5 cc. of this emulsion in each of five test tubes, and place the tubes in a water bath at 40° C. When the contents of the tubes have reached this temperature, add to tube (*a*) 1 cc. of the pancreatic extract and 1 cc. of water; to tube (*b*) 1 cc. of pancreatic extract and 1 cc. of a 1 per cent solution of bile salts; to tube (*c*) 1 cc. of *boiled* pancreatic extract and 1 cc. of the 1 per cent solution of bile salts; to tube (*d*) 1 cc. of bile salts; and to tube (*e*) no addition is made. Allow the tubes to remain at 40° C. overnight; then transfer the contents of each tube to a small beaker, washing out each tube with two 10-cc. portions of alcohol, and adding these washings to the appropriate beaker. Titrate the contents of each beaker with 0.01 *N* sodium hydroxide, using 5 drops of the phenolphthalein solution as the indicator. Tabulate and explain the results.

Experiment 20. Litmus Milk Test. Into each of two test tubes measure 5 cc. of milk and 1 cc. of litmus solution. Warm to 40° C. in a water bath. Add to one tube 2 cc. of pancreatic extract, and to the other 2 cc. of boiled pancreatic extract. Keep the tubes in the water bath at 40° C., and observe from time to time. Explain the results.

Experiment 21. The Hydrolysis of Ethyl Butyrate. Using an Ostwald-Folin pipette, add to each of two test tubes 1 cc. of ethyl butyrate. Then add to one of the tubes 4 cc. of pancreatic extract, and to the other 4 cc. of boiled pancreatic extract. Place in the incubator at 40° C. overnight. Remove, and add to the contents of each tube 5 cc. of water and 5 drops of phenolphthalein solution. Titrate with 0.01 *N* sodium hydroxide. What difference do you find in the two titrations? Explain fully.

[14] Compare the results obtained in this experiment with those of Walton, J. H., and Dittmar, H. R., *J. Biol. Chem.*, **70**, 713 (1926).

Experiment 22. The Course of Tryptic Digestion as measured by Sørensen's Formol Titration Method.[15] Sørensen's formol titration method is based on the well-known reaction of amino acids with formaldehyde.



The methylene derivatives of amino acids are strongly acid in reaction, because the basic properties of the amino groups have been destroyed. The amount of free carboxyl groups may be determined by titration with standard alkali.

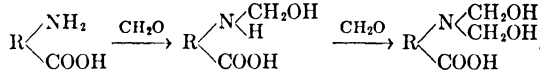
During the course of protein digestion, the number of free amino and carboxyl groups increases. To measure this increase quantitatively, samples of the digest are removed, formaldehyde is added, and the free carboxyl groups are titrated with a standard base.

Measure 180 cc. of the casein solution [16] into a 250-cc. flask, add 10 cc. of toluene, and place in the incubator at 40° C. When the solution reaches this temperature, the trypsin preparation will be added, but before this step is taken the student should make the necessary preparations to perform the formol titration.

Take four large, clean test tubes, of approximately the same diameter and of at least 100-cc. capacity, and label them 1, 2, 3, and 4.

[15] Based upon an experiment by Cole, in S. W. Cole's "Practical Physiological Chemistry," Seventh Edition, W. Heffer and Sons, Ltd., Cambridge (1926), page 261.

Recent studies have apparently proved that an amino acid may combine with either one or two formaldehyde groups as follows:



See Bodansky, "Introduction to Physiological Chemistry," 4th ed., p. 96.

[16] *Preparation of the Casein Solution.* Five hundred grams of commercial casein are worked into a paste with 2500 cc. of cold water. Transfer to a 6-liter flask with 2500 cc. of boiling water; add 125 cc. of 10 per cent sodium hydroxide and shake well. The casein should dissolve. Remove 5 cc. and titrate with 0.1 *N* sodium hydroxide to a reddish-purple color, using cresol red as the indicator. Calculate the quantity of *N* sodium hydroxide needed for the whole amount and add it with constant stirring. The *p*H of the mixture should now be about 8.1, which is midway in the *p*H range of cresol red and just acid to phenolphthalein. This *p*H is approximately the optimum for the action of trypsin.

Then make them up according to the following chart, omitting for the time being the addition of the digest in tubes 2 and 3.

Tube No	1	2	3	4
Digest, cc	0	20	20	0
Water, cc	50	55	25	75
Buffer solution (pH8.5)*, cc	25	0	0	0
Phenolphthalein (0.5 per cent), drops	20	0	20	0
Neutral formol,† cc	0	0	30	0

* The buffer solution may be prepared as follows. To 50 cc of 0.2 *M* boric acid in 0.2 *M* potassium chloride, add 10.4 cc of 0.2 *N* sodium hydroxide and dilute to 200 cc

† Titrate 5 cc of formol, 10–15 cc of water and 2 drops of phenolphthalein, to a faint pink color with 0.2 *N* sodium hydroxide. Calculate the amount of base needed to neutralize enough formol for the day's experiment. Prepare fresh each day.

When the casein solution has reached the temperature of the incubator (40° C.), add to it 20 cc. of pancreatic extract,[17] shake thoroughly, remove *immediately* two 20-cc. portions of the mixture by means of a pipette, and deliver into tubes 2 and 3. Replace the remainder of the digest in the incubator.

Titration. Titrate tube 3 with 0.2 *N* sodium hydroxide until the color effect appears the same on looking horizontally through tubes 4 and 3 (4 in front of 3) as on looking through the control tubes 1 and 2 (1 in front of 2).[18]

If more than 2 cc. of alkali is required in tube 3, add a similar volume of water to each of tubes 1 and 2. (Thus, if 5 cc. of 0.1 *N* sodium hydroxide was added to tube 3 before the end point was reached, add 5 cc. of water to each of tubes 1 and 2, and complete the titration. Very often it is difficult to obtain a satisfactory end point, owing to the opacity of the casein solutions. It is therefore recommended that after the titration has been completed, as has just been described, sufficient water should be added to each tube to bring up the volume to approximately 100 cc. The colors are again matched, and if a satisfactory end point was not obtained previously, the titration should be completed.)

At intervals of ½, 2, and 24 hours, remove additional samples of the digest and repeat the titration.

[17] Two per cent commercial trypsin in water; shake well before using.

[18] In matching the colors in this experiment it is convenient to place the tubes in a comparator block. For a description of Cole and Onslow's comparator, see S. W. Cole, "Practical Physiological Chemistry," W. Heffer and Sons, Ltd., Cambridge (1926), Fig. 45, page 331.

Calculation. One cubic centimeter of 0.1 *N* sodium hydroxide is equivalent to 1.4 mg. of amino-acid nitrogen.

If *a* is the amount of 0.1 *N* sodium hydroxide required for 20 cc. of the digestion mixture immediately after the addition of the enzyme, and *b* the amount after an interval of *t* minutes, then $(b - a) \times 5 \times 1.4$ equals the number of milligrams of amino-acid nitrogen liberated in 100 cc. of the digestion mixture in *t* minutes.

Plot a curve, based on your experimental data, representing the progress of tryptic digestion of casein, with time, in hours, as the abscissae and the milligrams of amino-acid nitrogen as the ordinates.

Experiment 23. Bromine Test for Free Tryptophane. Boil 15 cc. of the digest obtained in the preceding experiment after 24 hours of digestion. Acidify with acetic acid, while boiling. Cool and filter. To the filtrate add a few drops of saturated bromine water. The development of a pink, violet, or reddish color indicates the presence of tryptophane. What are the products of tryptic digestion?

Experiment 24. Preparation of Intestinal Extract. Scrape the mucous membrane of the washed duodenum and jejunum of a pig's intestine. Grind the scrapings with washed sand in a mortar, transfer to a flask, and add 50 cc. of 1 per cent sodium chloride and 5 cc. of toluene. Allow to stand for about 24 hours at room temperature, shaking frequently. (A commercial preparation of dried intestinal mucosa may be used instead of the fresh intestinal mucosa.)

Experiment 25. "Erepsin." The properties once attributed to erepsin are now ascribed to the two enzymes, aminopolypeptidase and dipeptidase.

Into each of two test tubes (*a* and *b*) introduce 5 cc. of a 1 per cent solution of peptone; into a third tube (*c*) introduce 5 cc. of a 1 per cent solution of egg albumin, and into a fourth tube (*d*), 5 cc. of a 1 per cent solution of casein.

To about 10 cc. of the intestinal extract prepared in the preceding experiment, add a dilute solution of sodium carbonate, drop by drop, until the reaction becomes *faintly* alkaline. Add 2 cc. of this alkaline extract to each of tubes *a*, *c*, and *d*. Heat the remainder of the alkaline extract to boiling, cool, and add 2 cc. to the contents of tube *b*. Add toluene to the contents of each tube, stopper the tubes, and set them away in an incubator at 40° C. for 2–3 days. Then test the contents of each tube by means of the biuret reaction, using in each test the same amounts of digestion mixture, sodium hydroxide, and dilute copper sulfate. Compare the intensities of the colors produced. Also

test the contents of each tube for free tryptophane. Record and explain the results.

Experiment 26. Sucrase in Intestinal Extract. To each of two test tubes add 5 cc. of a dilute solution of sucrose. To one, add 2 cc. of intestinal extract, and to the other 2 cc. of *boiled* intestinal extract. Incubate the tubes at 40° C., in the presence of toluene as a preservative, until just before the end of the laboratory period. At this time, test for reducing sugar (Benedict's test), using a small portion of the contents of each tube. If inversion has not proceeded far enough, replace the tubes in the incubator. At the next laboratory period, test again for reducing sugar. Explain the results.

Experiment 27. Lactase in Intestinal Extract. This experiment is not likely to be successful unless the intestinal mucosa (or commercial preparation) used is that of a young, suckling animal. To determine whether lactase is present, proceed as follows: To each of two test tubes, add 5 cc. of a dilute solution of lactose. To one add 2 cc. of intestinal extract, and to the other 2 cc. of *boiled* intestinal extract. Place the tubes in the incubator for about 24 hours. Then transfer the contents of each tube to a fermentation tube, after adding a small amount of yeast. Set aside in a warm place. Observe from time to time. Explain the results.

BILE

Experiment 28. Reaction. Test the reaction of fresh ox bile to phenolphthalein, litmus, and Congo red.

Experiment 29. Gmelin's Test for Bile Pigments. Place 3–5 cc. of concentrated nitric acid in a test tube. By means of a pipette introduce 2–3 cc. of diluted bile into the tube so as to form a layer on top of the acid. Note the succession of colored rings within the zone of contact of the two fluids.

Filter a small amount of the diluted bile through a small filter paper. Unfold the paper and place a drop of concentrated nitric acid in the center. Note the succession of colors produced. This is Rosenbach's modification of Gmelin's test.

Explain the chemistry of Gmelin's test.

Experiment 30. Hammarsten's Reaction for Bilirubin. To 1 volume of Hammarsten's acid mixture,[19] add 4 volumes of 95 per cent

[19] Hammarsten's acid mixture is prepared by mixing 1 volume of 25 per cent nitric acid with 19 volumes of 25 per cent hydrochloric acid, and allowing to stand until yellow. The solution will keep for a year.

alcohol. Now add a drop of diluted bile. A green color develops, due to the oxidation of bilirubin to biliverdin.

Experiment 31. Ehrlich's Diazo Reaction. Shake 4 cc. of diluted bile (1 part in 10,000) with 2 cc. of chloroform. Remove 1 cc. of the chloroform layer and add to it 3 cc. of 95 per cent alcohol and 1 cc. of the diazobenzenesulfonic acid reagent.[20] A pink to red color develops in the presence of bile pigment. Although this reaction is not specific it has found extensive use in the estimation of bile pigment in serum.

Experiment 32. Pettenkofer's Test for Bile Acids and Their Salts. To 5 cc. of diluted bile, contained in a test tube, add a few drops of a 5 to 10 per cent solution of sucrose, and mix.[21] Then carefully pour a few cubic centimeters of concentrated sulfuric acid down the side of the tube, to form a layer under the bile solution. A red ring forms at the point of contact of the two liquids.

Experiment 33. Hay's Surface-tension Test. The bile acids and their salts have the property of reducing surface tension. Introduce into a clean test tube a dilute solution of bile (or a solution of bile salts in distilled water), and into a second tube an equal volume of distilled water. Sprinkle on top of each fluid a small amount of flowers of sulfur. Note that the sulfur sinks in the dilute solution of bile or bile salts, but floats on the surface of the water. Explain.

Experiment 34. Cholesterol in Bile. Evaporate 10–15 cc. of undiluted bile to dryness on the water bath. Extract the dry residue several times with small quantities of ether, and combine the extracts in a small evaporating dish. Allow the ether to evaporate. **Caution: Do not evaporate ether near a flame!** Dissolve the residue in 5 cc. of chloroform and divide into two portions, introducing each portion into a *dry* test tube. To one portion apply Salkowski's test; to the other the Liebermann-Burchard reaction (page 56).

[20] *Ehrlich's Diazo Reagent* consists of two solutions. A: Sulfanilic acid, 1 g.; concentrated HCl, 15 cc.; diluted to 1 liter with distilled water. B: Sodium nitrite 0.5 g., diluted to 100 cc. These solutions are kept separately, but before use they are combined to form the diazobenzene sulfonic acid reagent as follows: Mix the two reagents in the proportions of 25 cc. of A and 0.75 cc. of B.

[21] Instead of the sucrose solution, 3 drops of a 1 : 1000 aqueous solution of furfural may be added to the bile. This is Mylius' modification of Pettenkofer's test. Both tests depend upon the formation of condensation products of furfural or its derivatives (methyl-hydroxy-furfural is one of the substances formed by the action of sulfuric acid on sucrose) with the bile salts.

Experiment 35. Composition of a Biliary Calculus.[22] Grind a gall-stone in a dry mortar with about 10 cc. of ether. Filter, using a small filter paper. To the filtrate add an equal volume of alcohol and allow the mixture to evaporate at room temperature. Cholesterol may crystallize out. Examine the crystals microscopically. Dissolve the crystals in a little chloroform and apply Salkowski's or the Liebermann-Burchard test to the solution.

To the ether-insoluble residue on the filter paper add dilute hydrochloric acid. Test the filtrate for the presence of calcium, phosphates, and iron.

Wash the acid-insoluble residue on the filter paper with a little water, and dry. Dissolve in a little chloroform and apply Gmelin's test for bile pigments.

[22] For an excellent discussion of the formation and chemistry of biliary concretions, see H. G. Wells, "Chemical Pathology," W. B. Saunders Company, Philadelphia, Fifth Edition (1925), pages 505–512.

CHAPTER VII

THE URINE

The following experiments demonstrate the presence of some of the more important nitrogenous constituents of urine. Other properties of these, as well as of other constituents, both organic and inorganic, will be brought out in connection with the qualitative and quantitative analysis of urine.

Experiment 1. Urea. (*a*) *Isolation.* Evaporate 500 cc. to 1 liter of urine to dryness. At first the evaporation may be conducted over a free flame, but to avoid charring it should be completed on the water bath under the hood. *The flame is now turned off.* The dry, warm residue is extracted several times with warm acetone, the acetone being allowed to come to a boil each time. Filter or decant the acetone solutions and set aside to cool. Urea crystallizes out. Dry the urea between filter paper and examine microscopically.

(*b*) *Urea Nitrate.* Dissolve a few crystals of urea[1] on a watch glass or glass slide in a drop or two of water. Add a small drop of concentrated nitric acid. Examine microscopically, and sketch the urea nitrate crystals that form.

(*c*) *Urea Oxalate.* Repeat, using, instead of nitric acid, a drop of oxalic acid solution. Examine microscopically and sketch the crystals of urea oxalate that form.

(*d*) *Reaction with Nitrous Acid.* To a few drops of concentrated nitric acid in a test tube add a minute quantity of arsenic trioxide and warm. Brown oxides of nitrogen and nitrous acid are formed. Write equation for the reaction. Now add to the contents of the tube a few crystals of urea. Note the evolution of nitrogen. What other compounds react with nitrous acid, liberating nitrogen?

(*e*) *Reaction with Sodium Hypobromite.* Add a drop of bromine to 2–3 cc. of 5 per cent sodium hydroxide. Warm gently. Sodium hypobromite (NaBrO) is formed. To this add a few crystals of urea and note the evolution of nitrogen. What is the reaction? One of the

[1] In this and subsequent experiments, the use of a purified preparation of urea is to be preferred.

older methods for the quantitative estimation of urea was based on this reaction.

(*f*) *Formation of Biuret.* Heat gently a little urea in a dry test tube. At 132° C., the urea melts and ammonia is given off. Continue heating gently until the molten mass is solidified. After cooling, dissolve the residue in a little water (2–3 cc.), and treat with an equal volume of 20 per cent sodium hydroxide and a drop of dilute copper sulfate. Mix. Note color. Explain.

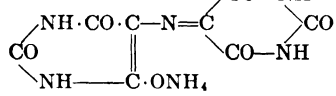
Experiment 2. Uric Acid. (*a*) *Isolation.* Treat filtered urine with 20–30 cc. of 25 per cent hydrochloric acid for each liter of urine. After 48 hours, collect the crystals of uric acid and examine them microscopically. (Make a sketch of the crystals.) Suspend the pigmented crystals in 50 cc. of water and dissolve them by adding dilute sodium hydroxide. Decolorize the solution by warming with animal charcoal. Filter. Acidify the filtrate with hydrochloric acid and set aside for 24 hours in a cool place. Filter off the crystals; wash with ice-cold water, and dry. Examine microscopically, and sketch the crystals of pure uric acid.

(*b*) *Murexide Test.* In a porcelain dish, treat a small amount of uric acid with 2–3 drops of concentrated nitric acid. Evaporate to dryness on the water bath until *all* the nitric acid has been removed and a reddish or yellowish residue remains. Treat this residue, after cooling, with a drop of dilute ammonia (5 drops of concentrated ammonia solution in 20–30 cc. of water). The residue turns reddish-violet in color owing to the formation of murexide (ammonium purpurate[2]). A purplish or bluish-violet color is obtained when the residue is treated with dilute sodium or potassium hydroxide.

(*c*) *Reducing Properties of Uric Acid.* Dissolve some uric acid in dilute sodium carbonate solution. (The salts of uric acid are much more soluble than the free acid.) Test the reducing properties of this solution with (*a*) Fehling's solution; (*b*) Benedict's solution (see page 22. Explain the significance of your observations.

(*d*) *Phosphotungstic Acid Reaction (Folin).* Dissolve a few par-

[2] Several formulas for murexide, the ammonium salt of purpuric acid, have been suggested. One of these is:



See Organic Chemistry, edited by H. Gilman, John Wiley & Sons, New York, p. 990 (1938).

ticles of uric acid in a saturated solution of sodium carbonate. Add 1 cc. of the uric acid reagent of Folin and Marenzi (page 234) and note the pronounced blue color that develops. Repeat the experiment, using 5 cc. of urine instead of uric acid.

Experiment 3. Creatinine. (*a*) *Isolation.* (*Folin-Benedict Method.*) See O. Folin, *J. Biol. Chem.*, **17**, 463 (1914), and S. R. Benedict, *ibid.*, **18**, 182 (1914).

(*b*) *Jaffe's Reaction for Creatinine.* Treat 5 cc. of urine in a test tube with 3 cc. of a saturated solution of picric acid. Render the solution alkaline with sodium hydroxide (10 per cent solution). A red color develops, which is believed to be due to the formation of a red tautomer of creatinine picrate. The reaction has been applied to the quantitative estimation of creatinine in blood and urine.

Experiment 4. Isolation of Hippuric Acid. Treat fresh horse or cow urine with saturated calcium hydrate until strongly alkaline; heat to boiling, filter, evaporate the filtrate to a syrupy consistency, and acidify strongly with hydrochloric acid. (The solution should be kept cool at this point.) Separate the hippuric acid thus precipitated, wash with cold water, dry between filter papers, dissolve in as small a quantity of boiling water as possible, and treat the boiling-hot filtrate with chlorine gas until the color of the solution is pale yellow. Cool quickly, filter, wash the hippuric acid several times with cold water, and crystallize from boiling water after treating the solution with animal charcoal.[3]

Treat some of the hippuric acid with several drops of concentrated nitric acid (fuming nitric acid may be used). Evaporate to dryness on the water bath. Mix the residue with sand, and heat in a dry test tube. Note the odor of nitrobenzene (resembles the odor of oil of bitter almonds). Explain the reaction that takes place.

Experiment 5. Indican. (*a*) *Obermayer's Test.* Treat 5 cc. of urine with an equal volume of Obermayer's reagent (2–4 g. of ferric chloride in 1 liter of concentrated hydrochloric acid). Add 2–3 cc. of chloroform and shake the contents of the tube thoroughly. If indican is present in the urine, the chloroform acquires an indigo blue color,

[3] *Isolation of Hippuric Acid (Alternate Method).* Ingest 2 g. of ammonium or sodium benzoate with the evening meal. Collect the urine the following morning and evaporate to a small volume. Acidify with hydrochloric or sulfuric acid and set aside in a cool place. After 24 hours, filter, and dry the precipitate. In addition to hippuric acid, the precipitate consists of uric acid and other substances. The hippuric acid is extracted with ether. Allow the extract to evaporate spontaneously. Hippuric acid separates out. Examine the crystals microscopically.

the depth of the color depending on the amount of indican present. Explain the reaction.[4]

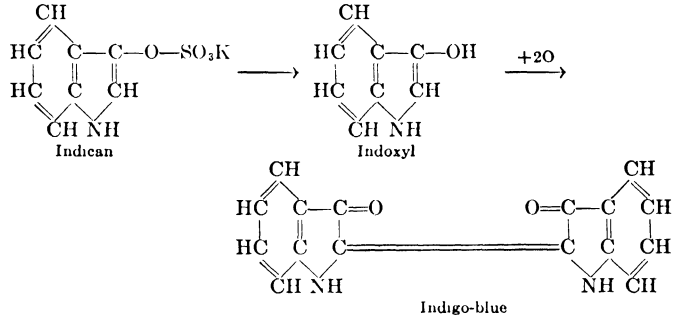
(*b*) *Jaffe's Test for Indican.* Treat 5 cc. of urine with 5–10 cc. of concentrated hydrochloric acid. Add 2–3 cc. of chloroform and a few drops of calcium hypochlorite solution (or 1–2 drops of 5 per cent potassium chlorate solution). Mix the contents of the tube thoroughly. If indican is present the chloroform layer will acquire a blue color, the intensity of which will depend upon the amount of indican present in the urine; in the absence of indican the chloroform will remain colorless.

ABNORMAL CONSTITUENTS OF THE URINE

Experiment 6. Glucose.[5] **Benedict's Test.** Heat 5 cc. of Benedict's reagent (qualitative reagent for sugar, page 22), add 8 drops of urine and boil vigorously for 2 minutes. Set the tube aside to cool spontaneously. The amount of precipitate and its color (red, yellow, or green) depend on the quantity of glucose present in the urine.

Experiment 7. Protein.[6] **Coagulation Test.** Fill a test tube

[4] The formation of indigo-blue from indican may be represented as follows:



Indigo-blue

Rarely, a reddish coloration is produced, due to the formation of indigo-red.

[5] A positive reduction test is usually due to the presence of glucose. Rarely other sugars may occur, such as l-xyloketose (in pentosuria), fructose (in fructosuria, or levulosuria) and lactose (in lactosuria). During pregnancy and lactation, or soon after weaning, lactose may be found. It may be distinguished from glucose by subjecting the urine to the action of pure yeast. Whereas glucose is fermented, lactose is not. The two sugars may also be distinguished by their osazones (page 28) and by the mucic acid test. The latter tests are not so reliable as the fermentation test, particularly when small amounts of lactose are present. Cole's test for the separation and differentiation of glucose and lactose (page 32) is of value.

[6] Albumins and globulins are meant here. Certain other proteins, such as Bence-Jones' protein, may occur in the urine. To test for the presence of this

three-fourths full of filtered or centrifuged urine. Holding the tube at an angle over the flame of a Bunsen burner, heat the upper third of the liquid to boiling. The clear lower portion will offer a sharp contrast to any turbidity, however slight, that may form as a result of the heating. Should a turbidity or coagulum form, it may be due either to protein or to phosphates. Acidify the hot urine by adding one or more drops of dilute (2 per cent) acetic acid. If the coagulum or turbidity is due to phosphates, it will disappear; but if due to protein, the precipitate will become more flocculent in character.

Experiment 8. Heller's Nitric Acid Ring Test for Protein. Place 3–5 cc. of concentrated nitric acid in a test tube. By means of a pipette, deliver 5 cc. of urine along the side of the tube. If care is exercised to avoid mixing, the urine forms a layer above the nitric acid. A white zone of precipitated protein forms at the junction of the two liquids if the urine contains protein.[7]

Experiment 9. Sulfosalicylic Acid Test for Protein. To 2 or 3 cc. of urine add a few drops of sulfosalicylic acid solution (20 per cent). In the presence of protein, a turbidity or precipitate results.[8]

Experiment 10. Detection of Globulin. Add 1 cc. of urine to 30 cc. of 1.5 molar solution of sodium sulfate. The presence of globulin is indicated by the formation of a turbidity or precipitate.

Experiment 11. Legal's Test for Acetone. Treat 5 cc. of urine with a few drops of a *freshly prepared* aqueous solution of sodium nitroprusside (5 per cent). Render the solution alkaline with sodium hydroxide. A ruby-red color is produced. Now acidify with acetic acid. In the absence of acetone, the ruby-red color (which may be due either to acetone or to creatinine) gives way to a yellow color. In the presence of acetone, the red color is intensified.

Perform this test on normal urine and on urine containing acetone.

protein the suspected urine is rendered faintly acid with acetic acid. The urine is then heated in a test tube immersed in a beaker of warm water. The temperature is increased gradually. If Bence-Jones' protein is present, the urine becomes turbid at a temperature of 40–45° C. At 60° C., the protein forms a flocculent precipitate which may adhere to the sides of the test tube. As the temperature is raised to 100° C., the protein tends to dissolve. On cooling, it reappears.

Bence-Jones' protein occurs in the urine principally in multiple myeloma.

[7] The following is a modification of the nitric acid ring test. Stratify 5 cc. of urine above an equivalent volume of Roberts' reagent (1 volume of concentrated nitric acid and 5 volumes of a saturated solution of magnesium sulfate). This is known as Roberts' test. How does it compare with Heller's test?

[8] The presence of protein in urine may be detected by means of a large number of reagents, such as picric acid, phosphotungstic acid, and dinitrosalicylic acid.

Experiment 12. Gerhardt's Test for Acetoacetic Acid. To 5 cc. of urine in a test tube, add a solution of ferric chloride (5 per cent), drop by drop, until no more precipitate of ferric phosphate is formed. Filter and treat the filtrate with more ferric chloride. The presence of acetoacetic acid is indicated by the development of a deep-red color (Bordeaux red).

A similar color is given by urine containing aspirin, antipyrin, and other substances. These drugs appear in the urine after their administration in therapeutic doses.

If the test for acetoacetic acid is positive, it should be confirmed. This may be done by boiling a separate portion of the urine to decompose the acetoacetic acid. After cooling, the boiled urine should give a negative reaction, if the color in the original test was due to acetoacetic acid.

The presence of acetoacetic acid may also be confirmed as follows: Acidify a convenient volume of urine with sulfuric acid. This liberates the acid from its salt combinations. Extract with ether. Treat the ether extract with *very dilute* ferric chloride (dilute the 5 per cent solution 10 or 20 times). If the ether extract contains acetoacetic acid, the lower layer, as it accumulates at the bottom of the tube, will be colored violet or Bordeaux red.

Experiment 13. Rothera's Test for Acetone Bodies. This test is given both by acetone and by acetoacetic acid. Add solid ammonium sulfate to 5 cc. of urine contained in a test tube, until the urine is completely saturated and no more of the ammonium sulfate goes into solution. Now add 2–3 drops of a freshly prepared aqueous solution of sodium nitroprusside and 1–2 cc. of concentrated ammonia. Mix by tapping the bottom of the tube, and allow to stand undisturbed for about half an hour. The presence of acetone or acetoacetic acid, or both, is indicated by the development of a characteristic purplish coloration, resembling that of permanganate.

Experiment 14. Gmelin's Test for the Detection of Bile. This is essentially a test for bile pigment. Place 5 cc. of concentrated nitric acid in a test tube. By means of a pipette, deliver down the side of the tube 5 cc. of urine. Avoid mixing. Note the colored rings: green nearest the urine, then blue, violet, red, and reddish-yellow nearest the acid. Explain.

Experiment 15. Pettenkofer's Test. This is essentially a test for bile acids. To 5 cc. of urine contained in a test tube, add 5 drops of a 5 per cent solution of sucrose. Pour down the side of the tube 3–4 cc. of concentrated sulfuric acid. A red ring develops at the point of con-

tact of the two solutions. Stir the mixture and note the effect. Repeat the test, using normal urine.

Experiment 16. Test for Urobilin (McMaster and Elman [9]). If the urine is neutral or alkaline, acidify slightly with acetic acid. To 5 cc. add an equal volume of a saturated solution of zinc acetate in 95 per cent alcohol, followed by a pinch of solid zinc acetate. After shaking vigorously let stand for a few minutes and filter. The filtrate should be clear and slightly acid to litmus. If it is not acid, correct the reaction by adding dilute acetic acid. Treat with a drop of Lugol's solution (I in KI), or tincture of iodine, in order to oxidize all the urobilinogen to urobilin. The development of a green fluorescence indicates the presence of urobilin.

Experiment 17. Test for Urobilinogen (Wallace and Diamond [10]). Freshly voided urine should be used, since urobilinogen is oxidized to urobilin if urine is allowed to stand. To 10 cc. of urine add 1 cc. of Ehrlich's aldehyde reagent (2 per cent solution of *p*-dimethylaminobenzaldehyde in 20 per cent HCl). If urobilinogen is present, a cherry-red color will develop within 3–5 minutes. Normal urine gives a greenish-yellow color in the absence of urobilinogen, or a pale pink color in the presence of traces.

If the test is positive, add 3 cc. of chloroform, shake vigorously, and allow to stand. Is the colored derivative extracted by the chloroform?

Perform the test on urine from a patient with jaundice. Dilute the urine 1 : 20 and repeat the test.

Discuss the significance of the presence of bilirubin, urobilin, and urobilinogen in the urine.

Experiment 18. Benzidine Reaction. Heat 3 cc. of urine to boiling, cool, and treat with an equal volume of a saturated solution of benzidine in glacial acetic acid. Add 1 cc. of 3 per cent hydrogen peroxide. The development of a blue or green color indicates the presence of blood.

Experiment 19. Guaiac Reaction. Heat 3 cc. of urine to boiling, cool, and treat with a *freshly prepared* alcoholic solution of guaiac (approximately 2 per cent) until turbidity appears. Now add hydrogen peroxide, drop by drop. (Old turpentine may be used in place of hydrogen peroxide.) The development of a blue color indicates the presence of blood.

[9] *J. Exptl. Med.*, **41**, 503 (1925).
[10] *Arch. Int. Med.*, **35**, 698 (1925).

Experiment 20. Examination of Urinary Sediment. Fill a 15 cc. centrifuge tube with urine and centrifuge for 2 to 3 minutes at moderate speed. Pour off the urine, leaving a small residue of liquid in the bottom of the tube. Stir, place a drop of the fluid on a microscope slide and examine.[11]

QUANTITATIVE ANALYSIS OF URINE

Collection. Samples of urine collected at different intervals during the day usually show considerable variation in composition; hence the analysis of a specimen collected at random has limited significance or none at all. As a rule, the quantitative analysis of urine is performed on a *mixed* 24-hour sample. This may be collected as follows: The subject empties his bladder at a fixed time in the morning (7 A.M. or 8 A.M. is usually a convenient hour), discarding the urine. From this time on, all urine, including that voided at exactly the same hour the following morning, is collected in a *clean* bottle, containing 10–20 cc. of toluene. The toluene prevents bacterial action. As an additional precaution against deterioration, the urine may be kept in a refrigerator until shortly before it is needed for analysis.[12]

NOTE TO STUDENT: When urine is collected for a metabolism study it is necessary to keep a record of the *food consumption* throughout the 24 hours during which the collection of urine is made. Record the approximate amounts of food for each meal and any additions to the diet; calculate as accurately as possible (1) the caloric intake; (2) the protein intake.[13] From the latter figure determine the grams of nitrogen consumed in the 24 hours. How does this check with the total nitrogen contained in the 24-hour specimen (Experiment 22a or 22b)?

Energy Expenditure. Keep a record of your activities for the 24-hour period, noting exercise, rest, etc. Estimate the approximate

[11] The student will find the following reference helpful: J. C. Todd and A. H. Sanford, "Clinical Diagnosis by Laboratory Methods," W. B. Saunders Company, Philadelphia.

[12] For the purpose of simplifying calculations, it is the practice in some laboratories to dilute the urine to some convenient volume. Thus, if the volume of urine happens to be 967 cc., it may be diluted to 1000 cc. All calculations should then be made on the basis of 1000 cc. If this is done, be sure to determine the specific gravity of the urine before diluting, and to mix the urine and water thoroughly after diluting.

[13] For the calorific and protein values of the common foodstuffs, consult H. C. Sherman, "Chemistry of Food and Nutrition," 5th edition, Macmillan, 1937; M. S. Rose, "Laboratory Handbook for Dietetics," 4th edition, Macmillan, 1937.

caloric expenditure for the entire period. (Consult Table IV, p. 281.) How does this compare with the caloric intake?

Experiment 21. Volume. Before any urine is taken for analysis, the total excretion for the 24 hours should be measured. (A large graduated cylinder may be used for this purpose.) *Record the volume* and use it as a basis for calculating the daily excretion of the individual constituents that are to be determined in the analysis.

Specific Gravity. Determine the specific gravity of the urine at a temperature of about 25° C. (or at room temperature, for this is usually 22–26° C.), using an accurate hydrometer or urinometer. Before using the urinometer, determine its true zero point by immersing it in distilled water at about 25° C.

Reaction. Test the reaction of the urine with litmus paper.

Determination of *p*H. This determination should be made as soon as possible after the urine is collected. (Urine collected and kept under mineral oil is to be preferred in this determination. Why?)

The *p*H of normal urine usually varies between 5.4 and 8.0. This range is covered by the two indicators, brom-cresol purple (*p*H 5.4–7.0) and phenol red (*p*H 6.6–8.2).[14]

Introduce into each of two tubes 8 cc. of distilled water that has been recently boiled and cooled. Add 2 drops of brom-cresol purple indicator to the water in one tube, and a similar number of drops of phenol red to the water in the other tube. (The diluted indicator solutions in each tube may now be covered by a layer of mineral oil.) Introduce 2 cc. of urine into each tube and stir gently. Compare the colors produced with those in a series of indicator standards[15] and record the *p*H of the tube which matches the urine tube most nearly.

NOTE TO THE STUDENT: All quantitative determinations should be done in duplicate, and the results should check within the limits of error of the method.

Experiment 22a. Total Nitrogen (Kjeldahl Method). Introduce into a large Kjeldahl flask (or a 500-cc. flat-bottomed Pyrex or Jena flask) 5 cc. of urine, 20 cc. of concentrated sulfuric acid, 5–10 g. of sodium or potassium sulfate, and a small crystal of copper sulfate. Digest the mixture in the hood for about half an hour after it has

[14] Pathological urine, as well as urine collected during starvation, may be more acid than *p*H 5.4. In examining such urine, brom-cresol green (*p*H 4.0–5.6) or methyl red (*p*H 4.4–6.0) will be found useful.

[15] For the preparation of the buffer solutions for these standards, see pages 278 and 279. Directions for the preparation of the indicators are given briefly on page 280 and in W. M. Clark's "Determination of Hydrogen Ions" (1928), page 94.

turned clear and pale greenish-blue in color. Cool, dilute with 250–300 cc. of distilled (ammonia-free) water, and cool again. Add, in the usual way, 60–80 cc. of a saturated solution of sodium hydroxide (syrupy sodium hydroxide), some talc, and connect with a condenser. Distil 150–200 cc. of fluid into a measured volume [16] of 0.1 *N* acid (sulfuric acid is preferred) containing several drops of a suitable indicator. After the distillation has been discontinued, the residual acid is titrated with standard alkali. From the results obtained, corrected for the blank, calculate the total nitrogen contained in the 24-hour specimen of urine.[17]

NOTE: The "total nitrogen" represents all the non-pprotein nitrogenous constituents of the urine, such as urea, uric acid, creatinine, amino acids, etc. If albumin is present in the urine, it must be removed, and the nitrogen determined on the protein-free filtrate. The usual method for removing protein consists in heating the urine after acidifying it with acetic acid. The protein coagulates and is filtered off. The filtrate is then adjusted at room temperature to the original volume by adding water.

[16] Fifty cubic centimeters is usually sufficient. A larger or smaller volume should be measured depending on whether the nitrogen content is expected to be high or low.

[17] The following is an illustration of a method of calculating the results:

Blank:	Volume of 0.1 *N* H_2SO_4 placed in receiving flask	40 cc.
	Volume of 0.1 *N* NaOH used in neutralizing the residual acid	39.8 cc.
	Volume of 0.1 *N* H_2SO_4 neutralized by the ammonia in the reagents	0.2 cc.
Determination:	Total volume of urine for 24 hours	1600 cc.
	Volume of urine taken for analysis	5 cc.
	Volume of 0.1 *N* H_2SO_4 placed in receiving flask	40 cc.
	Volume of 0.1 *N* NaOH used in neutralizing the residual acid	12.5 cc.
	Volume of 0.1 *N* H_2SO_4 neutralized by the ammonia that distilled over	27.5 cc.
	Correction for blank	0.2 cc.
	Corrected volume of 0.1 *N* H_2SO_4	27.3 cc.

1 cc. of 0.1 *N* H_2SO_4 is equivalent to 1 cc. of 0.1 *N* NH_3 and is therefore equivalent to 0.0014 g. of nitrogen. (Explain.) Therefore, 27.3 cc. is equivalent to $27.3 \times 0.0014 = 0.0382$ g. of nitrogen. This is the amount of nitrogen contained in 5 cc. of urine. It therefore follows that 1600 cc., or the daily output, contains 12.22 g. of nitrogen.

Experiment 22b. Total Nitrogen. (The Koch-McMeekin Micro-Kjeldahl Method). If the specific gravity of the urine is 1.018, or over, dilute *accurately* 5 cc. of the well-mixed urine (measured with a calibrated pipette) to 100 cc. in a volumetric flask. If the specific gravity is less than 1.018 dilute 10 cc. to 100 cc. Mix thoroughly.

Using an Ostwald-Folin pipette, measure accurately 1 cc. of the diluted urine into a Pyrex test tube (25 by 200 mm.). To this add 1 cc. of 1 : 1 sulfuric acid and digest [18] until the liquid becomes charred and *dense white fumes* of sulfuric acid fill the tube. Cover the mouth of the tube with a watch glass and continue the heating for a few minutes, then set aside and add 1 drop of 30 per cent hydrogen peroxide (Merck's blue label Superoxol), letting it fall directly into the mixture. Vigorous oxidation occurs and the digest usually clears at once. Again boil for 2 to 5 minutes. Should the digest again char or become discolored, repeat the hydrogen peroxide treatment; otherwise allow to cool, dilute with distilled (nitrogen-free) water, and transfer quantitatively to a 100 cc. volumetric flask, diluting to about 75 cc.

At the same time, in another 100 cc. volumetric flask, prepare the standard. With a pipette measure 5 cc. of a standard ammonium sulfate solution [19] (5 cc. = 0.3 mg. nitrogen). Dilute with a little water, add 1 cc. of the 1 : 1 sulfuric acid, and again dilute to about 75 cc. Then add 15 cc. of the Nessler's reagent [20] to standard and to unknown, dilute to the mark, and mix well.

[18] This may be done over the free flame of a micro-burner, on a sand bath, or on an electric hot plate.

[19] *Standard Ammonium Sulfate.* Dissolve 0.283 g. of pure ammonium sulfate in 1 liter of distilled, ammonia-free water. 5 cc. of this solution contain 0.3 mg of nitrogen.

The standard solution may also be prepared by dissolving the 0.283 g. of ammonium sulfate in 200 cc. of water in a liter volumetric flask and diluting to the mark with *N* H_2SO_4. This acid solution keeps better. It is also the standard recommended in the estimation of urea in the blood by the method of Leiboff and Kahn (page 230).

[20] Nessler's reagent is an alkaline solution of the double iodide of mercury and potassium ($HgI_2 \cdot 2KI$). It gives a distinct color (yellow, orange-yellow, or brownish-yellow) even with the most minute traces of ammonia or ammonium salts. The reaction is represented as follows:

$$2(HgI_2 \cdot 2KI) + NH_3 + 3KOH = NH_2HgOHgI + 7KI + 2H_2O.$$

Nessler's Reagent (prepared according to Koch and McMeekin, *J. Am. Chem. Soc.*, **46**, 2066 [1924]). Dissolve 22.5 g. of iodine in 20 cc. of water containing 30 g. of KI. After the solution is complete, add 30 g. of pure metallic mercury, and shake the mixture well, keeping it from becoming *hot* by immersing in tap water from time to time. Continue this until the supernatant liquid has lost all the

Compare the two solutions in the colorimeter[21] and record the results.

Calculations. In colorimetry an inverse proportionality is assumed between the depth of color and the concentration (p. 3). This may be expressed by the following ratio:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} = \frac{\text{Concentration of unknown}}{\text{Concentration of standard}}$$

The concentrations must be expressed for equal volumes of standard and unknown.

From the value thus obtained for the nitrogen content of 1 cc. of the diluted urine, the concentration in 1 cc. of the undiluted urine may be computed, and from this the total nitrogen content in any given volume of the urine, such as that in the 24-hour specimen.

The student should note that any error made in the conduct of the

yellow color due to iodine. Decant the supernatant aqueous solution and test a portion by adding a few drops thereof to 1 cc. of a 1 per cent starch solution. Unless the starch test for iodine is obtained, the solution may contain mercurous compounds. To the remaining solution add a few drops of an iodine solution of the same concentration as employed above, until a faint excess of free iodine can be detected by adding a few drops of the solution to 1 cc. of the starch solution. Dilute to 200 cc. and mix well.

To 975 cc. of an accurately prepared 10 per cent solution of sodium hydroxide now add the entire solution of potassium mercuric iodide prepared above. Mix thoroughly and allow to clear by standing.

[21] *Use of the Colorimeter.* Read over the description of the colorimeter on page 3. Care should be taken not to fill the cups too full. Certain solutions, especially the molybdate reagents used in blood sugar or phosphate determinations, will react with the metal jacket of the colorimeter cup and the solution will become darkened as a result. In immersing the prisms in the liquid, be sure that no air is trapped beneath the prism. What effect would an air bubble have on the matching of colors?

As the proportionality between the depth of color and the column of solution is accurate only in a certain range, no reading should be accepted that is greater than one and a half times or less than two-thirds of the setting of the standard. If the reading is outside this range, the determination must be repeated, using more or less of the unknown material, or adjusting the strength of the standard used. It is sometimes convenient to run several standards of varying strengths.

An average of several readings should be taken, all of which agree within 0.2 mm. The zero point of the colorimeter should always be determined by matching the standard against itself in a preliminary reading. The correction must be entered in the notebook and used in the calculation. A correction greater than 0.2 mm. should be reported to the instructor, in order that the machine may be adjusted.

analysis, or in the calculations, is multiplied many times. Hence care and precision must be exercised at every step in the determination.

Experiment 23. Determination of Ammonia (Van Slyke and Cullen).[22] Ammonium salts are present in urine. To liberate the ammonia the addition of a base is all that is necessary. In order to determine ammonia nitrogen, arrange the Van Slyke-Cullen apparatus as shown in Fig. 2. Each tube is fitted with a long inlet tube and a short outlet bulb loosely filled with cotton to prevent any carrying over of spray. The wash bottle contains about 20 cc. of concentrated sulfuric acid to absorb any ammonia that may be contained in the air. Air passes through the long tube into the sulfuric acid, thence through the short outlet bulb to the inlet tube of *A*. The short tube of *A* is connected with the long tube of *B*. Another pair of tubes, *A'* and *B'*, are connected in series in the same way, *B* leading into *A'* and *A'* into *B'*. If suction is used, connect tube *B'* through a trap bottle to the suction outlet; or if compressed air is available, connect the air cock with the inlet tube of the wash bottle. Check the connections carefully before proceeding with the experiment.

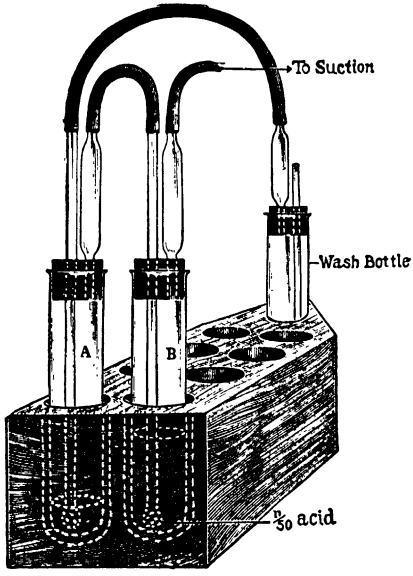


FIG. 2.—Van Slyke and Cullen Apparatus for the Determination of Urea.

Measure into tubes *B* and *B'* 25 cc. of *N*/50 sulfuric acid, 2–3 drops of caprylic alcohol (to prevent foaming during the aëration), and 1–2 drops of a suitable indicator. Into each of tubes *A* and *A'* measure 5 cc. of urine and 2–3 drops of caprylic alcohol. Connect in series.

[22] The Van Slyke and Cullen procedure is essentially a modification of Folin's method, *Am. J. Physiol.*, **13**, 45 (1915). Other methods for the determination of ammonia in urine are: "The Colorimetric Method of Folin and Macallum." *J. Biol. Chem.*, **11**, 523 (1912); "The Permutit Method of Folin and Bell," *J. Biol Chem.*, **29**, 329 (1917).

NOTE: In the following directions, such instructions as are indicated for *A* and *B* apply also to the duplicate set of tubes, *A'* and *B'*.

Then open *A*, add 4–5 g. of potassium carbonate, and close promptly. (Another procedure is to disconnect the rubber tubing from the inlet tube passing into *A* and to deliver, by means of a rapidly flowing pipette, 10 cc. of a saturated solution of potassium carbonate. The rubber tubing is then reconnected with the inlet tube.) Without delay, turn on the suction and pass an air current through the tubes until all the ammonia liberated in tube *A* has been aërated into the acid in tube *B*. The rate of aëration should be slow at first. Although most of the ammonia is usually carried over during the first 5 minutes, the aëration should be continued for at least 30 minutes to insure the complete removal of the ammonia from tubes *A* and *A'*.

When the aëration is complete, turn off the air current cautiously, at the same time disconnecting the tubing to tube *B* in order to avoid back suction. Before withdrawing the inlet tube, rinse it thoroughly on the inside and outside with a fine stream of distilled water, catching the washings in tube *B*. The excess of acid in tubes *B* and *B'* is titrated with *N*/50 sodium hydroxide. The difference between the acid originally contained in each tube and that remaining after the aëration represents the ammonia nitrogen contained in the sample of urine analyzed. Calculate the output of ammonia (both in terms of ammonia and ammonia nitrogen) for the 24-hour period.[23]

Experiment 24. Determination of Urea (Procedure of Van Slyke and Cullen).[24] Set up the Van Slyke-Cullen apparatus as directed in

[23] The *ammonia nitrogen* may be calculated as follows:

Given:	Total volume of urine for 24 hours	1600 cc.
	Volume of urine taken for analysis	5 cc.
	N/50 acid placed in tube *B*	25 cc.
	N/50 alkali required to neutralize excess after aëration	21 cc.
	N/50 acid neutralized by ammonia liberated in tube *A*	4 cc.

Since 1 cc. of *N*/50 acid is equivalent to 0.00028 g. of nitrogen, 4 cc. is equivalent to 0.00112 g. This is the amount of ammonia nitrogen contained in 5 cc. of urine; therefore 1600 cc. contains 0.3584 g. (0.36 g.).

[24] *J. Biol. Chem.*, **19**, 211 (1914).

NOTE: References to other procedures for the determination of urea in urine:

Marshall's Urease Method, *J. Biol. Chem.*, **14**, 283 (1913); **15**, 487, 495 (1913); **17**, 351 (1914). (The application of the enzyme *urease* to the determination of urea in blood, urine, etc., was first proposed by Marshall.)

Direct Nesslerization Method of Folin and Denis, *J. Biol. Chem.*, **26**, 501 (1916), and of Folin and Youngburg, **38**, 111 (1919).

Direct Nesslerization Method of Koch and McMeekin, *J. Am. Chem. Soc.*, **46**, 2066 (1924).

Sumner's "Rapid Method for the Determination of Urea in Urine," *J. Biol. Chem*, **38**, 57 (1919).

Youngburg's "Modification of the Van Slyke-Cullen Method," *J. Biol. Chem.*, **45**, 391 (1921).

Hydrolysis Method of Leiboff and Kahn, *J. Lab Clin. Med.*, **17**, 77 (1931).

Van Slyke's Manometric Method, *J. Biol. Chem.*, **73**, 695 (1927).

Experiment 23. (One may perform the ammonia determination and the urea-plus-ammonia procedure at the same time. One wash bottle and eight tubes for the two determinations may be set up in a large block containing nine holes, and the aëration carried out in series.)

Dilute 5 cc. of urine to 50 cc. in a volumetric flask with ammonia-free water. Disconnect tubes *A* and *A'* from the series and measure into each of them 5 cc. of the diluted urine, 1 cc. of a solution of urease, and 2 drops of buffer solution.[25] Stopper the tubes and place them in a beaker of warm water (not over 60° C.) for digestion. Measure into tubes *B* and *B'* 25 cc. of *N*/50 sulfuric acid, 2–3 drops of caprylic alcohol, and 1–2 drops of indicator. After 30 minutes remove tubes *A* and *A'* from the bath, add 5 drops of caprylic alcohol, connect in series, and aërate for about half a minute in order to draw over into the standard acid any free ammonia that may have collected in the air space during the digestion. Then add the carbonate and aërate as directed in Experiment 23.

The ammonia nitrogen determined by this procedure will represent the urea and the ammonia nitrogen contained in the sample of urine analyzed. From the titration figures obtained and the ammonia values

[25] Various procedures are followed in different laboratories. If buffer solution is not added separately, the urease preparation should be prepared as follows: Add 2 g. of urease preparation (jack-bean meal or soy-bean meal—this should be free from ammonia; see below), 0.6 g. of K_2HPO_4, and 0.4 g. of KH_2PO_4, to 10 cc. of water. Preserve with toluene and keep in a cool place. As this preparation contains the necessary buffer mixture for the action of the enzyme *urease*, it may be used in the procedure described here.

Ammonia may be removed from commercial preparations of urease (jack-bean meal and soy-bean meal) by means of permutit. Folin has recommended the following procedure: Wash 3 g. of permutit in a flask with a dilute solution of acetic acid (2 per cent). Drain off the acetic acid and wash twice with water. Now add 5 g. of jack-bean meal and 100 cc. of 15 per cent alcohol. Shake gently but continuously for 10 to 15 minutes, pour on a large filter paper and cover with a watch glass. The filtrate contains the urease and remains active for at least two weeks if kept in an ice box.

If this preparation of urease is used in the determination of urea, it is necessary to add a buffer mixture to the diluted urine in tube *A*. This may be prepared as follows: Dissolve 69 g. of crystallized mono-sodium phosphate ($NaH_2PO_4 \cdot H_2O$) and 179 g. of crystallized disodium phosphate ($Na_2HPO_4 \cdot 12H_2O$) in 800 cc. of distilled water. After cooling, dilute to 1 liter. Preserve with 1 or 2 cc. of toluene. In the determination of urea in urine, use 1, or at most 2, drops of this solution.

Urease tablets, such as those prepared by Squibb, are quite satisfactory. These tablets contain the necessary buffer agents, and one such tablet may be substituted for the 1 cc. of urease solution.

found in Experiment 23, calculate the amount of urea (as grams of urea and of urea nitrogen) excreted in the 24-hour period.[26]

Experiment 25a. Creatinine (Folin's Original Colorimetric Method).[27] This method is based on the color reaction given by creatinine with picric acid in an alkaline solution. Measure 10 cc. of urine into a 500-cc. volumetric flask, add 15 cc. of a saturated solution of picric acid and 5 cc. of a 10 per cent solution of sodium hydroxide, mix, and allow to stand for 5 minutes. During this interval pour into each cup of a colorimeter a little 0.5 *N* potassium bichromate solution and adjust the depth of the solution in one of the cups to the 8-mm. mark. With the solution in the other cup a few preliminary colorimetric readings are made, in order to determine the zero point and to obtain practice in comparing the solutions accurately. The bichromate solution in the two cups must, of course, be identical in color, and in taking the readings no two should differ more than 0.1 or 0.2 mm. from the true value (8 mm.). Four or more readings should be made, and an average taken of all but the first, which is likely to be less accurate than the succeeding readings.

At the end of the 5-minute interval referred to in the preceding paragraph, the contents of the flask are diluted to the 500-cc. mark.

The right-hand cup is emptied of the bichromate solution, rinsed with water, and then with the unknown solution. The plunger on that

[26] The following method may be used in calculating the results:

Given: Total volume of urine for 24 hours . . . . . 1600 cc.
Volume of urine taken for analysis . . . . . 0.5 cc.
N/50 acid placed in tube *B* . . . . . 25 cc.
N/50 NaOH used in neutralizing the acid after aëration 13.3 cc.
N/50 acid neutralized by the ammonia liberated in tube *A* 11.7 cc
One cc. of *N*/50 acid is equivalent to 0.00028 g. N (explain).
Therefore 11.7 cc. is equivalent to 0.003276 g. N.
This is the amount of *urea* plus *ammonia nitrogen* in 0.5 cc. of urine. Therefore, in 1600 cc., the amount of *urea* plus *ammonia nitrogen* is 10.48 g.
The ammonia nitrogen is 0.36 g. (see Experiment 24 and calculations for *ammonia nitrogen*).

Hence the urea nitrogen for the 24-hour period is 10.12 g. The results are stated to the second decimal place.

Calculate the urea output for this period.

[27] *Am. J. Physiol.*, **13**, 48 (1905). This method has been largely superseded by Folin's microchemical modification (Experiment 25*b*). The method, besides its historical interest, illustrates the use of an empirical standard.

side is rinsed with the solution also. Then the cup is partly filled and several readings are made at once with the standard set at the 8-mm. mark.

The calculation is based on the empirical observation that 10 mg. of pure creatinine gives, under the conditions of the determination, 500 cc. of a solution 8.1 mm. of which has exactly the same colorimetric value as 8 mm. of 0.5 *N* potassium bichromate solution (Folin).

The amount of urine taken for the determination is usually 10 cc.; but if this should contain more than 15 mg. or less than 5 mg. of creatinine, the determination should be repeated with a correspondingly different amount of urine, because outside of these limits the determination is much less accurate.

Calculate the quantity of creatinine in the 24-hour urine specimen.

Calculate the quantity of creatinine nitrogen. Calculate the creatinine coefficient.[28]

Use of Pure Creatinine Standards. A solution of pure creatinine is to be preferred as a standard solution. This may be prepared as directed in footnote 30. In carrying out the determination, 10 cc. of this solution is treated in exactly the same way as the 10 cc. of urine.

Experiment 25b. Creatinine (Folin's Microchemical Modification).[29] By means of an Ostwald pipette, measure 1 cc. of urine into a 100-cc. volumetric flask. Into another 100-cc. flask, measure 1 cc. of the standard creatinine solution (1 cc. of which contains 1 mg. of creatinine).[30] Twenty cubic centimeters of saturated picric acid solution[31] (measured from a pipette or burette) is added to each, and then 1.5 cc. of a 10 per cent solution of sodium hydroxide (measured *accurately* from a burette or pipette). At the end of 10 minutes the flasks are filled to the mark with water and the color compared in the

[28] The *creatinine coefficient* is calculated by dividing the milligrams of creatinine by the body weight in kilograms. The *creatinine nitrogen coefficient* may be figured in the same way, using the milligrams of creatinine nitrogen divided by the kilograms of body weight. What is its significance? What are the normal figures for men? For women? See Bodansky, "Introduction to Physiological Chemistry," 4th ed., p. 428.

[29] *J. Biol. Chem.*, **17**, 469 (1914).

[30] *Creatinine Standard.* Dissolve 1 g. of pure creatinine (or 1.61 g. of creatinine zinc chloride) in a liter of 0.1 *N* hydrochloric acid. One cubic centimeter of this solution contains 1 mg. of creatinine.

[31] Although a good grade of pure commercial picric acid meets the requirements of the determination of creatinine and creatine in urine, the repurified picric acid employed in blood analysis (page 238) is to be preferred. Saturated picric acid solution contains about 12 g. per liter.

colorimeter. Set the standard at a convenient level for color comparison. Make a series of readings and calculate the amount of creatinine and of creatinine nitrogen in the 24-hour specimen. Calculate the creatinine nitrogen coefficient.

Experiment 25c. Creatinine (Method of Langley and Evans).[32] To 1 cc. of creatinine standard (1 mg. of creatinine in 0.1 *N* HCl) in a tube graduated at 25 cc. and to 1 cc. of urine in another tube, add 20 cc. of 6 per cent sodium-3,5-dinitrobenzoate (measured with a cylinder) and 2 cc. of 5 per cent sodium hydroxide. Dilute each to the 25-cc. mark, mix, and at the end of 10 minutes compare in a colorimeter. The dinitrobenzoate reagent is somewhat more specific for creatinine than is picrate.

Experiment 26. Creatine (Microchemical Method of Folin).[33] Creatine, on boiling with acid, is transformed into creatinine. This property is the basis for the following method:

Enough urine to give 0.7 to 1.5 mg. of creatinine is measured into a weighed Pyrex Erlenmeyer flask (capacity 200 cc.). Saturated picric acid solution (20 cc.), about 130 cc. of water, and a few small pebbles to promote even boiling are added, and the mixture gently boiled, preferably over a micro-burner, for about one hour. At the end of this time the heat is increased and the solution is boiled down to rather less than 20 cc. The flask is transferred to the scales, and enough water is added to make the total solution equal to 20–25 g. The solution is cooled in running water and transferred to a 100-cc. volumetric flask. Then 1.5 cc. of 10 per cent sodium hydroxide is added, and the total creatinine is determined as in the preformed creatinine determination, 1 mg. of creatinine being used as a standard.

Calculate the content of total creatinine in the 24-hour specimen of urine. From the result thus obtained, subtract the value for preformed creatinine determined in Experiment 25b. The difference represents the content of *creatine* in terms of creatinine.[34]

[32] *J. Biol Chem.*, **115**, 333 (1936). The reagent is prepared by suspending 30 gm. of purified 3,5-dinitrobenzoic acid in 420 cc. of water and adding 80 cc. of 10 per cent sodium carbonate. When no more of the acid will dissolve, filter. For a discussion of the specificity of this reagent and its use, see B. F. Miller and R. Dubos, *J. Biol. Chem.*, **121**, 447 (1937).

[33] *J. Biol. Chem.*, **17**, 472 (1914).

[34] *Alternate Method.* Creatine may be converted to creatinine by evaporation to dryness with HCl and lead according to the method of Benedict (*J. Biol. Chem.*, **18**, 191 (1914). The total creatinine may then be determined by the method of Langley and Evans, or by a similar procedure proposed by Benedict and Behre (*J. Biol. Chem.*, 114, 515 (1936).

Experiment 27a. Uric Acid (Folin-Shaffer Method). Measure 100 cc. of urine into an Erlenmeyer flask and treat with 25 cc. of the Folin-Shaffer reagent.[35] Allow the precipitate to settle, and filter through a dry filter paper into a dry beaker or flask. The precipitate consists of phosphates and some organic matter which, if not removed, would interfere with the determination. Transfer 100 cc. of the filtrate (equivalent to 80 cc. of the original urine) to an Erlenmeyer flask, add 5 cc. of concentrated ammonium hydroxide, and allow the mixture to stand for 24 hours. The uric acid is thus converted into ammonium urate. This is now removed *quantitatively* to a filter paper.[36]

Rinse the flask several times with 10–20 cc. portions of 10 per cent ammonium sulfate solution and use the rinsings in washing the precipitate on the filter paper until the filtrate is approximately free from chloride. Now, holding the filter paper over the funnel, open it carefully and, by means of a stream of hot distilled water, wash the precipitate from the filter paper, through the funnel, and back into the flask in which the urate was originally precipitated (and which may still contain crystals of ammonium urate adhering to its sides). About 100 cc. of hot water is usually sufficient to accomplish this purpose. Cool the contents of the flask, add 15 cc. of concentrated sulfuric acid, and titrate while hot with $N/20$ potassium permanganate solution. The end point is reached when, despite stirring, the entire solution remains faintly pink for about 30 seconds. What is the chemistry of this reaction? Write the equations involved.

Calculation. One cubic centimeter of $N/20$ potassium permanganate solution is equivalent to 3.75 mg. of uric acid. Multiply this value by the number of cubic centimeters of permanganate used in the

[35] This consists of 500 g. of ammonium sulfate, 5 g. of uranium acetate, and 60 cc. of 10 per cent acetic acid dissolved in 650 cc. of distilled water.

[36] It is not essential that all of the crystals of ammonium urate be transferred to the filter paper. As much as adheres to the sides of the flask may be allowed to remain. It is important, however, that the flask and any ammonium urate that it may contain be thoroughly washed with small portions of 10 per cent ammonium sulfate until the rinsings are free from chloride. These rinsings may be poured by decantation on to the filter paper. It is of the utmost importance not to use ordinary filter paper in this filtration, for in the subsequent washing of the ammonium urate back into the flask, hot water is used, and sufficient of the filter paper may be removed to interfere with the permanganate titration. (Explain.) A hardened filter paper is therefore much safer, although a well-washed, properly prepared asbestos filter in a Gooch crucible is even more to be recommended.

titration, to obtain the amount of uric acid precipitated from 100 cc. of the urine after treatment with the Folin-Shaffer reagent, or from 80 cc. of the original urine. Multiply by 5/4 to obtain the uric acid precipitated from 100 cc. of the original urine. Since an amount of urate equivalent to 3 mg. of uric acid is soluble in 100 cc. and is therefore not precipitated, a correction of 3 mg. must be added to this value. Calculate the grams of uric acid and of uric acid nitrogen in the 24-hour sample of urine.

Experiment 27b. Uric Acid (Colorimetric Method of Benedict and Franke).[37] The urine is so diluted that 10 cc. will contain between 0.15 and 0.30 mg. of uric acid. (Usually a dilution of 1 to 20 is satisfactory.) Ten cubic centimeters of the diluted urine is measured into a 50-cc. volumetric flask, and 5 cc. of the 5 per cent sodium cyanide is added **from a burette (exercise caution, as sodium cyanide is very poisonous)**, followed by 1 cc. of the arseno-phosphotungstic acid reagent.[38] The contents of the flask are mixed by gentle shaking, and at the end of 5 minutes diluted to the 50-cc. mark with distilled water and mixed. The solution, which becomes blue, is compared in a colorimeter with a simultaneously prepared solution obtained by treating 10 cc. of the standard uric acid solution (0.2 mg. of uric acid) in a 50-cc. flask with 5 cc. of the sodium cyanide solution and 1 cc. of the arseno-phosphotungstic acid reagent, and diluting to the mark at the end of 5 minutes.

Calculate the output of uric acid and of uric acid nitrogen in the 24-hour specimen of urine.

Experiment 28. Titratable Acidity (Folin). Measure 25 cc. of urine into a flask and add 15–20 g. of finely pulverized neutral potassium oxalate and 1 or 2 drops of phenolphthalein solution (1 per cent). Shake the mixture and titrate with a standard solution (0.1 *N*) of sodium hydroxide. Calculate the acidity of the 24-hour specimen of urine in terms of cubic centimeters of 0.1 *N* acid.

NOTE: The oxalate is added to precipitate the calcium present in the urine. Otherwise, the calcium would form insoluble calcium phosphate as the urine approached the neutral point during the titration.

[37] *J. Biol. Chem.*, **52**, 387 (1922). For a modification of this method see A. A. Christman and S. Ravwitch, *J. Biol. Chem.*, **95**, 115 (1932).

[38] Benedict's uric acid reagent is prepared as follows: Introduce into a liter flask 100 g. of pure sodium tungstate, and dissolve in about 600 cc. of water. Add 50 g. of pure arsenic pentoxide, followed by 25 cc. of 85 per cent phosphoric acid and 20 cc. of concentrated hydrochloric acid. Boil the mixture for 20 minutes, cool, and dilute to 1 liter. The reagent appears to keep indefinitely.

The oxalate also diminishes the disturbing effect of ammonium salts. Omitting the addition of the potassium oxalate, repeat the determination and compare the results of the two titrations.

Experiment 29a. Determination of Total Phosphates by Titration with Uranium Acetate. Measure into an Erlenmeyer flask 50 cc. of urine (use a pipette). Add 5 cc. of "special" sodium acetate [39] solution, and heat to boiling. Maintaining the mixture at the boiling point, titrate with a standard solution of uranium acetate.[40] The standard solution should be added slowly as long as any precipitate is formed. From time to time, remove a drop of the mixture at the end of a glass rod and bring it into contact with a drop of 10 per cent potassium ferrocyanide. (This may be done conveniently on a porcelain test tablet.) The end point is indicated by the formation of a reddish-brown coloration.

From the uranium acetate used, calculate the excretion of phosphates in terms of grams of P_2O_5 for the 24-hour period. The standard uranium solution is usually prepared so that 1 cc. is equivalent to 5 mg. (0.005 g.) of P_2O_5. Calculate your results in terms of P, as well as in terms of H_3PO_4 (expressed in grams).

Calculate the results obtained above in terms of cubic centimeters of 0.1 *N* NaH_2PO_4, assuming that only one of the hydrogen atoms is replaceable during the titration with sodium hydroxide. Compare this result with the titratable acidity of the urine as determined in Experiment 28. What can you say regarding the condition of the phosphates in this particular specimen of urine?

[39] The sodium acetate solution is prepared as follows: Dissolve 100 g. of sodium acetate in 800 cc. of water. Add 100 cc. of 30 per cent acetic acid and dilute to 1 liter with water.

[40] The uranium acetate solution is prepared by dissolving 35 g. of uranium acetate in water (heat to facilitate solution) with the aid of 3–4 cc. of glacial acetic acid. The solution is cooled, diluted to 1 liter, and allowed to stand for several days, after which it is filtered. The solution is standardized by titration with a solution of sodium ammonium phosphate ($NaNH_4HPO_4 \cdot 4H_2O$), containing 14.721 g. of this salt per liter. The procedure for the titration is the same as that employed in the determination of phosphates in urine. The uranium acetate solution should be adjusted so that 1 cc. will be equivalent to 1 cc. of the phosphate solution, the latter being in turn equivalent to 0.005 g. of P_2O_5.

Uranium acetate forms with phosphates a precipitate of $(UO_4)HPO_2$. With potassium ferrocyanide, uranium acetate reacts to form

$$[Fe(CN)_6]\begin{matrix}\diagup\!\!\!\diagup UO_2 \\ -K \\ \diagdown\!\!\!\diagdown K\end{matrix} \quad \text{and} \quad [Fe(CN)_6][UO_2]_2.$$

By means of the same equation used in Experiment 7, Chapter I, calculate the *p*H of the urine specimen from the phosphate ratio. How does this compare with the *p*H found according to the method on p. 148.

Experiment 29b. Determination of Phosphate in Urine (Colorimetric Method of Fiske and Subbarow).[41] Measure into a 100-cc. volumetric flask enough urine to contain between 0.2 and 0.8 mg. of inorganic phosphorus (usually 1 or 2 cc.). Add water to bring the total volume to 70 cc., followed by 10 cc. of 2.5 per cent ammonium molybdate made up in 5 *N* sulfuric acid,[42] and 4 cc. of fresh 0.25 per cent amino-naphthol-sulfonic acid.[43] After the addition of each reagent, the solution should be mixed by gentle shaking.

At the same time, transfer to a similar flask 5 cc. of the standard phosphate solution (containing 0.4 mg. of phosphorus),[44] 65 cc. of water, and the same reagents that were added to the urine sample. Dilute the contents of each flask to the mark, mix, and compare in the colorimeter after 5 minutes. Compare the results obtained by this method with those obtained in Experiment 29a.

Experiment 30. Chlorides (Volhard-Arnold Method). Pipette 10 cc. of urine into a 100-cc. volumetric flask. Add 20–30 drops of nitric acid (sp. gr. 1.2) and 2 cc. of a cold, saturated solution of ferric alum. (Should a red color develop at this point it may be dissipated by the addition of a few drops of an 8 per cent solution of potassium permanganate.) While shaking the mixture gently, add slowly from a burette or pipette 20 cc. of standard silver nitrate solution.[45] (In the presence of excessive amounts of chloride, it may be necessary to use more than this volume of silver nitrate solution.)

[41] *J. Biol. Chem.*, **66**, 375, 389 (1925).

[42] Dissolve 25 g. of ammonium molybdate in 200 cc. of water. Rinse into a liter volumetric flask, containing 500 cc. of 10 *N* sulfuric acid. Dilute to the mark with water, and mix.

[43] Dissolve 0.5 g. of dry amino-naphthol-sulfonic acid in 195 cc. of 15 per cent sodium bisulfite, add 5 cc. of 20 per cent sodium sulfite, stopper, and shake until dissolved. If the bisulfite solution is old, more than 5 cc. of sulfite will be needed; in that event add more sulfite, 1 cc. at a time, shaking after each addition, until solution is complete. For further details, see the original paper of Fiske and Subbarow.[41]

[44] *Standard Phosphate Solution* (5 *cc.* = 0.4 *mg.* P). Dissolve 0.3509 g. of pure mono-potassium phosphate (KH_2PO_4) in water. Transfer quantitatively to a liter volumetric flask, add 10 cc. of 10 *N* sulfuric acid, dilute to the mark, and mix. The standard keeps indefinitely.

[45] *Standard Silver Nitrate Solution.* Dissolve 29.061 g. of silver nitrate in 1 liter of distilled water. One cubic centimeter of this solution is equivalent to 0.01 g. of sodium chloride or 0.006 g. of chlorine.

Allow the mixture to stand for 10 minutes, then dilute to the mark with distilled water. Mix the contents of the flask thoroughly and filter through a dry filter into a dry vessel. Of the filtrate, 50 cc. is removed (use a pipette) and titrated with a standardized solution of ammonium thiocyanate[46] (sodium or potassium thiocyanate may be used instead) until a faint but permanent red tinge is obtained. The ferric alum is the indicator. When all the excess silver nitrate is used up in the titration with ammonium thiocyanate, the addition of a slight excess of the latter results in the formation of red ferric thiocyanate. Write the equations representing these reactions.[47]

From the titration values calculate the amount of chloride eliminated in the 24-hour specimen of urine (*a*) in terms of NaCl, (*b*) in terms of Cl.

Experiment 31. Total Sulfur (Benedict's Method.[48] Givens' Modification).[49] Ten cubic centimeters of urine are delivered into a 150-cc. porcelain dish or casserole. Any trace of urine on the side of the dish is washed down with 10 cc. of Benedict's sulfur reagent.[50] The vessel is then placed on the electric hot plate, plugged in "low," without further attention; or, if it is desired to hasten the process but sacrifice some time in watching, one can plug in "medium heat." In about 45 minutes to an hour, the contents of the dish or casserole will be evaporated to dryness. The casserole or dish is then

[46] The thiocyanate (sulfocyanate) solution is prepared so that 1 cc. is equivalent to 1 cc. of the standard silver nitrate solution.

[47] *The Volhard-Harvey Method.* This is a simplified procedure for the estimation of chlorides in urine. Dilute 5 cc. of urine with 20 cc. of water. (A small flask or beaker may be used, but a small porcelain evaporating dish or casserole is to be preferred.) Add 10 cc. of silver nitrate and 2 cc. of acidified ferric alum indicator. (The indicator is prepared as follows: Dissolve 100 g. of crystalline ferric ammonium sulfate in a mixture containing 70 cc. of 33 per cent nitric acid (sp. gr. 1.2) and 30 cc. of distilled water. Filter.) The excess of silver nitrate is determined directly by titrating the mixture with standard ammonium thiocyanate until a faint red color is obtained. The same standard reagents may be used in this determination as are used in the Volhard-Arnold Method.

[48] *J. Biol. Chem.*, **6**, 363 (1909).

[49] *Ibid.*, **29**, 15 (1917).

[50] *Benedict's Sulfur Reagent:*

Crystallized copper nitrate	200 g.
Sodium or potassium chlorate	50 g.
Distilled water to	1000 cc.

This mixture oxidizes the organic matter present in the urine. The unoxidized sulfur is oxidized to sulfate. Barium chloride is then added to precipitate all of the sulfate as barium sulfate, in which form it is finally weighed.

removed from the hot plate, put directly over the Bunsen burner, and heated to the fullest extent of the burner for 10–12 minutes to drive off all NO_2 and decompose all chlorate. If there is any loss of material due to spattering, the determination should be repeated. The flame is then removed and the dish allowed to cool more or less completely. Ten to 20 cc. of dilute (1 : 4) hydrochloric acid is then added to the residue until the contents have completely dissolved and a perfectly clear solution is obtained. This dissolving of the residue requires scarcely 2 minutes. With the aid of a stirring rod,[51] the solution is washed into a 300 cc. Erlenmeyer flask and diluted with cold, distilled water to 100–150 cc. Ten cubic centimeters of 10 per cent barium chloride solution are now added, drop by drop, and the solution allowed to stand for about an hour. It is then shaken up and filtered under suction through a weighed Gooch crucible.[52] It is essential to make control analyses of the reagents used in this determination in order to ascertain their sulfur content.

Following the usual analytical procedure, the weight of the barium sulfate is determined.[53] From the weight of the barium sulfate, calculate the sulfur content of the urine taken for analysis (10 cc.) and the total sulfur output for the 24-hour period.

[51] Sometimes the porcelain glaze cracks during the heating, and it is then safer to filter the solution through a small folded filter into the flask, followed by a little wash water.

[52] The Gooch crucible has a perforated bottom which may be covered over by a layer of asbestos which serves as a filter. For the proper method of preparing such a filter, the student should consult the instructor as well as standard textbooks on quantitative analysis. The crucible containing the filter is dried in a hot-air oven at 110° C. for several hours and subsequently cooled in a desiccator. It is then weighed. The drying and cooling should be repeated until two successive weighings yield identical results. In the determination, the barium sulfate is filtered on to the asbestos filter in the crucible. It should be washed thoroughly with cold water. The crucible is then placed in the drying oven and alternately heated and cooled until successive weighings yield results that check.

The barium sulfate precipitate may be filtered on to an "ashless" filter paper. The precipitate is washed with cold water. The filter paper is then allowed to dry. It is then carefully folded (any loss of precipitate being avoided) and placed in a crucible that has been previously ignited, cooled in a desiccator, and weighed. The crucible is now heated, the heat being gradually increased until the filter paper is ignited. The heating is continued at a dull-red heat until only a white residue of barium sulfate remains in the crucible. The crucible is cooled in the desiccator and is finally weighed. The ignition and cooling should be repeated until the crucible attains constant weight.

[53] Subtract the weight of the empty crucible from the weight of the crucible plus barium sulfate. If an "ashless" filter paper was used in the analysis, correct for the weight of the ash of the filter paper.

NOTE: In the case of dilute urines, more than 10 cc. should be taken for analysis.

Experiment 32. Total Sulfates (after Folin).[54] Place 25 cc. of urine and 20 cc. of dilute hydrochloric acid (1 part of concentrated acid to 4 parts of water) in a 250-cc. Erlenmeyer flask and boil gently for 30 minutes, keeping the flask covered with a watch glass during the boiling. Cool under the tap and dilute to about 150 cc. with cold distilled water. Now add 10 cc. of 5 per cent barium chloride solution, drop by drop, taking care not to shake the solution during the addition of the barium chloride, nor for an hour afterward. After one hour, filter off the barium sulfate on to an ashless filter paper. (A Gooch crucible may be used instead.) Wash with about 250 cc. of cold water. Let the paper dry and then place it in a crucible which has been previously heated, cooled in a desiccator, and weighed. Ignite until a white or nearly white residue remains. Cool in the desiccator and weigh. Reheat and weigh again until a constant weight is obtained. Calculate, from the weight of the barium sulfate, the amount of sulfur present as sulfate in the 24-hour specimen of urine. Calculate your results also in terms of SO_3 and H_2SO_4.

Experiment 33. Inorganic Sulfates (after Folin).[54] Place 25 cc. of urine, 100 cc. of water, and 10 cc. of hydrochloric acid (1 : 4) in a 250-cc. Erlenmeyer flask. Add, drop by drop, 10 cc. of 5 per cent barium chloride, taking care to avoid shaking during the addition of the barium chloride and for at least one hour afterward. From this point, proceed as in the determination of total sulfates (Experiment 32).

Calculate the quantity of inorganic sulfates in the 24-hour specimen of urine, expressing the results (*a*) as S, (*b*) as SO_3, (*c*) as H_2SO_4.

Neutral or Unoxidized Sulfur. From the result obtained in Experiment 31 for total sulfur, subtract the value obtained in Experiment 32 for the sulfur present as "total sulfates." The difference is the amount of sulfur present in the unoxidized or so-called neutral form.

Ethereal Sulfates. From the result obtained in Experiment 32 for total sulfates subtract the value obtained in Experiment 33 for inorganic sulfates. The difference represents the ethereal sulfates. (What is the chemical nature of these substances?)

Experiment 34a. Volumetric Method for the Determination of Sulfates in Urine (Method of Rosenheim and Drummond).[55] **Inorganic Sulfates.** With a pipette measure 25 cc. of urine into a 250-cc.

[54] *J. Biol. Chem.*, **1**, 131 (1905–06).

[55] *Biochem. J.*, **8**, 143 (1914–15).

Erlenmeyer flask; add dilute (1 : 4) hydrochloric acid until the reaction is distinctly acid to Congo red paper (1–2 cc. of the acid is usually sufficient). Add 100 cc. of the benzidine solution [56] and allow the precipitate to settle for 10 minutes. Filter through a Büchner funnel, under suction; wash with 10–20 cc. of water saturated with benzidine sulfate; transfer the precipitate and filter paper to the original flask with about 50 cc. of hot water. Titrate hot with 0.1 *N* NaOH after adding a few drops of a saturated solution of phenolphthalein.

Calculate the amount of inorganic sulfate present in the 24-hour specimen of urine in grams of H_2SO_4, SO_3, and S.

Total Sulfates. Measure 25 cc. of urine into a 250-cc. Erlenmeyer flask; add 2–2.5 cc. of 1 : 4 hydrochloric acid solution; cover with a watch glass and boil for 15 to 20 minutes to hydrolyze the ethereal sulfates. Carefully neutralize the acid, after boiling, with a solution of sodium hydroxide, and then add hydrochloric acid until the reaction is just acid to Congo red paper. Cool the solution and precipitate the sulfates, as before, with benzidine. Continue the procedure as outlined above.

Calculate the amount of total sulfate present in the 24-hour specimen in grams of H_2SO_4, SO_3, and S.

The difference between the total and inorganic sulfates represents the *ethereal* sulfates.

Experiment 34b. Volumetric Method for the Determination of Total Sulfur, Sulfates, etc., in Urine (Fiske's [57] Modification of the Method of Rosenheim and Drummond). A preliminary step in these determinations is the removal of phosphates, as follows: Transfer to a 50-cc. volumetric flask sufficient urine to contain between 5 and 10 mg. of sulfur (usually 5–10 cc. of urine is taken) in the form of inorganic sulfate, and dilute to about 25 cc. with water. Add 1 drop of phenolphthalein solution and 1 drop of concentrated ammonium hydroxide (or as much as is necessary to make the solution faintly pink), followed by 5 cc. of a 5 per cent solution of ammonium chloride. Make up to the mark, mix, and pour the solution into a dry Erlenmeyer flask containing about 0.75 g. of finely powdered basic magnesium carbonate (sulfate-free). Shake for 1 minute, and transfer to a 9 cm. filter paper enough of the suspension to fill the paper nearly to the top. Allow

[56] *Preparation of the Benzidine Solution.* Four grams of benzidine are rubbed into a fine paste with about 10 cc. of water and transferred with about 500 cc. of water into a 2-liter flask. Five cubic centimeters of concentrated HCl are added, the flask is shaken until the benzidine dissolves, and the solution is made up to 2 liters with distilled water.

[57] *J. Biol. Chem.*, **47**, 59 (1921).

this first filtrate to drain back into the Erlenmeyer flask, and then filter the entire suspension through the same paper into a dry container.

Inorganic Sulfate. Pipette 5 cc. of the filtrate into a 100-cc. beaker. Add 2 drops of 0.04 per cent alcoholic solution of bromphenol blue and 5 cc. of water. Then add approximately *N* HCl, drop by drop, until the solution is yellow without a trace of blue. Run in, from a pipette, 2 cc. of benzidine reagent [58] and let stand for 2 minutes. Finally, add 4 cc. of 95 per cent acetone, and let stand for 10 minutes more. Filter through a mat of paper pulp in a special filtration tube (Fig. 3), using suction. Wash the beaker and the filter, first with three 1-cc. portions of 95 per cent acetone, and then once with 5 cc. Transfer about 2 cc. of water to the filtration tube, and poke the precipitate and mat through the hole in the lower end into a large Pyrex test tube (200 by 20 mm.), using a sharpened nichrome wire. Rinse off the wire with a few drops of water, and heat the contents of the test tube just to boiling, leaving the filtration tube suspended in the mouth of the test tube. Add 2 drops of a 0.05 per cent aqueous solution of phenol red (mono-sodium salt) and run in from a microburette, through the filtration tube, about 1 cc. of 0.02 *N* NaOH. Rinse down the wall of the filtration tube with 2 or 3 cc. of water from a wash bottle, heat again to boiling until steam escapes actively from the test tube, and rinse a second time with sufficient water to bring the total volume up to about 10 cc. This treatment should suffice to remove all traces of precipitate from the filtration tube, which may now be removed, and the titration of 0.02 *N* NaOH continued. When the color begins to change from yellow to red, again heat to boiling, and pour the hot solution into the beaker (in which the precipitation took place) and back. This will decompose any trace of precipitate that may have adhered to the wall of the beaker. From this point on, the standard alkali should be added, not more than 0.02 cc. at a time, until the solution acquires a definite pink color, which further boiling does not discharge.

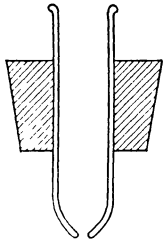


FIG. 3—Filtration Tube (One-half Natural size).

Each cubic centimeter of 0.02 *N* NaOH is equivalent to 0.32 mg. S. Multiply the titration value by 0.32. The result is the number of milligrams of S present as *inorganic sulfate* in 5 cc. of urine. Calculate

[58] Suspend 4 g. of benzidine in about 150 cc. of water in a 250-cc. volumetric flask. Add 50 cc. of *N* HCl. Shake until dissolved, and make up to volume. Filter if necessary.

the inorganic sulfate output for the 24-hour period in terms of S, SO_3, and H_2SO_4.

Total Sulfate. To 5 cc. of the filtrate in a 100-cc. beaker, add 1 cc. of 3 *N* HCl (approximate). Heat on the water bath until the solution has evaporated to dryness, and for 10 minutes longer. Immediately add 10 cc. of water, and break up the residue by rotating the beaker. Add 2 cc. of the benzidine reagent and (2 minutes later) 4 cc. of acetone, exactly as in the method for inorganic sulfate, and complete the determination as described above.

The calculation is the same as for inorganic sulfate.

Ethereal Sulfate. The *ethereal sulfate* is the difference between the total sulfate and the inorganic sulfate.

Total Sulfur. Transfer 0.25 cc. of Benedict's sulfur reagent (page 176) to a 6-cm. evaporating dish, and add 5 cc. of the urine filtrate. Evaporate to dryness, preferably on an electric hot plate at low heat. When the mixture has become dry, increase the heat by steps to the maximum, and finish the ignition with a micro-burner, allowing 2 minutes at red heat after the contents of the dish have become thoroughly black. Cool for 5 minutes. Add 1 cc. of 3 *N* HCl, and evaporate to dryness on the hot plate (low heat). When the residue is thoroughly dry, dissolve and wash into a 100-cc. beaker with five 2-cc. portions of water. Add 1 drop of *N* HCl, and precipitate with the benzidine reagent and acetone as in the other two methods. The rest of the determination is likewise the same as before, with the single exception that 2 cc. of 50 per cent acetone should be used in place of the *first* of the three 1-cc. portions of 95 per cent acetone. Otherwise, it would be impossible to wash the filter free from copper.

The calculation is the same as before.

Neutral or Unoxidized Sulfur. The difference between total sulfur and total sulfate sulfur represents the neutral or unoxidized sulfur.

NOTES: Kahn and Leiboff, *J. Biol. Chem.*, **30**, 623 (1928), have devised a colorimetric method for the determination of inorganic sulfates in small amounts of urine. The sulfate is precipitated as benzidine sulfate; the precipitate is diazotized and coupled with phenol in an alkaline medium to produce a yellow color which is proportional to the amount of benzidine.

Wakefield, *J. Biol. Chem.*, **81**, 713 (1929), has described a colorimetric method for the determination of total and inorganic sulfate in blood, serum and urine. The sulfate is precipitated with benzidine; the precipitate is washed and dissolved in dilute hydrochloric acid and then treated with hydrogen peroxide and ferric chloride. A yellow solution is produced which is compared with known standards.

Experiment 35a. Determination of Glucose in Urine (Benedict's Method). This method is described on page 42. If the sugar content

of the urine is low, it is not necessary to dilute it. On the other hand, if the concentration is high, dilute 10 cc. of the urine in a volumetric flask to 100 cc. with distilled water. The contents of the flask should be thoroughly mixed before analyzing.

Experiment 35b. Microchemical Adaptation of Benedict's Method. Accurately measure 1 cc. of Benedict's reagent into a test tube.[59] Add about 0.5 g. of anhydrous sodium carbonate and a small, well-dried pebble or piece of quartz. Heat the mixture to boiling, then add the urine (diluted if necessary) from a micro-burette [60] at intervals, a drop at a time, until reduction is complete, as evidenced by the disappearance of the blue color. When nearing the end-point the urine should be added very slowly and sufficient time allowed for boiling after each addition to insure the qualitative reduction of the reagent.

Calculation. 1 cc. of Benedict's reagent is reduced by 2 mg. of glucose. Hence the volume of urine used in the titration presumably contains 2 mg. of glucose. From the result of the titration calculate the percentage of sugar in the urine. If the urine has been diluted, the result is multiplied by the dilution. If a 24-hour specimen has been analyzed, calculate the total glucose excretion.

Experiment 35c. Sumner's Dinitrosalicylic Acid Method.[61] Pipette into a Folin-Wu sugar tube (see page 212) 1 cc. of urine (diluted if the qualitative sugar test indicates that the concentration is high) and 3 cc. of the dinitrosalicylic acid reagent.[62]

At the same time, either one or several standards may be prepared, containing 1 mg., 0.5 mg., or 0.25 mg. of glucose per 1 cc.

To 1 cc. of the standard glucose solution add 3 cc. of the dinitrosalicylic acid reagent. Mix and immerse the tubes in boiling water for 5 minutes. Remove and cool in running water for 3 minutes, dilute to the 25-cc. mark, mix, and compare in the colorimeter in the usual way.

[59] These directions are based on those given by Smith, *J. Lab. Clin. Med.*, **7**, 364 (1921–22), who employs a specially designed test tube.

[60] A 1-cc. or 2-cc. micro-burette, calibrated at intervals of 0.01 or 0.02 cc., is satisfactory. Smith recommends a specially designed 0.4-cc. micro-pipette.

[61] *J. Biol. Chem.*, **65**, 393 (1925).

[62] *Dinitrosalicylic Acid Reagent of Sumner.* To 10 g. of crystallized phenol add 22 cc. of 10 per cent NaOH. Dissolve in a little water and dilute to 100 cc. Weigh out 6.9 g. of sodium bisulfite and add to it 69 cc. of the alkaline phenol reagent. Then add a solution containing the following:

300 cc. of 4.5 per cent NaOH
255 g. of Rochelle salt ($KNaC_4H_4O_6 \cdot 4H_2O$)
800 cc. 1 per cent dinitrosalicylic acid solution.

Mix and keep tightly stoppered in well-filled bottles.

Calculate the percentage of sugar in the urine.

Experiment 36a. Folin's Gravimetric Method for the Determination of Albumin in Urine.[63] Pipette 10 cc. of urine into an ordinary conical centrifuge tube, which has been previously weighed; add 1 cc. of 5 per cent acetic acid, and let stand for 15 minutes in a beaker of boiling water. At the end of this time remove the tube from the water bath and centrifuge for a few minutes. Pour off the supernatant liquid, stir up the precipitate in the tube with about 10 cc. of boiling 0.5 per cent acetic acid, and again centrifuge. Remove the supernatant liquid from the precipitate in the tube and wash once more, this time with 50 per cent alcohol. After centrifuging and pouring off the supernatant alcohol, place the tube for 2 hours in an air bath at 100 to 110° C., then cool in a desiccator and weigh.[64] Multiply the weight by 10 to obtain the percentage of protein in the urine.

Experiment 36b. Freeing Urine from Albumin, and Kjeldahl Determination of the Albumin.[65] Take 100 cc. of urine. If necessary, make it faintly acid with dilute acetic acid, and heat on the water bath until the albumin begins to separate in flakes. After drying the outside of the beaker, boil for 2 minutes over a free flame. If the albumin does not coagulate well, carefully add a drop or two of dilute acetic acid. Excess of acid may cause some of the albumin to stay in solution. Filter, while still hot, through a nitrogen-free filter. If further quantitative determinations are to be made on the urine, filter into a measuring flask, and wash the beaker and filter paper with small amounts of distilled water until the volume at room temperature is brought up to its original amount. Wash the precipitate on the filter with more warm water and then determine the nitrogen of the filter paper and its contents by the Kjeldahl method. The amount of nitrogen contained in the reagents and in the filter paper should be determined by making a control analysis. The nitrogen of the precipitate multiplied by 6.25 is equal to the percentage of albumin in the urine.

Experiment 36c. Esbach's Method. This method for the determination of protein in urine though it gives at best only approximate values is still employed quite extensively in clinical laboratories. Esbach's albuminometer is used in this determination. Urine is added to

[63] After O. Folin, "Laboratory Manual of Biological Chemistry," D. Appleton & Co., New York, 1926 edition, page 211.

[64] Instead of weighing the protein precipitate, its nitrogen content may be determined by the Kjeldahl method, or else the precipitate may be first weighed and subsequently analyzed for nitrogen. The amount of nitrogen, multiplied by 6.25, multiplied by 10, gives the percentage of protein in the urine.

[65] After Van Slyke, "Medical War Manual," No. 6, page 105, published by Lea & Febiger, 1918.

the mark U. Esbach's reagent (10 g. of picric acid and 20 g. of citric acid in 1 liter of water) is then added to the mark R. The tube is now inverted several times and set aside in a cool place. At the end of 24 hours, the height of the precipitate is read off. The reading corresponds to the grams of protein per liter of urine. Accordingly, the reading, divided by 10, gives the percentage of protein in the urine.

Experiment 36d. Tsuchiya's Method. If the urine is alkaline, acidify with acetic acid. Proceed as in Experiment 36*c*, using Tsuchiya's reagent [66] in place of Esbach's.

Experiment 37. Determination of the Acetone Bodies (Van Slyke's Methods).[67,68] *Removal of Glucose and Other Interfering Substances*

[66] *Tsuchiya's reagent:*

Phosphotungstic acid	1.5 g.
Hydrochloric acid, concentrated	5.0 cc.
Alcohol (96 per cent)	95.0 cc.

[67] *J. Biol. Chem.*, **32**, 455 (1917); published with the permission of Dr. D. D. Van Slyke and the *Journal of Biological Chemistry.*

These methods are based on a combination of Shaffer's oxidation of β-hydroxybutyric acid to acetone and Denigès' precipitation of acetone as a basic mercuric sulfate compound. Oxidation and precipitation are carried out simultaneously in the same solution, so that the technique is simplified to boiling the mixture for an hour and a half under a reflux condenser, and weighing the precipitate which forms. The acetone and acetoacetic acid may be determined either with the β-hydroxybutyric acid or separately. Neither the size of sample nor mode of procedure has required variation for different urines; the same process may be used for the smallest significant amounts of acetone bodies and likewise for the largest that are encountered. The precipitate is crystalline and beautifully adapted to quick drying and accurate weighing; but when facilities for weighing are absent the precipitate can be redissolved in dilute hydrochloric acid and the mercury titrated with potassium iodide by the method of Personne (1863).

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

J. A. Behre and S. R. Benedict, *J. Biol. Chem.*, **70**, 487 (1926) have described a colorimetric method for the determination of acetone bodies in urine and blood, based on the reaction of acetone with salicylic aldehyde in alkaline solution.

[68] *Reagents required for Van Slyke's Methods:*

1. 20 per cent copper sulfate ($CuSO_4 \cdot 5H_2O$).
2. 10 per cent mercuric sulfate (dissolve 73 g. of pure red mercuric oxide in 1 liter of 4 *N* H_2SO_4).
3. 17 *N* sulfuric acid.
4. 10 per cent calcium hydrate suspension. Mix 100 g. of Merck's fine light "reagent" $Ca(OH)_2$ with 1 liter of water.
5. 5 per cent potassium bichromate solution in water.

The "combined reagents" for the total acetone body determination contain the above reagents in the following proportions:

17 *N* H_2SO_4, 1 liter; mercuric sulfate, 3.5 liters; water, 10 liters.

from Urine. With a pipette measure 25 cc. of urine into a 250-cc. volumetric flask. Using a graduate, add 100 cc. of water and 50 cc. of copper sulfate solution, and mix. Then add 50 cc. of 10 per cent calcium hydroxide, 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.

Simultaneous Determination of Total Acetone Bodies (Acetone, Acetoacetic, and Hydroxybutyric Acid) in One Operation. Place in a 500 cc. Erlenmeyer flask 25 cc. of urine filtrate. Add 100 cc. of water, 10 cc. of 50 per cent sulfuric acid, and 35 cc. of the 10 per cent mercuric sulfate. Or, instead of adding the water and reagents separately, add 145 cc. 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 cc. of the 5 per cent dichromate through the condenser tube. Continue boiling gently 1½ hours. The yellow precipitate which forms consists of the mercury sulfate-chromate compound of the preformed acetone,[69] and of the acetone which has been formed by decomposition of acetoacetic acid and by oxidation of the hydroxybutyric acid. It is collected in a Gooch, or "medium density" alundum crucible, washed with 200 cc. 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.

Acetone and Acetoacetic Acid. The acetone plus the acetoacetic acid, which completely decomposes into acetone and carbon dioxide on heating, is determined without the hydroxybutyric acid, exactly as the total acetone bodies, except that (1) no dichromate is added to oxidize the hydrobutyric acid and (2) the boiling must continue for not less

[69] The approximate composition of this compound is

$$2HgSO_4\,HgCrO_4\,5HgO\,2(CH_3)_2CO.$$

than 30 nor more than 45 minutes. Boiling for more than 45 minutes splits off a little acetone from hydroxybutyric acid even in the absence of chromic acid.

β-Hydroxybutyric Acid. The 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 cc. of urine filtrate plus 100 cc. of water is treated with 2 cc. of the 50 per cent sulfuric 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 cc. Then 8 cc. of the 50 per cent sulfuric acid and 35 cc. of mercuric sulfate are added. The flask is connected under the condenser and the determination is continued as described for total acetone bodies.

Blank Determination of Precipitate from Substances in Urine Other than the Acetone Bodies. The 25 cc. aliquot of urine filtrate is treated with sulfuric acid and water and boiled 10 minutes to drive off acetone. The residue is made up to 175 cc. with the same amounts of mercuric sulfate and sulfuric acid used in the above determinations, but without chromate, and is boiled under the reflux for 45 minutes. Longer boiling splits off some acetone from β-hydroxybutyric acid, and must therefore be avoided. The weight of precipitate obtained may be subtracted from that obtained in the above determination.

The blank is so small that it is relatively significant only when compared with the small amounts of acetone bodies found in normal or nearly normal urines. In routine analyses of diabetic urines it need not be determined.

Tests of Reagents. When the complete total-acetone-bodies determination, including the preliminary copper sulfate treatment, is performed on a sample of distilled water instead of urine, no precipitate whatever should be obtained. This test must not be omitted.

Titration of the Precipitate. Instead of weighing the precipitate, one may wash the contents of the Gooch crucible, including the abbestos, into a small beaker with as little water as possible, and add 15 cc. of 1 *N* 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 precipitate to the beaker and broken up with a rod in 15 cc. of 1 *N* 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 addition of excess sodium acetate has been found the most satisfactory means. Six to 7 cc. of 3 *M* acetate is 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 cc. of KI in excess of the amount required to form the soluble K_2HgI_4 has been added. If only a few milligrams of mercury are present, the excess of KI may be added before the HgI_2 has had time to precipitate, so that the titrated solution remains clear. In this case not less than 5 cc. of the 0.2 *M* KI is added, as it has been found that the final titration is not satisfactory if less is present. The excess of KI is titrated back by adding 0.05 *M* $HgCl_2$ from another burette until a permanent red precipitate forms. Since the reaction utilized is $HgCl_2+4KI=K_2HgI_4+2KCl$, 1 cc. of 0.5 *M* $HgCl_2$ is equivalent in the titration to 1 cc. of the 0.2 *M* KI.

In preparing the two standard solutions, the 0.05 *M* $HgCl_2$ is standardized by the sulfide method, and the iodide is standardized by titration against it. A slight error appears to be introduced if the iodide solution is gravimetrically standardized and used for checking the mercury solution, instead of *vice versa*.[70]

Both by gravimetric analysis of the basic mercuric sulfate-acetone precipitate and by titration, the mercury content of the precipitate has been found to average 76.9 per cent. On this basis, each cubic centimeter of 0.2 *M* KI solution, being equivalent to 10.0 mg. of Hg, is equivalent to $\frac{10.0}{0.769} = 13.0$ 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.

[70] In standardizing the mercuric chloride the following procedure is convenient: 25 cc. of 0.05 *M* $HgCl_2$ is measured with a calibrated pipette, diluted to about 100 cc., 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 g. if the solution is accurate.

Factors for Calculating Results

1 mg. of β-hydroxybutyric acid yields 8.45 mg. of precipitate.

1 mg. of acetone yields 20.0 mg. of precipitate.

1 cc. of 0.2 *M* KI solution is equivalent to 13 mg. of precipitate in titration of the latter.

From these values the factors of the table below have been calculated.

In order to calculate the acetone bodies as *β-hydroxybutyric acid* rather than acetone, multiply the factors in the table below by the ratio of the molecular weights $\frac{\beta\text{-acid}}{\text{acetone}} = \frac{104}{58} = 1.793$. In order to calculate the acetone bodies in terms of molecular concentration, divide the factors in the table by 58. To calculate cubic centimeters of 0.1 *M* acetone bodies per liter of urine, use the factors multiplied by $\frac{10{,}000}{58} = 172.4$.

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

Determination Performed	Acetone Bodies, Calculated as Grams Acetone per Liter of Urine, Indicated by	
	1 G. of Precipitate	1 Cc. of 0.2 *M* KI Solution
Total acetone bodies*	24.8	0.322
β-hydroxybutyric acid	26 4	0.344
Acetone plus acetoacetic acid	20.0	0.260

* The "total acetone bodies" factor is calculated on the assumption that the molecular proportion of β-hydroxybutyric acid is 75 per cent of the total. This is the proportion usually approximated in acetonuria. Because hydroxybutyric acid yields only 0.75 molecule of acetone, the factors are strictly accurate only when this proportion is present, but the error introduced by the use of the approximate factors is for ordinary purposes not serious. The actual errors in percentages of the amounts determined are as follows: molecular proportion of acetone bodies as β-acid 0.50, error −6.5 per cent; β-acid 0.60, error −3.8 per cent; β-acid 0.80, error −1.3 per cent.

CHAPTER VIII

THE BLOOD

Experiment 1. Guaiac Test for Blood. Treat a dilute solution of blood with a freshly prepared alcoholic solution of guaiac (approximately 2 per cent) until a turbidity appears. Now add hydrogen peroxide drop by drop (old turpentine may be used in place of hydrogen peroxide) until a blue color develops.

Experiment 2. Benzidine Reaction. Treat a dilute solution of blood with an equal volume of a saturated solution of benzidine in glacial acetic acid. Add 1 cc. of 3 per cent hydrogen peroxide and note the development of a blue or green color.

Experiment 3. Hemolysis. Place a small drop of blood on a microscope slide or watch glass and examine microscopically. Note the individual red corpuscles. Add a few drops of water and note change. Determine the effect of ether, chloroform, bile salts, 1 per cent saponin, dilute acid and alkali, heat, alternate freezing and thawing, and 5 per cent sodium chloride solution.

Experiment 4. The Clotting of Blood and Its Prevention. This experiment may be conducted by small groups of students working together. Prepare a series of small test tubes as follows:

Tube 1 to contain a small amount of powdered potassium oxalate (about 20–50 mg.).
Tube 2 to contain powdered sodium citrate (or 2 drops of a 2 per cent solution).
Tube 3 to contain sodium fluoride (a small amount of the powdered salt or 2 drops of a 2 per cent solution).
Tube 4 to contain a few milligrams of heparin.
Tube 5 (empty), kept with tubes 1 to 4 at room temperature.
Tube 6 cooled in ice.
Tube 7 lined with paraffin.

Blood may be collected fresh from a canulated artery (the cannula should be paraffined) of a dog, or from the heart by means of a syringe.

Without delay distribute approximately 2 cc. of blood into each tube. After mixing the contents of tubes 1, 2, 3, and 4 gently but thoroughly to dissolve the anticoagulant, set all tubes in a rack.

The tubes are not to be disturbed any more than is required to make the necessary observations. Note the time required for clotting in tube 5; in tube 6; in tube 7. Explain why the blood does not clot in tube 1, tube 2, tube 3, tube 4. To each of tubes 1, 2, 3, and 4 add 1 to 2 drops of 5 per cent calcium chloride, mix, and allow to stand for 15 minutes. Explain the results.

Stopper tube 5 and allow it to remain in the incubator at 37° C. overnight. Note the retraction of the clot and the separation of the serum.

Experiment 5. Fibrinogen. Take 10 cc. of oxalated blood and separate the plasma from the corpuscles by centrifuging. Remove the plasma with a pipette and add a measured amount (3 or 5 cc.) to 100 cc. of physiological saline solution (0.9 per cent sodium chloride). Without stirring, add 5 cc. of a 5 per cent solution of calcium chloride. Let stand undisturbed for 10 to 15 minutes. Then shake the flask. Note the separation of the fibrin. Filter it off and test the solid material for protein with Millon's reagent.

Experiment 6. Hemoglobin Crystals. Mix a drop of defibrinated blood taken from a guinea pig or a white rat with a drop of water on a microscope slide, and cover with a cover glass. Examine the crystals of hemoglobin that form after a few minutes. Sketch the crystals.

Hemolyze 5 cc. of defibrinated blood (dog, rat, or guinea pig) with ether; add about 0.2 gm. of solid ammonium oxalate, shake vigorously, and place in the refrigerator for several hours to cool. Note the appearance of the contents of the tube. Transfer a drop to a slide and examine microscopically.

Experiment 7. Hemoglobin (Oxyhemoglobin and Reduced Hemoglobin). Examine spectroscopically a dilute solution of blood (1 drop of blood added to 5 cc. of distilled water). A direct-vision spectroscope is convenient for this purpose. Note the two absorption bands. The α band is near the D line. The middle of band α is about $\lambda 579$, and of band β, about $\lambda 542$. Plot the spectrum, indicating the approximate positions of the absorption bands.

Examine similarly more dilute, as well as more concentrated, solutions of blood.

To 5 cc. of a solution of blood so dilute that the two absorption bands of oxyhemoglobin are fairly far apart, add one or more drops of

Stokes' reagent[1] to which the ammonia has just been added, and without shaking note the change in the spectrum. Now shake thoroughly with air and examine the spectrum. Explain.[2] To 1 cc. of blood add an equal volume of water to produce hemolysis. Then add 2 to 3 cc. of 20 per cent potassium ferricyanide and mix by inverting. Note the liberation of gas.[3] This reaction is the basis of a method for the quantitative estimation of oxygen in blood.

Experiment 8. Carbon-monoxide Hemoglobin. Through a dilute solution of oxyhemoglobin (diluted blood) pass a current of illuminating (carbon-monoxide) gas for a few minutes. Examine spectroscopically. Add Stokes' reagent. Explain the results.

Tests to Distinguish Carbon-monoxide Hemoglobin from Oxyhemoglobin.[4] Into each of two test tubes place 1 cc. of a dilute solution of oxyhemoglobin (diluted, hemolyzed blood). Pass carbon monoxide gas through the contents of one tube. Note change in color. Now add water in equal amounts to the contents of each tube and observe that while the oxyhemoglobin on dilution becomes yellowish in color, carbon-monoxide hemoglobin retains a carmine tint.

Into each of two test tubes measure 1 cc. of blood. Pass carbon monoxide gas through one. To each tube add 3 cc. of water, followed by 4 cc. of a freshly prepared 1 per cent tannic acid solution. Mix,

[1] Stokes' reagent is prepared as follows: Dissolve 2 g. of ferrous sulfate in cold water. To this add an aqueous solution containing 3 g. of tartaric acid. Mix and dilute to 100 cc. with water. Just before using, add just enough ammonia to all or to a portion of this solution to redissolve the precipitate that is formed at first. Ammonium ferrotartrate is formed by this procedure. It is a reducing agent.

[2] If a spectroscope is not available, the reversible change from oxyhemoglobin to hemoglobin may be shown by adding to dilute blood several drops of Stokes' reagent (or strong ammonium sulfide). Note change in color. Now shake the solution with air. Note change in color. Again add Stokes' reagent. Repeat several times and note the ease with which the reversible reaction Oxyhemoglobin $\rightleftarrows$ Reduced hemoglobin takes place.

[3] This reaction involves the conversion of oxyhemoglobin to methemoglobin; it has been represented by the following equation:

$$HbO_2 + K_3Fe(CN)_6 + H_2O = HbOH + K_2HFe(CN)_6 + O_2.$$

[4] Christman and Randall, *J. Biol. Chem.*, **102**, 595 (1933), have devised a method for the detection as well as for the quantitative estimation of carbon monoxide in blood. The procedure is based on the release of carbon monoxide by the action of acid ferricyanide solution and the reduction of palladium chloride by the gas thus liberated. The residual palladium chloride is converted into palladous iodide, which gives a red solution and may be estimated colorimetrically.

stopper, and allow to stand overnight. Note the difference in the colors developed in the two tubes.

Experiment 9. Methemoglobin. Treat 5 cc. of a dilute solution of blood with 2 drops of a freshly prepared solution of sodium nitrite. Examine the spectrum of the resulting solution. Try to reduce the solution with Stokes' reagent. Explain your results.

Experiment 10. Hemin Crystals. Place a small drop of blood upon a microscope slide and allow it to dry. Add a very small crystal of sodium chloride and a drop of glacial acetic acid.[5] Cover with a cover glass and heat to boiling over a small flame. Allow to cool. Examine under the microscope. Note the dark brown prisms and plates. Sketch some of the crystals.[6]

QUANTITATIVE ANALYSIS

Foreword. In 1919, Folin and Wu[7] published their now widely adopted system of blood analysis. The preliminary step in this system consists in the preparation of a protein-free filtrate. This is done usually by diluting 1 volume of the blood with 7 volumes of water, adding 1 volume of 10 per cent sodium tungstate, followed by 1 volume of 2 3 *N* sulfuric acid. The proteins which are thus precipitated are removed by filtration and the water-clear filtrate is analyzed. Methods have been devised for the following determinations: non-protein nitrogen, urea, uric acid, sugar, creatine, creatinine, amino acids, chlorides. Since 1919, numerous modifications and improvements have been introduced by Folin and other investigators.[8]

[5] Instead of adding sodium chloride and then glacial acetic acid, the blood after drying on the slide may be treated with 2 drops of a solution containing 0.1 g. each of KCl, KI, and KBr in 100 cc. of glacial acetic acid (Nippe's solution). Cover with a cover glass and heat gently over a small flame until the solution boils. An additional drop or two of the reagent may be run in under the cover glass before examining under the microscope.

[6] Compare the crystals with those on page 244 in Bodansky's "Introduction to Physiological Chemistry," Fourth Edition.

[7] *J. Biol. Chem.*, **38**, 81 (1919).

[8] According to Folin, *J. Biol. Chem.*, **86**, 173 (1930), blood filtrates prepared by the original method contain products of red cell disintegration which interfere with the determination of uric acid and sugar. Folin has therefore proposed a new method for the preparation of blood filtrate without laking the blood. The use of unlaked blood as a basis for analysis, while possibly obviating some of the difficulties of the older method, has not, however, superseded the original technique.

Preparation of Protein-free Extract from Unlaked Blood (Folin). Transfer 40 cc. (8 volumes) of the sulfate-tungstate solution (a solution containing 15 g.

Experiment 11. Preparation of Protein-free Blood Filtrate (Method of Folin and Wu). Blood collected for analysis should not contain an excessive amount of oxalate. To prevent coagulation, 20 mg. of sodium or potassium oxalate for 10 cc. of blood will be found sufficient. Transfer a measured amount of blood (5 to 15 cc.) into a flask having a capacity of 15 to 20 times that of the volume taken. For this work a special pipette has been devised by Folin and Wu (Fig. 4).[9] Ordinary pipettes may be used, however. Dilute the blood with 7 volumes of distilled water, and mix. With an appropriate pipette add 1 volume of 10 per cent solution of sodium tungstate ($Na_2WO_4 \cdot 2H_2O$), and mix. With another suitable pipette add to the contents of the flask 1 volume of 2/3 *N* sulfuric acid, slowly, carefully shaking after each addition. Close the mouth of the flask with a rubber stopper and shake vigorously a few times. Air bubbles should be absent and a "metallic" sound should be heard during the shaking. Let stand for at least 5 minutes. The color of the coagulum gradually changes from bright red to dark brown. If this change in color does not occur, the coagulation is incomplete, usually because too much oxalate is present. In such an emergency the sample may be saved by adding 10 per cent sulfuric acid, one drop at a time, shaking vigorously after each drop, and continuing until there is practically 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. If the filtration is begun by pouring the first few cubic centimeters of the mixture down the double portion of the filter paper and withholding the remainder until the whole filter has been wet, the filtrates are almost invariably

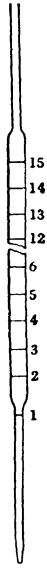


FIG. 4.—Folin-Wu Pipette.

of anhydrous sodium sulfate and 6 g. of sodium tungstate per liter) to a small flask. With a pipette, add 5 cc. of blood. Mix without any rough shaking, so as not to damage the cells mechanically, and let stand, with occasional very gentle shaking, for 5 minutes, or as much longer as may be convenient. With a pipette, add slowly, with constant but gentle mixing, 5 cc. (1 volume) of 1/3 *N* sulfuric acid. Transfer the mixture to 15-cc. centrifuge tubes, and centrifuge for 10 minutes, at a moderate speed. The supernatant liquid, which should be perfectly colorless and clear as water, is used in the analyses.

[9] It is desirable to have three such pipettes, one for measuring the blood, one for the sodium tungstate solution, and the third for the sulfuric acid. The pipette

as clear as water from the first drop. If the filtrate is not perfectly clear, the first 2 or 3 cc. may have to be returned to the funnel.

If the filtrate is to be kept for any length of time, some preservative (a few drops of toluene) should be added to prevent bacterial action. The filtrate may be set away in a refrigerator. The blood-sugar determination is to be made without undue delay, as glucose decreases on standing. Sodium fluoride (10 mgm. per cc.) will prevent glycolysis, as well as prevent coagulation.

NOTE: This filtrate has been prepared by diluting the blood ten times; 1 cc. of the filtrate is thus equivalent to 0.1 cc. of blood, a point to be remembered in all subsequent calculations.

Instructions to students: All determinations are to be done in duplicate.

Experiment 12a. Determination of Sugar in Blood (Folin's Modification [10] of the Folin-Wu Method). Transfer 2 cc. of the Folin-Wu filtrate to a Folin-Wu sugar tube (Fig. 5), or 1 cc. plus 1 cc. of water if very high blood-sugar values are expected. Transfer 2 cc. of the sugar standard (0.1 mg. glucose per cubic centimeter) [11] to another sugar tube. Add 2 cc. of freshly mixed copper tartrate reagent [12] to each tube and heat in rapidly boiling water for 8 minutes. Cool in running water. Add 4 cc. of the acid molybdate reagent [13] and after waiting about 1 minute dilute to volume with diluted acid molybdate reagent,[14] mix, and make the color comparison in a colorimeter.[15]

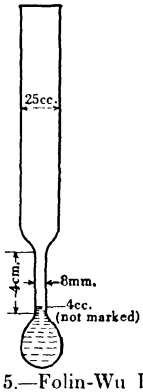


FIG. 5.—Folin-Wu Blood Sugar Tube.

Calculation: Note the glucose content of the standard, on the basis of which calculate the glucose content of the blood in milligrams per 100 cc. Is the value thus obtained within normal limits?

used for the blood may also be used in measuring the 7 volumes of water. In that case, since the volume of the blood is increased by the blood adhering to the walls of the pipette, the pipette should be wetted with distilled water (and blown out) just before it is used for measuring the water.

It is very important that the sodium tungstate should be practically free from carbonate.

The following procedure is recommended for plasma or serum: Dilute 1 volume of plasma or serum with 8 volumes of water, then add ½ volume of the sodium tungstate solution and ½ volume of ⅔ *N* sulfuric acid. From this point proceed as with whole blood.

[10] *J. Biol. Chem.*, **82**, 92 (1929).

[11] *Standard Sugar Solution.* Dissolve 2.5 g. of benzoic acid in 1 liter of boil-

NOTE: Any departure from the procedure, whether in the use of only 1 cc. of filtrate (diluted with 1 cc. of water), or in the use of a

ing water, and cool. Transfer to a bottle; this solution will keep indefinitely. Dissolve 1 g. of pure glucose in about 50 cc. of the benzoic acid solution. Transfer to a 100-cc. volumetric flask, rinse, and fill to the mark with the benzoic acid solution. Label and preserve. This *stock* solution seems to keep indefinitely.

Transfer 1 cc. of the stock solution, by means of an Ostwald pipette, to a 100-cc. volumetric flask; fill to the mark with distilled water, and mix. The diluted solution so obtained, which contains 0.1 mg. of glucose per cubic centimeter, is a standard suitable for most blood-sugar determinations. Another standard, twice as strong, is, however, occasionally needed. This is made by diluting 2 cc. of the stock solution to 100 cc. These standard solutions may be preserved by a few drops of toluene, but it is preferable to add no preservative and to make up fresh standards at frequent intervals (once or twice a week).

[12] *Solution A.* Transfer 35 g. of anhydrous sodium carbonate to a volumetric liter flask, add 175 to 200 cc. of water, and shake for a few moments. Then add 13 g. of sodium tartrate and 11 g. of sodium bicarbonate. Add water to a volume of about 800 cc., and shake until a clear solution is obtained. Dilute to volume and mix.

Solution B. A 5 per cent solution of C.P. crystallized copper sulfate to which has been added a trace of concentrated sulfuric acid to prevent the formation of a copper sediment.

For use the copper tartrate reagent is prepared as follows: Half fill a 50-cc. volumetric flask with the alkaline tartrate solution (*A*). Add 5 cc. of the 5 per cent copper sulfate solution (*B*), dilute to volume with the tartrate solution, and mix.

[13] Folin has modified his original method for the preparation of the acid molybdate reagent. Two methods are now offered: (1) for the preparation of a so-called temporary reagent, and (2) the preparation of a purified reagent which has better keeping qualities.

(1) *The Temporary Acid Molybdate Reagent.* Dissolve 40 g. of sodium molybdate in 100 cc. of distilled water in a 500-cc. beaker. The molybdate dissolves very quickly (2 to 3 minutes), but a certain turbidity is left which does not clear up. To the turbid solution add, with stirring, 55 cc. of 85 per cent phosphoric acid, 40 cc. of cool sulfuric acid (25 per cent, 1 volume of H_2SO_4 to 3 volumes of water), and finally 20 cc. of 99 per cent acetic acid. The resulting mixture is at once ready for use.

This reagent should be renewed at frequent intervals, depending on the intensity of the blue color which develops. The reagent keeps longer if placed in a refrigerator.

(2) *The Purified Acid Molybdate Reagent.* For the preparation of this reagent it is advantageous to keep on hand a brominated 30 per cent solution of sodium molybdate. By means of a large funnel and fine glass rod, transfer 600 g. of sodium molybdate to a 2-liter volumetric flask. Add much water and shake until solution is complete except for the turbidity. Dilute to volume, mix, and transfer this stock solution to a large flask or bottle. Add about 0.5 cc.

stronger standard (2 cc. = 0.4 mg. glucose), should be taken into account in calculating the results. With a blood of unknown sugar content, time may be saved by preparing three standards, one of low, one of normal, and one of high sugar content. Upon inspection the standard most nearly matching the unknown is selected for the colorimetric comparison.

Blood Sugar Values. The normal range for blood sugar with this method is 90 to 120 mgm. per 100 cc. This includes about 20 to 30 mgm. of non-sugar reducing substances. By the use of zinc hydroxide precipitation for blood proteins, or more specific reagents, the "true sugar" of the blood is found to be between 70 and 90 mgm. per cent.

Experiment 12b. Determination of Sugar in Blood (Benedict's Method).[16] To each of two (or more) Folin-Wu blood sugar tubes add 2 drops of a 1 per cent solution of sodium bisulfite.[17] Measure into one tube 2 cc. of the standard glucose solution (2 cc. = 0.2 mg. for ordinary cases, or 2 cc. = 0.4 mg. when diabetic blood is examined). Into the other tube measure 2 cc. of the Folin-Wu blood filtrate.[18]

of liquid bromine, shake, and set aside. Transfer 500 cc. of the clear supernatant solution to a Florence flask, capacity 1000 to 1500 cc. Add with stirring 225 cc. of 85 per cent phosphoric acid. Some bromine is set free and imparts a yellow color to the solution. Next add 150 cc. of cool sulfuric acid (25 volumes per cent). Remove the bromine by means of an air current either immediately while the solution is still warm or better still the next day. Then add 75 cc. of 99 per cent acetic acid. Mix and dilute to 1 liter. If protected from organic matter this reagent will remain colorless for months.

[14] The diluted acid molybdate reagent is prepared by adding to 1 volume of the reagent 4 volumes of water and mixing.

[15] This method is also applicable to the unlaked blood filtrate (see footnote, page 208). Folin states that the sugar values found with this are 10 to 15 mg. per cent lower than with the Folin-Wu filtrate, owing to the fact that the non-sugar reducing material is eliminated. In using this filtrate, it is recommended that the sugar standard be made up to contain 2 per cent sodium sulfate in order to equalize, approximately, the salt concentration in the blood filtrate.

[16] *J. Biol. Chem.*, **92**, 141 (1931). The principle of Benedict's method is similar to that of the Folin-Wu procedure, except that the reagent (Benedict's copper reagent combined with bisulfite) is more specific than the alkaline copper tartrate of the older procedure and is therefore believed to give a closer approximation of the true glucose content of the blood. By this procedure, 65 to 90 mg. of glucose per 100 cc. of blood are considered normal fasting values.

[17] The bisulfite solution should be prepared fresh once every 2 to 3 weeks.

[18] The Folin-Wu filtrate may be used with complete satisfaction. However, Benedict has described a slightly modified technique for precipitation of blood proteins with tungstomolybdic acid. *J. Biol. Chem.*, **92**, 135 (1931).

Then add to each tube 2 cc. of the copper reagent [19] and mix by careful lateral shaking. Place the tubes in vigorously boiling water for exactly 5½–6 minutes, then remove them to a large beaker of cold water. Cool for 1 minute, add 2 cc. of the color reagent,[20] mix by vigorous lateral shaking, and after 1 minute dilute the contents of each tube with water to the 25-cc. mark. Mix by inversion and read in the colorimeter, preferably within 10 minutes after dilution.

The calculations are the same as in Experiment 12a.

Experiment 13a. Determination of Non-protein Nitrogen (Folin and Wu). A portion of the Folin-Wu blood filtrate is digested with a mixture of sulfuric and phosphoric acids (this is essentially a micro-Kjeldahl digestion). The nitrogenous constituents are thus converted into ammonium salts. The latter are then quantitatively estimated by nesslerization.

Introduce 5 cc. of the protein-free filtrate (prepared in Experiment 11) into a *dry* 75-cc. test tube (Pyrex, 200 mm. by 25 mm.) graduated at 35 cc. and 50 cc. These tubes should have been washed the day before and dried *thoroughly* overnight. Add 1 cc. of the sulfuric-phosphoric acid mixture [21] and a dry quartz pebble. Place upright in

[19] *Benedict's Blood Sugar Reagent:*

Sodium carbonate (anhydrous)	15 g.
Alanine	3 g.
Rochelle salt	2 g.
Copper sulfate (crystallized)	3 g.
Distilled water to make 500 cc.	

The alanine, Rochelle salt, and copper sulfate should be weighed accurately. The sodium carbonate may be weighed more roughly.

Dissolve the carbonate, alanine, and Rochelle salt in 300 to 400 cc. of distilled water. Dissolve the copper sulfate in 50 to 75 cc. of water and add this to the other solution with constant stirring. Dilute the deep blue solution to 500 cc. If kept cool the mixed solution will keep without appreciable deterioration for at least 6 to 8 weeks.

[20] *Color Reagent.* Place 150 gms. of pure molybdic acid anhydride (free from ammonia) in a large Erlenmeyer flask and add 75 g. of pure anhydrous sodium carbonate. Add water in small portions, with shaking, until about 500 cc. have been added. Shake thoroughly and heat the mixture to boiling or until nearly all the molybdic acid has been dissolved. An appreciable amount of insoluble material remains, which is filtered off. Wash the residue on the filter with hot water until the total volume of filtrate and washings is about 600 cc. Add 300 cc. of 85 per cent phosphoric acid to the total filtrate, cool, and dilute to 1 liter.

[21] To 300 cc. of 85 per cent phosphoric acid add 50 cc. of a 5 per cent solution of copper sulfate. Mix. Now add 100 cc. of concentrated sulfuric acid (free from the least trace of ammonia). Mix again and set aside for the sedimentation

a clamp on a ring stand and adjust a low flame preferably of a microburner beneath so that the flame just covers evenly the entire bottom of the tube. Avoid a single jet of flame on one spot of the tube as this encourages violent bumping with loss of liquid. Boil vigorously until the excess water is removed, when characteristic dense fumes begin to fill the tube. At this point cut down the size of the flame so that the contents of the tube are just visibly boiling, and cover the mouth of the tube with a watch glass. Continue the heating until the contents char and then become clear, taking care not to evaporate to dryness. Extreme caution is needed in order to obtain complete digestion without allowing excessive evaporation. Allow the contents to cool for 70 to 90 seconds and then add 15 to 25 cc. of water. Cool further, approximately to room temperature, and add water to the 35-cc. mark.

The standard most commonly required is 0.3 mg. of N (in the form of ammonium sulfate).[22] Measure 3 cc. of the standard solution into a 100-cc. volumetric flask. Add to it 2 cc. of the sulfuric-phosphoric acid mixture and about 50 cc. of water.

Add, with a pipette, 15 cc. of Nessler's solution [23] to the unknown

of calcium sulfate. This sedimentation is very slow, but in the course of a week or so the top part becomes clear and 50 to 100 cc. can be removed by means of a pipette. (It is not absolutely necessary that the calcium should be thus removed, but it is probably a little safer to do so.) For use in the determination of nonprotein nitrogen, dilute the digestion mixture with an equal volume of water. Keep the solution well protected from ammonia fumes.

[22] The standard solution is prepared by dissolving 0.4716 g. of purified ammonium sulfate in a liter of ammonia-free water. Three cubic centimeters of this solution is equivalent to 0.3 mg. of N.

[23] *Nessler's Reagent (Prepared According to Folin and Wu).* Transfer 150 g. of potassium iodide and 110 g. of iodine to a 500-cc. Florence flask; add 100 cc. of water and an excess of metallic mercury, 140 to 150 g. Shake the flask continuously and vigorously for 7 to 15 minutes or until the dissolved iodine has nearly disappeared. The solution becomes quite hot. When the iodine solution, though still red, has begun to pale visibly, cool in running water and continue the shaking until the reddish color of the iodine has been replaced by the greenish color of the double iodide. This whole operation usually does not take more than 15 minutes. Now separate the solution from the surplus mercury by decantation and washing with liberal quantities of distilled water. Dilute the solution and washings to a volume of 2 liters. If the cooling is begun in time, the resulting reagent is clear enough for immediate dilution with 10 per cent alkali and water, and the finished solution can at once be used for nesslerizations.

From the stock solution of mercuric potassium iodide, made according to the process described above, the final Nessler's solution is prepared as follows: From completely saturated caustic soda solution containing about 75 g. of NaOH per 100 cc., decant the clear supernatant liquid and dilute to a concentration of 10 per cent. (It is worth while to determine by titration that a 10 per cent solution

and 30 cc. to the standard. Dilute the standard to the mark and mix. Insert a clean rubber stopper in the tube containing the unknown and mix. If the solution is turbid, filter through glass wool or centrifuge a portion before making the color comparison with the standard.

The unknown and the standard are now compared in the colorimeter. Calculate the milligrams of non-protein nitrogen in 100 cc. of blood remembering that the colorimetric calculation is based on *equivalent volumes* of unknown and standard.

NOTE: The procedure may be modified, depending on circumstances, by using a somewhat more concentrated standard, or less than 5 cc. of blood filtrate in the digestion, the latter being recommended when the N.P.N. is abnormally high.

Experiment 13b. Determination of Non-protein Nitrogen (Procedure of Koch and McMeekin[24]). Transfer 5 cc. of the Folin-Wu protein-free blood filtrate into a Pyrex tube (200 by 25 mm.). Add 1 cc. of 1 : 1 sulfuric acid,[25] and evaporate off the water in a sand bath, on an electric hot plate, or over a free flame. After the water has evaporated continue heating over the free flame of a micro-burner (or on the electric hot plate, plugged in high) until the liquid becomes charred and dense white fumes fill the tube. Cover the mouth of the tube with a watch glass and continue the heating for a few minutes, then remove and add 1 drop of 30 per cent hydrogen peroxide (Merck's blue label Superoxol),[26] letting it fall directly into the mixture. Vigorous oxidation occurs and the digest usually clears at once. Again boil for 2 to 5 minutes. Should the digest again become discolored, repeat the hydrogen peroxide treatment; otherwise allow to cool, dilute with distilled water, transfer quantitatively to a 100-cc. volumetric flask, diluting to about 75 cc.

At the same time prepare two standards, one containing 0.1 mg.

has been obtained within an error of not over 5 per cent.) Introduce into a large bottle 3500 cc. of 10 per cent sodium hydroxide solution, and add 750 cc. of the double iodide solution and 750 cc. of distilled water, giving 5 liters of Nessler's solution.

The Nessler's solution so obtained contains enough alkali in 15 cc. to neutralize 1 cc. of the diluted phosphoric-sulfuric acid mixture and to give a suitable degree of alkalinity for the development of the color given by ammonia at a volume of 50 cc.

[24] *J. Am. Chem. Soc.*, **46**, 2066 (1924).

[25] To distilled water add an equal volume of concentrated, C.P. (ammonia-free) sulfuric acid.

[26] Although the hydrogen peroxide is usually free from nitrogen, this should be established by blank analyses of the reagents.

and the other 0.3 mg. of nitrogen (1 cc. and 3 cc. respectively of the ammonium sulfate solution given on page 220).

Transfer the requisite amount of the standard ammonium sulfate solution to a 100-cc. volumetric flask, add 1 cc. of the 1 : 1 sulfuric acid, and dilute to 75 cc. To standards and unknown add 15 cc. of Nessler's reagent (prepared according to Koch and McMeekin, page 152), mix, dilute to the mark, and mix again.

Compare in the colorimeter after 5 to 20 minutes. Calculate the non-protein nitrogen in milligrams per 100 cc. of blood.

NOTE: The technique may be varied somewhat by using digestion tubes calibrated at 35 cc. and 50 cc. and by diluting the digest (after completion) to the 35-cc. mark with water, then adding 15 cc. of Nessler's reagent. No change need be made in the preparation of the standard, except that 30 cc. of Nessler's reagent should be used. The weaker standard (containing 0.1 mg. nitrogen) will be found unnecessary.

Experiment 14a. Determination of Urea (Folin and Svedberg's Modification of the Folin-Wu Method).[27] Transfer 5 cc. of tungstic acid blood filtrate to a Pyrex or Jena test tube, having a capacity of 50–75 cc. Add 2 drops of the acetate buffer solution [28] and 1 cc. of a freshly prepared jack-bean extract.[29] Insert a cork, and then either immerse the tube in a 600-cc. beaker filled with water having an initial temperature of about 45° C. or let the tube stand at room temperature. The time allowed for the digestion should be 10 minutes in the warm water or 25 minutes at room temperature. A longer digestion period causes no error.

The ammonia formed from the urea is most conveniently obtained by distillation, without a condenser, by using a test tube graduated at 25 cc. and containing 2 cc. of 0.05 *N* hydrochloric acid as the receiver. The illustration (Fig. 6) shows a compact and convenient arrangement for this distillation.

Cool the tube (if warm), remove the cork, and add (*a*) an anti-bumping tube (Fig. 6), (*b*) 2 drops of the anti-foaming oil mix-

[27] *J. Biol. Chem.*, **38**, 91 (1919); **88**, 77 (1930).

[28] *Buffer Mixture.* Dissolve 15 g. of crystallized sodium acetate in 50 to 75 cc. of water in a 100-cc. volumetric flask. Add 1 cc. of glacial acetic acid, dilute to the mark with water, and mix.

[29] Transfer 0.5 g. of jack-bean meal to a clean 50-cc. flask; add 20 cc. of 30 per cent (by volume) alcohol. Shake for 10 minutes and filter or centrifuge. This extract should always be prepared on the day it is to be used.

Instead of jack-bean extract, a permanent and convenient urease preparation in the form of filter paper impregnated with a strong urease solution may be used. For the method of preparation, consult the paper of Folin and Svedberg.

ture,[30] and finally 2 cc. of saturated borax solution.[31] Connect at once with the delivery tube and a test tube receiver graduated at 25 cc. The latter contains 1 cc. of 0.1 *N* acid and 1 cc. of water (or 2 cc. of 0.05 *N* acid). Fasten the boiling tube in a clamp and start the distillation by the help of a small microburner whose flame can be well regulated. As soon as the contents are nearly boiling, cut the flame down sharply so that the first minute of actual boiling is very gentle. Then boil briskly for about 3 minutes. Loosen the stopper from the delivery tube, raise the latter above the surface of the liquid, and continue boiling for another minute.

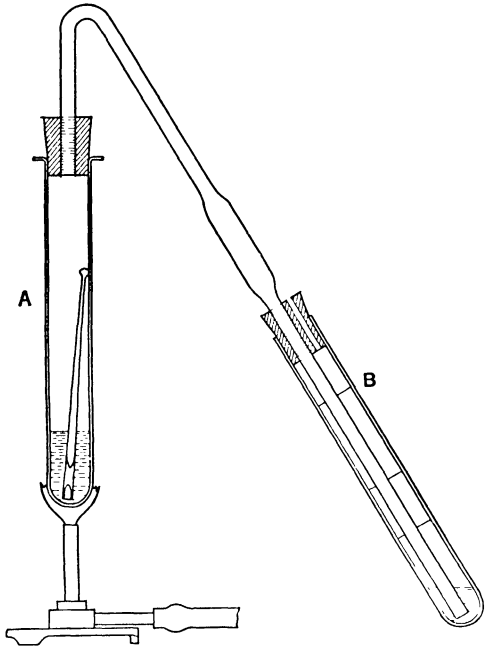


FIG. 6.—Arrangement of the Distillation Apparatus in the Determination of Urea. Note Antibumping Tube in Tube *A*.

Transfer 0.1 mg. of ammonium sulfate N (1 cc. of the standard solution) [32] to another test tube, which, like the receiver, is graduated at 25 cc., add 1 cc. of gum ghatti solution [33] to each, dilute both to a

[30] *Antifoaming Oil Mixture.* To 1 volume of crude fuel oil add about 10 volumes of toluene. Two drops of this mixture will completely prevent the foaming if the boiling is started slowly.

[31] The solution is prepared by dissolving 4 g. of anhydrous borax (sodium borate, $Na_2B_4O_7$) in 100 cc. of water.

[32] The same standard as used in the determination of non-protein nitrogen (0.4716 g. of purified ammonium sulfate in a liter of ammonia-free water).

[33] *Gum Ghatti Solution.* Fill a 500-cc. cylinder with water, and suspend at the top just below the surface of the water in a wire basket (galvanized iron) 10 g. of gum ghatti. Leave it overnight, but not for 24 hours, and then remove the basket with undissolved material. A little dirt may get into the solution when the wire basket is disturbed, but this soon settles and the clear solution may be used without any further purification.

volume of about 20 cc., and add 2.5 cc. of Nessler's reagent. Dilute to the mark, mix, and make the color comparison.

Calculate the amount of urea in 100 cc. of the blood.

Experiment 14b. Determination of Urea in Blood (after the Method of Van Slyke and Cullen).[34] This procedure is similar to that employed in the determination of urea in urine (page 158). Measure into tube *A* 3 cc. of whole blood to which oxalate or citrate has been added to prevent coagulation. Add 1 cc. of a strong urease solution and 2 or 3 drops of buffer solution (page 160). A urease tablet or powdered urease may be used in place of the urease and buffer solutions. Stopper the tube and set aside in a beaker of warm water (about 50° C.) for 30 minutes. In the meanwhile measure accurately into tube *B* 15 cc. of 0.01 *N* acid. Add 1 or 2 drops of indicator and 2 or more drops of caprylic alcohol. At the end of 30 minutes restore tube *A* to its place in the block, make sure of all connections, add through the inlet tube 5 or more drops of caprylic alcohol to the blood mixture, and pass a current of air for about a half minute, in order to aspirate into the acid in tube *B* any ammonia that may have escaped into the air space of tube *A*. Disconnect the rubber tubing from the inlet tube passing into *A* and deliver by means of a rapidly flowing pipette 10 cc. of a saturated solution of potassium carbonate. The rubber tubing is then reconnected with the inlet tube. Turn on the suction and aërate for at least 30 minutes. When aëration is complete, titrate the excess of acid in tube *B* with 0.01 *N* alkali.

Report the result as milligrams of urea per 100 cc. of blood and as milligrams of urea nitrogen per 100 cc. of blood. Show all the steps in the calculation.

The concentration of ammonia in the blood[35] is exceedingly small, being but a few hundredths of a milligram per 100 cc. Hence it may be disregarded in this calculation.

Experiment 14c. Determination of Urea in Blood (Method of Leiboff and Kahn).[36] The principle of this method is the hydrolysis of the urea under pressure in a suitably constructed tube, in the presence of sulfuric acid, followed by direct nesslerization.

[34] *J. Am. Med. Assoc.*, **62**, 1558 (1914).

[35] Methods for the determination of ammonia in blood: see O. Folin and W. Denis, *J. Biol. Chem.*, **11**, 527 (1912); T. P. Nash and S. R. Benedict, *ibid.*, **48**, 463 (1921).

[36] *J Biol. Chem.*, **83**, 347 (1929).

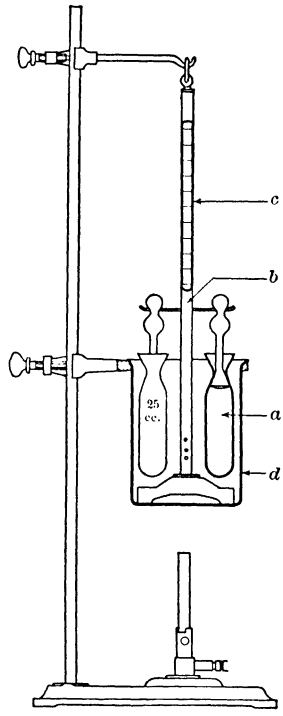


FIG. 7.—Leiboff-Kahn Urea Apparatus.

Suspend the pressure tube in the groove of the disk as shown in the diagram (Fig. 7).[37] Insert the stopper halfway in the tube by raising the tube, and introduce 5 cc. of the Folin-Wu filtrate (page 210). Add 1 cc. of N H_2SO_4 and wash down the stopper with 1 cc. of water. The solutions are best introduced into the tube by holding the tip of the pipette close to the lower end of the stopper. Close the tube by holding the stopper in its place with one hand and pulling down the tube with the other hand, turning it slightly, thus making it fit snugly. Place the rack holding the tube in the oil bath in such a way that the liquid in the tube is somewhat below the level of the oil.[38]

Heat the oil bath with a Bunsen burner, raising the temperature to 150° C. and keeping it there for 10 minutes. A variation of a few degrees does not matter. Remove the tube from the bath, cool to room temperature, and add 13 cc. of water, followed by 3 cc. of modified Nessler's reagent.[39] Dilute to the 25-cc. mark with distilled water.

[37] The pressure tube, *a*, is made of heavy Pyrex glass to withstand high pressure. The upper portion of the tube tapers off to a funnel shape and is ground on the inside at the tapered portion. The wide portion of the glass stopper inside the tube is ground in such a way that when the stopper is raised the two ground surfaces fit very snugly. When the temperature is raised to above 100° C. the pressure produced within the tube tightens the stopper very closely.

The heating is done in an oil bath, *d*. The bath consists of a metal container capable of holding six tubes, thus allowing simultaneous determinations. In the container is placed a removable rack, the center of which holds a 200° thermometer, *c*, inclosed in a metal jacket to prevent breakage. To the upper part of the thermometer jacket is attached a circular disk, *b*, with six grooves into which the external ends of the stoppers slip, thus holding the pressure tubes suspended in the oil. The edges around the grooves are slightly turned to prevent the tubes from falling off.

[38] Any oil of high boiling-point will serve the purpose. Nujol oil is satisfactory and produces little odor when heated.

[39] Nessler's reagent as prepared by Koch and McMeekin (*J. Am. Chem. Soc.*, **46**, 2066 [1924]) is recommended. See page 152 for its preparation.

Prepare the standard by introducing 5 cc. of the standard ammonium sulfate solution[40] in a 100-cc. volumetric flask two-thirds filled with water. While rotating the flask, add 12 cc. of the modified Nessler's reagent and fill with water to the mark.

Make the color comparison in a colorimeter. Calculate the urea nitrogen in milligrams per 100 cc. of blood.[41]

Experiment 15a. Determination of Uric Acid (Folin).[42] Transfer 5 cc. of the blood filtrate[43] to a test tube graduated at 25 cc. In two similar test tubes set up two standards; one containing 3 cc. of the uric acid solution[44] and 2 cc. of water; the other 5 cc. of the uric

[40] *Standard Ammonium Sulfate.* Dissolve 0.238 g. of pure ammonium sulfate in 200 cc. of water in a liter volumetric flask and add *N* sulfuric acid to the liter mark. Five cubic centimeters of this solution contain 0.3 mg. of nitrogen.

[41] The values for urea obtained by the Leiboff-Kahn method are somewhat higher than those obtained by the other procedures which have been described.

Calculation: Since 100 cc. of the standard are equivalent to 0.3 mg. N, 25 cc. is equivalent to 0.075 mg. In the determination 5 cc. of the filtrate were used, this being equivalent to 0.5 cc. of the original blood. Accordingly the following formula may be used in calculating the urea N in 100 cc. of blood.

$$\frac{\text{Reading of the standard}}{\text{Reading of the unknown}} \times 15 = \text{mg. urea N per 100 cc. of blood.}$$

NOTE: If the urea concentration is high, less than 5 cc. of the blood filtrate (diluted to 5 cc. with water) may be used and an appropriate correction introduced in the calculation.

[42] *J. Biol. Chem.*, **86**, 173 (1930).

[43] Although this method is applicable to both the Folin-Wu filtrate and the unlaked blood filtrate, Folin recommends the use of the latter.

[44] *Preparation of Uric Acid Standard. Stock Solution.* Transfer 1 g. of uric acid to a liter volumetric flask. Transfer 0.6 g. of lithium carbonate to a 250-cc. Florence flask, add 150 cc. of water; shake until solution is obtained (5 minutes). Some insoluble material remains and it may be removed by filtering. Heat the solution (or filtrate) to 60° C. Also, warm the liter flask under running warm water. Pour the warm lithium carbonate solution into the liter flask, and shake. The uric acid is dissolved. The lithium carbonate solution is not always perfectly clear, even when filtered, and one should not mistake this little turbidity for undissolved uric acid and keep warming and shaking too long. In 5 minutes all the uric acid should be dissolved. Shake the flask under cold running water without undue delay. Add 20 cc. of 40 per cent formalin, and half fill the flask with distilled water. Add a few drops of methyl orange solution and finally add, from a pipette, rather slowly and with shaking, 25 cc. of normal sulfuric acid. The solution should turn pink, while 2 or 3 cc. are still left in the pipette. Dilute to volume, mix thoroughly, and transfer to a clean, tightly stoppered bottle. This stock solution, containing 1 mg. of uric acid per cc., should be kept away from light.

Standard Solution. Dilute 1 cc. of the stock solution with water to 250 cc. It keeps well for many days, especially if kept in the refrigerator.

acid solution. To each of the tubes add 5 cc. of the cyanide-urea solution[45] (**measured from a burette** or a small graduate cylinder) and mix thoroughly. Add 1 cc. of the uric acid reagent[46] to each tube,

[45] *Sodium Cyanide-Urea Solution.* Transfer 50 g. of sodium cyanide to a 2-liter beaker. Add 700 cc. of water, and stir continuously until substantially complete solution is obtained. Add 300 g. of C.P. urea (Merck's) and stir; complete solution is obtained in a couple of minutes, but the solution is not clear, mostly because of impurities in the urea. Transfer the solution to a 2-liter flask, add 5–6 g. of C.P. calcium oxide and shake moderately for 4 or 5 minutes. Filter. The filtered solution contains calcium hydroxide. This may be removed by adding for each 100 cc. of the solution, 1 g. of disodium phosphate, $Na_2HPO_4 \cdot 5H_2O$, in *finely powdered form*, shaking and filtering or centrifuging.

It is not essential to remove the calcium hydroxide. If it is not removed, turbidity due to calcium phosphate will form in the determination and this may be centrifuged off.

The cyanide solution will remain satisfactory for 2 months, or longer, if kept in the refrigerator.

For further details consult Folin's paper to which reference has been made.

[46] *Preparation of Uric Acid Reagent.* Folin and Marenzi (*J. Biol. Chem.*, **83**, 109 [1929]). Transfer 100 g. of sodium tungstate and 200 cc. of water to a 500-cc. Florence flask. Shake until the tungstate is dissolved. Add slowly, with shaking and cooling, 20 cc. of 85 per cent phosphoric acid. The solution must not be allowed to become warm from the heat of the reaction with the phosphoric acid. Pass H_2S into the phosphotungstate solution at a very moderate rate for 20 minutes. At the end of the first 3 or 4 minutes, add gradually and slowly another 10 cc. of 85 per cent phosphoric acid without interrupting the H_2S current. The 30 cc. of phosphoric acid should be just sufficient to render the solution slightly acid to Congo red paper. At the end of 20 minutes, filter the solution through a good grade of quantitative filter paper. It is advisable to collect the first 40 cc. of filtrate in a 50-cc. cylinder because the first portion may be a little turbid, and it may need to pass through the filter a second time. The filtrate should be clear and have a greenish color. Transfer the filtrate to a separatory funnel (capacity 1 liter) and add, with shaking, 300 cc. (1.5 volumes) of alcohol. The mixture separates at once into a reddish or slightly greenish supernatant solution, and a bluish, very heavy solution at the bottom. The latter contains all of the phosphotungstic acid in a supersaturated solution, and it is best to withdraw it rather soon into a *weighed* 500-cc. Florence flask. If left too long in the separatory funnel, it sometimes forms crystal deposits which block the exit through the stopcock.

In so far as any insoluble molybdenum sulfide happens to be present, this will be floating between the two layers of liquid in the separatory funnel, and these solid aggregates must not be allowed to pass through the stopcock and into the phosphotungstic acid solution. The mixture remaining in the separatory funnel is discarded. Add water to the concentrated phosphotungstic acid in the 500-cc. flask until the weight of the contents amounts to 300 g. Boil the solution over a micro-burner for a few minutes, until a paper moistened with lead

mix well, and note the time. Let stand for 4 minutes, then heat in boiling water for 2 minutes, cool, dilute to volume, mix, and make the color comparison between the nearest standard and the unknown.

The 5 cc. standard contains 0.02 mg. uric acid and the 3 cc. standard contains 0.012 mg. of uric acid.

Report in milligrams of uric acid contained in 100 cc. of blood.

Experiment 15b. Determination of Uric Acid (Method of Benedict and Behre).[47] Transfer with a pipette 5 cc. of the protein-free filtrate [48] into a 15-cc. centrifuge tube. Add 2.5 cc. of the acid lithium chloride solution [49] and mix by inverting the tube. Add 2.5 cc. of the silver nitrate solution [50] and shake the contents of the tube thoroughly (using a tight rubber stopper). Centrifuge for about ½ minute or longer and pour off *all* of the clear supernatant liquid into a test tube.

acetate solution shows that the H_2S has been removed. Then, but not until then, cut down the flame, and add 20 cc. of 85 per cent phosphoric acid. Insert a 10-cm. funnel into the 500-cc. flask to hold a 200-cc. flask filled with cold water, and boil gently for 1 hour. At the end of this time, the reaction is finished. Cut down the flame, remove the condenser (funnel and flask), filter, and add to the filtrate a few drops of bromine, and boil to remove the blue color of the solution. When the blue color is gone, boil rapidly for a few minutes to remove the bromine, then cover the mouth of the flask with a beaker and cool under running water.

Transfer 25 g. of lithium carbonate to a liter beaker, add first 50 cc. of phosphoric acid, then add slowly 250 cc. of water and boil to remove the CO_2. Cool the resulting lithium phosphate solution; add to it the concentrated uric acid reagent in the 500-cc. flask and dilute to 1 liter.

[47] *J. Biol. Chem.*, **92**, 161 (1931).

[48] The Folin-Wu filtrate may be used. Protein-free blood filtrate may also be prepared according to the modified method of Benedict, *J. Biol. Chem.*, **92**, 135 (1931). Dilute 1 volume of blood with 7 volumes of water, then add 1 volume of tungstomolybdate solution and 1 volume of 0.62 *N* sulfuric acid. Filter after a few minutes, as in the Folin-Wu precipitation. For the precipitation of the proteins in plasma or serum, dilute with 8 volumes of water and add 0.5 volume of the tungstomolybdate solution and 0.5 volume of sulfuric acid.

Preparation of the Tungstomolybdate Reagent. Treat 10 g. of pure, ammonia-free molybdic acid with 50 cc. of *N* sodium hydroxide solution, then boil the mixture gently for 4 to 5 minutes. Filter, washing the filter with about 150 cc. of hot water. Cool the combined filtrate and washings and mix with a solution of 80 g. of sodium tungstate dissolved in about 60 cc. of water. Dilute to 1 liter.

[49] *Acid Lithium Chloride Solution.* A solution containing 3 g. of lithium chloride and 20 cc. of concentrated hydrochloric acid per liter.

[50] *Silver Nitrate Solution.* A solution containing 11.6 g. of silver nitrate per liter.

Transfer 5 cc. of the standard uric acid solution [51] to another test tube and add 5 cc. of water. Add 4 cc. of the sodium cyanide solution [52] (**measured from a burette**) to each tube, followed by 1 cc. of the color reagent.[53] Invert each tube once immediately after the addition of the reagent and place in a boiling water bath for 3 minutes. Remove tubes and allow to stand at room temperature for 2 minutes, after which compare in the colorimeter while still warm or even hot.

Experiment 16a. Determination of Preformed Creatinine (Folin and Wu).[54] Transfer 25 (or 50) cc. of a saturated solution of purified picric acid [55] to a small, clean flask, add 5 (or 10) cc. of 10 per cent

[51] *Uric Acid Standard Stock Solution (Prepared according to Benedict and Hitchcock).* Dissolve 9 g. of pure crystallized disodium phosphate ($Na_2HPO_4 \cdot 12H_2O$) and 1 g. of sodium dihydrogen phosphate ($NaH_2PO_4 \cdot H_2O$) in about 200 cc. of hot water. Filter if not perfectly clear, and dilute to 500 cc. with hot water. Pour this hot solution on 0.2 g. of uric acid (accurately weighed), contained in a 1-liter volumetric flask and suspended in a little water. When all the uric acid has dissolved, add 1.4 cc. of glacial acetic acid, dilute to 1 liter, and mix. Add 5 cc. of chloroform as a preservative and keep in a cool place. The stock solution should be renewed about once every 2 months.

Measure 10 cc. of this stock solution (containing 2 mg.) into a 500-cc. volumetric flask and dilute to about 400 cc. with water. Add 5 cc. of concentrated hydrochloric acid and dilute to the mark with water. Mix. This solution should be prepared fresh about once a week.

[52] *Cyanide Solution.* Five per cent sodium cyanide solution, to which concentrated ammonia is added in the proportion of 2 cc. per liter. This solution improves during the first 2 or 3 weeks after its preparation, but should not be used after 6 to 7 weeks.

[53] The *Arsenotungstic Color Reagent* is prepared as follows: 100 g. of pure sodium tungstate are placed in a liter flask and dissolved in about 600 cc. of water; 50 g. of pure arsenic pentoxide are now added, followed by 25 cc. of 85 per cent phosphoric acid, and 20 cc. of concentrated hydrochloric acid. The mixture is boiled for 20 minutes, cooled, and diluted to 1 liter.

[54] *J. Biol. Chem.*, **38**, 81 (1919).

[55] To be suitable for use in the determination of creatinine in blood, the picric acid must be of the highest purity and should fulfil the following test of Folin and Doisy: "To 20 cc. of a saturated (1.2 per cent) solution of picric acid add 1 cc. of 10 per cent sodium hydroxide and let it stand for 15 minutes. The color of the alkaline picrate solution thus obtained 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 a half times as deep as that of a saturated picric acid solution; i.e., by setting the picric acid solution at 20 mm. in the Duboscq colorimeter, the picrate will give a reading of 13 to 14 mm."

The following is one of the two methods which S. R. Benedict and E. B.

sodium hydroxide, and mix. Transfer 10 cc. of blood filtrate to a small flask or to a test tube, transfer 5 cc. of the standard creatinine solution described below [56] to another flask, and dilute the standard to 20 cc. Then add 5 cc. of the freshly prepared alkaline picrate solution to the blood filtrate, and 10 cc. to the diluted creatinine solution. Let stand for 8 to 10 minutes and make the color comparison in the usual man-

Newton, *J. Biol. Chem.*, **82**, 1 (1929), have recommended for the preparation of pure picric acid.

The technical picric acid must be dried thoroughly before being used in this procedure. Dissolve 100 g. of dry picric acid with the aid of heat in 150 cc. of glacial acetic acid, and continue the heating until the mixture boils. (The mixture should be heated in an Erlenmeyer flask upon an electric plate.) Pour the hot solution upon a fluted filter contained in a dry funnel which has been previously heated, and collect the filtrate in a dry beaker. Cover the beaker with a watch glass and allow to stand for some hours, or overnight at room temperature (not in a refrigerator). At the end of this time if picric acid has not crystallized out, stir the mixture vigorously, or better, seed with a minute crystal of pure picric acid. Crystallization will begin at once and is complete within 2 hours or less. At the end of 2 hours filter with suction on a hardened filter and wash with about 35 cc. of cold glacial acetic acid. Suck as free from acetic acid as possible and dry at about 80–90°, with occasional stirring, until there is no odor of acetic acid. It is best to conduct all these operations in a good draft of air. The yield is about 60 g. of pure picric acid, which should read 12.5 to 13.5 mm. against 20 mm. by the Folin-Doisy test.

[56] When the amount of blood filtrate available for the creatinine determination is too small to permit repetition, it is, of course, advantageous or necessary to start with more than one standard. If a high creatinine should be encountered unexpectedly without several standards ready, the determination can be saved by diluting the unknown with an appropriate amount of the alkaline picrate solution, using for such dilution a picrate solution first diluted with two volumes of water so as to preserve equality between the standard and the unknown in relation to the concentration of picric acid and sodium hydroxide.

One standard creatinine solution, suitable both for creatinine and for creatine determinations in blood, can be made as follows: Transfer to a liter flask 6 cc. of the standard creatinine solution used for urine analysis (which contains 6 mg. of creatinine); add 100 cc. of normal hydrochloric acid, dilute to the mark with water, and mix. Transfer to a bottle and add four or five drops of toluene or xylene. Five cubic centimeters of this solution contain 0.03 mg. of creatinine, and this amount plus 15 cc. of water, represents the standard needed for the vast majority of human bloods, for it covers the range of 1 to 2 mg. per 100 cc. In the case of unusual bloods representing retention of creatinine, take 10 cc. of the standard plus 10 cc. of water, which covers the range of 2 to 4 mg. of creatinine per 100 cc. of blood; or 15 cc. of the standard plus 5 cc. of water by which 4 to 6 mg. can be estimated. By taking the full 20 cc. volume from the standard solution at least 8 mg. can be estimated; but when working with such blood it is well to consider whether it may not be more advantageous to substitute 5 cc. of blood filtrate plus 5 cc. of water for the usual 10 cc. of blood filtrate.

ner, never omitting first to ascertain that the two fields of the colorimeter are equal when both cups contain the standard creatinine picrate solution. The color comparison should be completed within 15 minutes from the time the alkaline picrate is added; it is never advisable, therefore, to work with more than three to five blood filtrates at a time.

Calculation. In connection with the calculation, it is to be noted that the standard is made up to twice the volume of the unknown, so that each 5 cc. of the standard creatinine solution, while containing 0.03 mg., corresponds to 0.015 mg. in the blood filtrate. Calculate the amount of creatinine in 100 cc. of blood.

Experiment 16b. Determination of Creatinine (Langley and Evans).[57] To 10 cc. of Folin-Wu blood filtrate in a test tube and to 0.01 (or 0.005) mg. of creatinine in 10 cc. of solution in another tube, add 3-cc. portions of saturated sodium dinitrobenzoate[58] and 0.5 cc. of 10 per cent sodium hydroxide. Shake, allow tubes to stand for 10 minutes, and without further delay compare in a colorimeter. Calculate. If the reading of the unknown is not close to that of the standard, repeat the determination, using a more appropriate standard.[59] Compare the result by this method with the one obtained in Experiment 16a.

Experiment 17. Determination of Creatine plus Creatinine (Folin and Wu).[54] Transfer 5 cc. of blood filtrate to a test tube graduated at 25 cc. Add 1 cc. of normal hydrochloric acid. Cover the mouth of the test tube with tinfoil and heat in the autoclave to 130° C. for 20 minutes or to 155° C. for 10 minutes. Cool. Add 5 cc. of the alkaline picrate solution and let stand for 8 to 10 minutes, then dilute to 25 cc. The standard solution required is 10 cc. of creatinine solution in a 50-cc. volumetric flask. Add 2 cc. of normal acid and 10 cc. of the alkaline picrate solution, and after 10 minutes' standing dilute to 50 cc.

In calculating the result account is taken of the fact that 10 cc. of the standard solution contains 0.06 mg. of creatinine. If the color developed by the blood filtrate were identical with that developed in the standard, it would mean that 5 cc. of the blood filtrate contained

[57] *J. Biol. Chem.*, **115**, 333 (1936).

[58] This solution is prepared from 20 gm. of purified 3,5-dinitrobenzoic acid, 150 cc. of water, and 50 cc. of 10 per cent sodium carbonate.

[59] Langley and Evans recommend the use of a graph from which the creatinine content of the filtrate may be established. When the determination of creatinine is made on less than 0.03 mg. per 100 cc. of solution, the color formed is not proportional to the creatinine content. The graph may be based on a series of determinations on solutions containing 0.003 to 0.03 mg. per 10 cc. and the correlation of these concentrations with the colorimeter readings. See also J. E. Andes, *Am. J. Clin. Pathol.*, **8**, Technical Suppl., p. 12 (1938).

0.03 mg. of "total creatinine." Explain by performing the proper calculations.

Calculate the "total creatinine" per 100 cc. of blood.

The normal value for "total creatinine" given by this method is about 6 mg. per 100 cc. of blood.

In the case of uremic bloods containing large amounts of creatinine, 1, 2, or 3 cc. of blood filtrate, and enough water to make approximately 5 cc., are substituted for 5 cc. of the undiluted filtrate.

Experiment 18a. Chlorides (Whitehorn's Method).[60] Pipette 10 cc. of the protein-free Folin-Wu filtrate into a porcelain dish. Add with a pipette 5 cc. of the standard silver nitrate solution ($M/35.46$) and stir thoroughly. Add about 5 cc. of concentrated nitric acid, mix, and let stand for 5 minutes, to permit the flocking out of the silver chloride. Then add with a spatula an abundant amount of powdered ferric ammonium sulfate (about 0.3 g.), which is here used as the indicator, and titrate the excess of silver nitrate with the standard sulfocyanate solution ($M/35.46$) until a definite salmon-red color is formed which persists, in spite of stirring, for at least 15 seconds. This is the end point in the titration, the red color being due to the formation of ferric thiocyanate.

Calculation. One cubic centimeter of $M/35.46$ $AgNO_3 \rightleftharpoons 1$ mg. of Cl. (Why?) Calculate the amount, in milligrams, of Cl and NaCl in 100 cc. of blood (or plasma). Calculate the concentration in milliequivalents.[61]

NOTE: Chloride determinations are usually made on plasma rather than on whole blood. If the plasma is to be analyzed, the blood should be collected without a tourniquet into a tube containing oxalate and paraffin oil. Why? The plasma should be separated from the corpuscles as soon as possible in order to avoid the loss of carbon dioxide and the transfer of chlorides from the corpuscles to the plasma.

Experiment 18b. Chlorides (Method of Wilson and Ball [62]). With

[60] *J. Biol. Chem.*, **45**, 449 (1921).

[61] See page 273, Bodansky's "Introduction to Physiological Chemistry," Fourth Edition, 1938.

[62] *J. Biol. Chem.*, **79**, 221 (1928). This is a modification of the method of Van Slyke, *J. Biol. Chem.*, **58**, 523 (1923). The original procedure, according to Van Slyke, follows: Measure 1 cc. of blood (plasma or serum) into a 100 cc. Pyrex test tube. Add, with stirring, 3 cc. of 0.05 *N* silver nitrate in concentrated nitric acid (8.495 gm. of fused silver nitrate dissolved in a minimum amount of water and made up to 1 liter with concentrated nitric acid [sp. gr. 1.4], or 5.394 gm. of pure silver dissolved in 1 liter of nitric acid). Cover the tube with a watch glass and heat the mixture on a water bath until the solution above the silver chloride precipitate is clear and light yellow in color. The remainder of the technique is essentially as in Experiment 18b.

an Ostwald pipette measure accurately 1 cc. of plasma (whole blood, or serum) into a 50-cc. Pyrex flask. Using another 1 cc. Ostwald pipette add, while mixing, 1 cc. of the silver nitrate solution (0.15 *N*). Then add 3 cc. of concentrated nitric acid (C.P. free from chlorides). Place the flask on a water bath and digest until the protein has completely dissolved (15 minutes are usually required for serum or plasma and 40 minutes for whole blood). After digestion is complete add 6 cc. of the ferric alum solution (5 per cent) and cool to room temperature or lower. At high temperatures the end point is not sharp. Using a micro-burette, titrate with 0.02 *N* ammonium sulfocyanate solution until 1 drop causes a color change which persists for about 1 minute. The titration should be performed in strong daylight, as the end point cannot be determined sharply by artificial light. To correct for the amount of sulfocyanate required to give a suitable end point in the presence of the reagents, 0.03 cc. is subtracted from the titration reading.[63]

Calculation. One cubic centimeter of the silver nitrate is equivalent to 7.5 cc. of the sulfocyanate; 1 cc. of 0.02 sulfocyanate is equivalent to 0.7092 mg. Cl and 1.17 mg. NaCl. Calculate the chloride concentration as Cl and as NaCl in milligrams per 100 cc. and in milliequivalents.[61]

Experiment 19. Determination of Calcium in Serum (Clark-Collip Modification[64] of the Kramer-Tisdall Method). Two cubic centimeters of clear serum, 2 cc. of distilled water, and 1 cc. of 4 per cent ammonium oxalate solution are thoroughly mixed in a 15-cc. graduated centrifuge tube.[65] The tube is covered and allowed to stand overnight and then centrifuged until the precipitate is well packed in the bottom of the tube. The supernatant liquid is carefully poured off, and, while the tube is still inverted, it is placed in a rack for 5 minutes to drain, the mouth of the tube resting on a pad of filter paper.[66] The mouth of the tube is wiped dry with a soft cloth and the

[63] A control titration should be made in which 1 cc. of the 0.15 *N* silver nitrate, 3 cc. of nitric acid, plus 6 cc. of ferric alum, are titrated directly with 0.02 *N* sulfocyanate solution. If the titration figure varies from 7.50 cc. (after deducting the 0.3 cc. correction), the number of cubic centimeters of sulfocyanate in the formula is to be multiplied by the factor $\frac{7.50}{\text{cc. of sulfocyanate used in control}}$

[64] *J. Biol. Chem.*, **63**, 461 (1925).

[65] The outside diameter of the tube should be 6 to 7 mm. at the 0.1-cc. mark.

[66] To insure uniform drainage, the tubes are always cleaned thoroughly by heating at approximately 100° for a few minutes in a cleaning mixture made by adding 1500 cc. of concentrated sulfuric acid to a solution of 200 g. of sodium dichromate in 100 cc. of water.

precipitate is stirred up and the sides of the tube washed with 3 cc. of dilute ammonia water (2 cc. of concentrated ammonia and 98 cc. of water), directed in a very fine stream from a wash bottle. The suspension is centrifuged and drained again as just described; the washing with ammonia water is repeated and the calcium oxalate again separated by centrifuging; then 2 cc. of approximately normal sulfuric acid is added. The acid is blown from a pipette directly upon the precipitate so as to break up the mat and facilitate solution. The tube and contents are placed in a boiling water bath for about 1 minute, and the oxalic acid is then titrated with 0.01 *N* potassium permanganate,[67] using a micro-burette. The titration is best carried out in a water bath at a temperature of 70 to 75° C. A blank determination should be made to test the purity of the reagents.

From the number of cubic centimeters of 0.01 *N* permanganate used (1 cc. is equivalent to 0.2 mg. of calcium) calculate the calcium content in milligrams per 100 cc. of serum.

NOTE: See also the method of Roe, J. H., and Kahn, B. S., *J. Biol. Chem.*, **81**, 1 (1929). Koch has recommended a procedure which consists in the precipitation of the serum proteins with trichloracetic acid. The calcium in the filtrate is precipitated as the oxalate; this is separated, washed, dissolved in sulfuric acid, and titrated with permanganate. F. C. Koch, "Practical Methods in Biochemistry," William Wood & Co., 2d ed. p. 160, 1937.

See also ceric sulfate titration method of C. E. Larson and D. M. Greenberg, *J. Biol. Chem.*, **123**, 199 (1938).

Experiment 20a. Determination of Inorganic Phosphate in Serum (Method of Benedict and Theis).[68] Dilute 2 cc. of clear serum (serum showing hemolysis should not be used) with a little water in a 10-cc. volumetric flask. Add 4 cc. of 20 per cent trichloracetic acid and dilute to the mark with water. Mix, let stand for at least 10 minutes, and then filter through an ashless filter. Place 5 cc. of the filtrate in a tube, add 3 cc. of water, 1 cc. of the molybdic acid reagent,[69] and 1 cc. of the bisulfite-hydroquinone solution.[70]

[67] The potassium permanganate, sufficient for the day's use, may be prepared by diluting 0.1 *N* solution. The diluted solution may then be checked against a standard 0.01 *N* solution of sodium oxalate.

[68] *J. Biol. Chem.*, **61**, 63 (1924). This method is also applicable to the analysis of plasma.

[69] *Molybdic Acid Reagent.* To 20 g. of pure molybdic acid (MoO_3) in a flask add 25 cc. of 20 per cent sodium hydroxide solution and warm gently until the molybdic acid dissolves. Cool and dilute to 250 cc. Filter if necessary. A small quantity of this reagent (enough for a few days' use) is diluted 1 : 1 with concentrated sulfuric acid as it is needed.

The molybdic acid used should be strictly pure and free from ammonia.

[70] *Bisulfite-Hydroquinone Reagent.* Dissolve 15 g. of sodium bisulfite and 0.5 g. of hydroquinone in 100 cc. of water.

Treat 5 cc. of the standard phosphate solution [71] similarly.

Mix the tubes, stopper loosely, and place in a boiling water bath for 10 minutes. Cool and compare in the colorimeter.

Five cubic centimeters of the standard solution are equivalent to 0.025 mg. of phosphorus. Calculate in terms of milligrams of P per 100 cc. of serum.

Experiment 20b. Determination of Inorganic Phosphate in Serum (Method of Fiske and Subbarow).[72] Transfer to an Erlenmeyer flask 4 volumes (16 cc.) of 10 per cent trichloracetic acid. While the flask is being gently rotated, run in 1 volume (4 cc.) of serum (or plasma) from a pipette. Close the mouth of the flask with a clean, dry rubber stopper, and shake vigorously for a few minutes. Filter through an ashless filter paper.

Measure 5 cc. of the filtrate into a tube graduated at 10 cc. (or a 10-cc. volumetric flask). Add 1 cc. of 2.5 per cent ammonium molybdate in 3 *N* H_2SO_4,[73] and finally (after mixing), 0.4 cc. of the aminonaphthol-sulfonic acid reagent (page 174). Dilute to the mark and mix. The standard is prepared as nearly as possible at the same time. It is identical with the standard used for urine (page 174) and contains 0.4 mg. of phosphorus in a volume of 100 cc. It should be noted that the molybdate reagent added to the standard is always the one containing 5 *N* H_2SO_4 and is different from the one used for the blood filtrate. The purpose of this is to compensate for the high concentration of trichloracetic acid in the filtrate.

Let stand for 5 minutes and make the color comparison. If the color of the unknown is particularly strong, make a second reading a few minutes later and base the calculation on the second reading.[74]

Experiment 20c. Determination of Inorganic Phosphate (Method of A. Bodansky [75]**).** With an Ostwald-Folin pipette measure 1 cc. of serum (or plasma) into a small flask containing 9 cc. of 5 per cent trichloracetic acid. Mix, let stand a few minutes, and filter through an ashless filter paper.

[71] *Stock Solution.* Dissolve 0.4389 g. of KH_2PO_4 in a liter of water. Add chloroform as a preservative.

Standard Solution. Dilute 5 cc. of the stock solution to 100 cc. Add 2 cc. of chloroform as a preservative and shake.

[72] *J. Biol. Chem.*, **66**, 375 (1925).

[73] The reagents are the same as those employed in the determination of inorganic phosphate in urine (page 174).

[74] Calculate the result in milligrams of phosphorus per 100 cc. of serum (or plasma). From the figure so obtained should be subtracted the correction for any phosphate which may be contained in the trichloracetic acid.

[75] *J. Biol. Chem.*, **99**, 197 (1932). For the determination of the enzyme phosphatase in serum refer to A. Bodansky, *ibid.*, **101**, 93 (1933); **120**, 167 (1937).

Transfer 5 cc. of the clear filtrate to a test tube, add 4 cc. of the molybdate reagent,[76] mix, then add 1 cc. of the stannous chloride solution.[77] Mix immediately by a single inversion.

INORGANIC P IN ALIQUOT, AT STATED COLORIMETRIC READINGS, CORRECTED FOR DEVIATION FROM BEER'S LAW. 0.02 MG. STANDARD SET AT 20 MM.

Mm.....	0 0	0.1	0 2	0 3	0 4	0 5	0 6	0.7	0.8	0.9
mm.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.	mg.
8	0.0560	0 0552	0 0545	0 0538	0 0531	0 0525	0 0518	0 0512	0.0505	0.0499
9	493	487	482	476	471	465	460	455	450	445
10	440	435	431	426	422	417	413	408	404	400
11	396	392	389	385	381	377	373	370	367	363
12	360	357	353	350	347	344	341	338	335	332
13	329	326	324	321	318	316	313	310	308	305
14	303	301	298	296	293	291	289	287	284	282
15	280	278	276	274	272	270	268	266	264	262
16	260	258	256	254	253	251	249	248	246	244
17	242	241	239	237	236	234	233	231	230	228
18	227	225	224	222	221	220	218	217	215	214
19	213	211	210	209	207	206	205	204	203	201
20	200	199	198	197	196	194	193	192	191	190
21	189	188	186	185	184	183	182	181	180	179
22	178	177	176	175	174	173	172	171	170	170
23	169	168	167	166	165	164	163	163	162	161
24	160	159	158	158	157	156	155	154	153	152
25	152	151	151	150	149	148	147	147	146	145
26	145	144	143	143	142	141	140	140	139	138
27	138	137	137	136	135	135	134	134	133	132
28	132	131	130	130	129	128	128	127	127	126
29	126	125	124	124	123	123	122	122	121	121
30	120	119	119	118	118	117	117	116	116	115
31	115	114	114	113	113	112	112	111	111	110
32	110	110	109	109	108	108	107	107	106	106
33	105	105	105	104	104	103	103	102	102	102
34	101	101	100	100	100	099	099	098	098	098
35	097	097	096	096	096	095	095	095	094	094
36	093	093	093	092	092	092	091	091	090	090

[76] *Molybdate Reagent.* This is prepared fresh before use from the following stock reagents:

1. Sulfuric acid 10 *N*. Keep in the refrigerator.

2. Sodium molybdate solution, 7.5 per cent. In a 2-liter volumetric flask dissolve 90 g. of pure molybdic acid anhydride (ammonia and phosphate free) in 250 cc. of 5 *N* sodium hydroxide. Dilute to volume and mix. The solution should be faintly alkaline to phenophthalein. Let stand and decant for use.

Preparation of the Molybdate Reagent. To 1 volume of the cold 10 *N* sulfuric acid add 1 volume of the 7.5 per cent molybdate stock reagent, and while mixing add 2 volumes of water.

[77] *Stannous Chloride. Stock Reagent.* Dissolve 15 g. of stannous chloride in

At the same time prepare one or more standards. A convenient standard is one containing 0.02 mg. of P in 5 cc. Transfer 5 cc. of the standard phosphate solution [78] to a test tube, add 4 cc. of the molybdate reagent, then add 1 cc. of the stannous chloride solution. Mix by a single inversion.

Compare in the colorimeter. Calculate in the usual way the concentration of inorganic phosphate in terms of milligrams of P per 100 cc. of serum (or plasma).[79]

NOTE: The usual formula for calculation does not take into account the possible lack of proportionality between concentration and the depth of color developed. The deviation from this proportionality is taken into account in the table on page 252. Referring to this table, recalculate the concentration of P.

Experiment 21. Determination of Proteins in Serum (or Plasma) (Micro-Kjeldahl Digestion According to Koch and McMeekin). Total Protein. Measure accurately 1 cc. of serum (or plasma) into a 50-cc. volumetric flask and dilute to the mark with 0.9 per cent sodium chloride solution. Mix thoroughly, then transfer exactly 1 cc. of the solution to a Pyrex test tube (200 by 25 mm.). Add 1 cc. of 1 : 1 sulfuric acid and proceed exactly as in the determination of non-protein nitrogen outlined on page 222. A suitable standard is one containing 0.2 mg. of nitrogen. If the protein is likely to be low, prepare a second standard containing 0.1 mg.

NOTE: If serum is analyzed only albumin and globulin are included in the "total protein." If plasma is used, fibrinogen is also included. Theoretically, therefore, the difference between plasma protein and serum protein should represent the fibrinogen fraction. However, it

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concentrated hydrochloric acid, making the total volume 25 cc. Keep in the refrigerator. For use the stock reagent is diluted 200 times with water (0.1 cc. to 20 cc. of water).

[78] *Standard Phosphate Solution.* The stock solution is prepared by dissolving 110 mg. of potassium acid phosphate (highest reagent quality) in water, adding 1 cc. of concentrated sulfuric acid, and diluting to 250 cc. 10 cc. contain 1 mg. of P. Toluene may be added as a preservative.

From this the standard solution is prepared by diluting 10 cc. to 250 cc.; 5 cc. contain 0.02 mg.

[79] To test the reagents treat 5 cc. of the trichloracetic acid with 4 cc. of the molybdate and 1 cc. of the stannous chloride solution. The resulting mixture should be colorless, or at most faintly blue or green.

should be noted that through the use of an excessive amount of oxalate to prevent clotting, the plasma may be sufficiently diluted through the withdrawal of water from the corpuscles to yield a lower total protein value for the plasma than that obtained on analysis of the corresponding serum.

Calculation. The 1 cc. of the diluted serum taken for analysis is equivalent to 0.02 cc. of serum (or plasma). From the colorimeter reading calculate the total nitrogen in 100 cc. (A correction may be required for the nitrogen content of the hydrogen peroxide. With pure preparations, the error is usually negligible.) From the total nitrogen per 100 cc. subtract the value for non-protein nitrogen per 100 cc. of serum (or plasma) determined as outlined on page 222. The difference, representing the proteins, is multiplied by the factor 6.25. This gives the value for total protein in grams per 100 cc.

Albumin and Globulin: The serum globulins are precipitated in a sodium sulfate solution, approximately 1.5 *M*. The albumins remain in solution.

Accurately transfer 1 cc. of serum (or plasma) to a small Erlenmeyer flask or to a 50-cc. glass-stoppered cylinder. Add exactly 30 cc. of 22.2 per cent sodium sulfate, warmed in the incubator to 37° C. Stopper the flask (or cylinder) and keep in the incubator for 3 hours. Fit a small funnel with a filter paper of fine grade, and together with a small flask place in the incubator. With all the apparatus at a temperature of 37° C., filter the solution in the incubator, covering the funnel with a watch glass to prevent evaporation. The filtrate should be absolutely clear; if not, pour back on the filter paper, or use a double filter. Transfer 1 cc. of the *clear* filtrate to a Pyrex digestion tube, add 1 cc. of the 1 : 1 sulfuric acid and proceed as in the determination of total protein.

Calculation. One cc. of the filtrate represents 0.0323 cc. of the original serum (or plasma). Calculate the total nitrogen present in terms of 100 cc. of the original serum (or plasma). Deduct the non-protein nitrogen. The difference, multiplied by the factor 6.25, gives the albumin content per 100 cc. Subtract the albumin from the total protein to obtain the globulin. Calculate the albumin/globulin ratio.

Experiment 22a. Determination of Cholesterol in Plasma or Serum (Method of Bloor).[80] From a pipette slowly run 3 cc. of

[80] *J. Biol. Chem.*, **77**, 53 (1928). In this paper are also described methods for determining total lipids and total fatty acids. Bloor's method for the determina-

plasma (or serum) into a 50-cc. volumetric flask containing about 40 cc. of an alcohol-ether mixture,[81] rotating the flask during the process so that a finely flocculent precipitate of the protein is obtained. Immerse the flask in boiling water, rotating it continually, until the liquid boils. Keep at boiling temperature for a few seconds, after which allow the liquid to cool to room temperature. Dilute to volume with the alcohol-ether mixture, mix, and filter through a fat-free filter.

Saponification. Measure 15 cc. of the alcohol-ether extract into a 100-cc. Erlenmeyer flask; add 2 cc. of sodium ethylate,[82] and evaporate on the water bath until the residue is pasty, but not dry, and the alcohol is completely evaporated (as determined by the absence of odor). Acidify with 1 cc. of dilute H_2SO_4 (1 part of concentrated acid and 3 parts of water). Heat the acidified mixture on the water bath for 1 minute; then add to the hot mixture 10 cc. of petroleum ether. The mixture is thus made to boil. Gently rotate the flask at the boiling temperature on the water bath for 2 or 3 minutes. Then pour off the solvent as completely as possible from the watery residue into a 25-cc. volumetric flask. Repeat the heating and extraction several times with 5-cc. portions of the petroleum ether, each time pouring off the latter into the 25-cc. flask until it is nearly full. Cool the contents of the flask to room temperature, fill to the mark with petroleum ether, and stopper tightly.

Determination. Measure 10 cc. of the petroleum ether extract into a small Erlenmeyer flask and evaporate to dryness on the water bath. Add chloroform in successive *small* portions (at least three times), gently warming to dissolve the residue and decant into a 10-cc. glass-stoppered cylinder. After the chloroform solution reaches room temperature, add chloroform to the 5-cc. mark, followed by 1 cc. of acetic anhydride (highest purity) and 0.1 cc. of C.P. concentrated H_2SO_4. Stopper the cylinder and mix the contents well.

tion of phospholipids is described in the *J. Biol. Chem.*, **82**, 273 (1929). A micro-method for the estimation of cholesterol by the oxidation of the digitonide is described by Okey in the same journal, **88**, 367 (1930).

[81] The alcohol-ether mixture contains 3 parts of alcohol and 1 part of ether (both redistilled).

[82] The sodium ethylate is made by dissolving 2 to 3 g. of cleaned metallic sodium in 100 cc. of absolute alcohol, the solution being kept cool during the process. This reagent should be kept in a cool, dark place and discarded when it becomes much colored.

The standard should be prepared at the same time. Transfer 5 cc. of the standard solution,[83] containing 0.5 mg. of cholesterol, into a similar 10-cc. cylinder. As in the determination, add 1 cc. of acetic anhydride and 0.1 cc. of concentrated H_2SO_4, stopper the cylinder and mix.

Set the cylinders away in the laboratory under the same light conditions in which the reading is to be made. After 15 minutes compare in the colorimeter.[84]

Experiment 22b. Determination of Cholesterol in Blood, Plasma, or Serum (Sackett's Modification [85] of Bloor's Original Method [86]). Into a 15-cc. graduated centrifuge tube, measure 9 cc. of alcohol and 3 cc. of ether and mix by inverting.[87] Run in slowly 0.2 cc. of whole blood, serum, or plasma. Cork tightly and shake vigorously for about 1 minute. Let lie horizontally, with the sediment evenly distributed along the tube, for 30 minutes. Centrifuge rapidly for 3 minutes and decant into a small beaker. Evaporate just to dryness in a water bath. Extract the cholesterol two or three times with *small* portions of chloroform and decant into a 10-cc. glass-stoppered graduated cylinder. Let cool and make up to 5 cc. with chloroform.

Measure 5 cc. of a standard cholesterol solution in chloroform (containing 0.4 mg. of cholesterol) into a similar 10-cc. cylinder. To each of the solutions add 2 cc. of acetic anhydride and 0.1 cc. of concentrated H_2SO_4. Mix by inverting several times and then set away in the dark for 15 minutes. Transfer to the cups of the colorimeter and compare, setting the standard at 12 or 15 mm. Calculate in milligrams of cholesterol per 100 cc. of blood (serum or plasma).

NOTE: A convenient micromethod for the determination of free and combined cholesterol is that of R. Schoenheimer and W. M. Sperry, *J. Biol. Chem.*, **106**, 745 (1934), see also W. M. Sperry, *ibid.*, **118**, 377 (1937).

[83] *Standard Cholesterol Solution.* This is a solution of cholesterol in chloroform containing from 0.5 to 1 mg. of cholesterol in 5 cc., depending on the cholesterol content in the blood which is being measured. For most purposes a standard containing 0.5 mg. of cholesterol in 5 cc. of solution will be found suitable. For convenience in weighing the cholesterol, a standard twenty times the strength of the final standard is prepared, and this is diluted as needed.

[84] If the directions are followed as regards the volume of plasma or serum taken for the analysis (3 cc.), and the volume of the alcohol-ether extract saponified (15 cc.), the 10 cc. of the petroleum ether extract, used in the determination, represent 0.36 cc. of the original plasma or serum.

[85] *J. Biol. Chem.*, **64**, 203 (1925).

[86] *Ibid*, **24**, 227 (1916).

[87] The alcohol and ether should be redistilled. 12 cc. of Bloor's 3 : 1 alcohol-ether mixture may be used.

Experiment 23. Determination of Hemoglobin (Newcomer's Method).[88] *Principle.* Blood is diluted with hydrochloric acid. The resulting color of acid hematin is compared with that of a standard brown-glass plate.[89]

Procedure. By means of a capillary pipette, measure 20 cu. mm. of blood (40 cu. mm. or more may be used in severe anemia) into 5 cc. of 0.1 *N* hydrochloric acid, and rinse the pipette twice with the acid. After allowing the solution to stand for 40 minutes or longer, transfer it to the right-hand cup of a colorimeter. Partly fill the left-hand cup with distilled water. Insert the Newcomer plate in its proper place and match the two colors.

Calculation. Tables to aid in the calculation are supplied by the manufacturers with the Newcomer plate. The plate, if 1 mm. in thickness, is equivalent to 0.038 per cent hemoglobin solution. Accordingly,

$$\frac{10}{\text{Reading}} \times .038 \times \text{dilution} = \text{grams of hemoglobin per 100 cc. of blood.}$$

When 20 cu. mm. of blood are used, the dilution is 250.

Experiment 24. Carbon Dioxide Combining Capacity of the Plasma (Van Slyke and Cullen[90]**).** *Principle.* Blood is collected with minimum exposure to air, the plasma separated by centrifugalization and transferred to a separatory funnel where it is equilibrated with air containing approximately 5.5 per cent carbon dioxide, corresponding to the carbon dioxide tension of alveolar air. A definite volume of the saturated plasma is transferred to a suitable apparatus where it is submitted to the action of acid and a partial vacuum. The

[88] *J. Biol. Chem.*, **37**, 465 (1919); **55**, 569 (1923).

[89] The Newcomer plate may be secured from A. H. Thomas & Co., Philadelphia, or from Bausch & Lomb. Bausch & Lomb also manufacture a specially constructed colorimeter for hemoglobin determinations. In this form of colorimeter, the Newcomer plate is contained within the prism box.

The manufacturers supply directions as well as a table of data that is useful in the calculations of results, especially when the plate is not exactly 1.0 mm. in thickness.

For use with the Duboscq colorimeter (page 4) there is also manufactured a special Newcomer hemoglobinometer attachment. This consists of yellow and blue filters, the yellow filter mounted for insertion in the recess of the cup carrier underneath the cup, and the blue filter for insertion in the recess over the upper lens of the eyepiece. A special mixing pipette is required, and a conversion chart is also supplied which permits translating millimeter readings on the colorimeter scale into readings in grams of hemoglobin.

Standard solutions of hematin may be used in place of the Newcomer plate. See Cohen and Smith, *J. Biol. Chem.*, **39**, 489 (1919); and Terrill, *J. Biol. Chem.*, **53**, 179 (1922).

[90] *J. Biol. Chem.*, **30**, 289, 347 (1917).

carbon dioxide which is thus liberated is measured. Corrections are made for barometric pressure and temperature, the air liberated by the fluid contained in the apparatus, and especially for the carbon dioxide physically dissolved in the plasma. The carbon dioxide combined as bicarbonate is thus determined. This gives a measure of the "alkali reserve" of the blood, which in turn reflects more or less closely the reserve of available base present in the body as a whole.[91]

Apparatus. The apparatus used in the estimation of the carbon dioxide content of the blood (or plasma) is illustrated in Fig. 8. It is made of strong glass in order to stand the weight of mercury without danger of breaking, and is held in a strong screw clamp, the jaws of which are lined with thick pads of rubber. In order to prevent accidental slipping of the apparatus from the clamp, an iron rod of 6 or 8 mm. diameter should be so arranged as to project under cock *f*, between *c* and *d*.

When mounted on a board, the apparatus is less likely to break.

Two hooks or rings at the levels 1 and 2 serve to hold the leveling bulb at different stages of the analysis. The bulb is connected with the bottom of the apparatus by a heavy-walled rubber tube.

It is necessary, of course, that both stopcocks be properly greased and air-tight, and it is also essential that they (especially *f*) be held in place so that they cannot be forced out by pressure of the mercury. Rubber bands may be used for this purpose, but elastic cords of fine wire spirals, applied in the same manner as rubber bands, are stronger and more durable. In later models of this apparatus the stopcocks are provided with devices to hold them in place.

After a determination has been finished, the leveling bulb is lowered without opening the upper cock, and most of the mercury is withdrawn from the pipette through *c*. The water solution from *d* is readmitted, and, the leveling bulb being raised to position 1, the water solution, together with a little mercury, is forced out of the apparatus through *a*. (It is well to have outlet *a* connected by means of rubber tubing to a vessel to catch the water residues and mercury overflow from *a*. A considerable amount of mercury is thus regained if many analyses are run. It requires only washing with water and straining through cloth

[91] Refer to Bodansky's "Introduction to Physiological Chemistry, Fourth Edition," page 269.

The alkali reserve may also be determined by titration as described by D. D. Van Slyke, E. Stillman, and G. E. Cullen, *J. Biol. Chem.*, **38**, 167 (1919); See also H. D. Haskins and E. E. Osgood, *J. Lab. Clin. Med.*, **6**, 37 (1920).

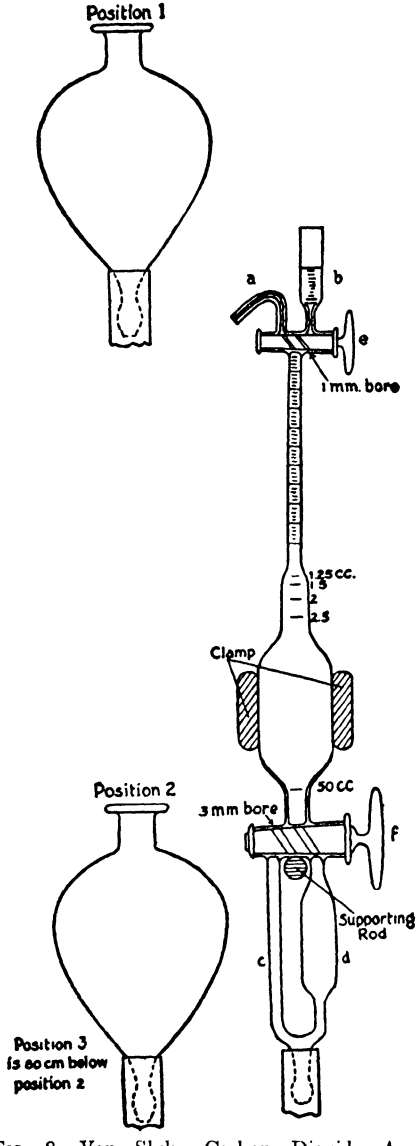


FIG. 8.—Van Slyke Carbon Dioxide Apparatus.

or chamois skin to prepare it for use again.) The stopcock is reversed, water admitted from *b*, and the pipette thoroughly rinsed, the washings being forced out through *a*. The process may be repeated several times.

Procedure. For at least an hour before the blood is drawn, the subject should avoid vigorous muscular exertion, as this lowers the bicarbonate content of the blood presumably because of the lactic acid formed. The blood (6–8 cc.) is drawn from an arm vein and transferred or aspirated into a centrifuge tube containing a small amount of neutral potassium oxalate (20–30 mg.) and some paraffin oil.[92] The tube is subjected to a minimum of agitation after the blood is in it. The slight amount of agitation necessary to assure mixture with the oxalate is accomplished by stirring gently with the needle attached to the syringe, or a thin glass rod, or with the inlet tube in case the blood is aspirated directly into the centrifuge tube.

Without unnecessary delay the blood is centrifuged, the plasma separated and transferred to another tube. The carbon dioxide capacity of separated plasma remains unchanged for several hours, at

[92] Paraffin oil though essential in the collection of blood for the determination

least. As much of the plasma as is available (3 to 4 cc.) is transferred to a 300-cc. separatory funnel, arranged as in Fig. 9, and the air within the funnel is displaced either by alveolar air from the lungs of the operator or by a 5.5 per cent carbon dioxide-air mixture from a tank. In both cases the gas mixture must be passed over glass beads (these may be slightly moist) before it enters the funnel. This prevents the condensation of moisture in the funnel. If dry air were blown through the funnel, slight evaporation would occur.

When alveolar air is used, the operator, without inspiring more deeply than normal, expires as quickly and as completely as possible through the glass beads and separatory funnel. The stopper of the funnel should be inserted just before the expiration is finished, so that there is no opportunity for air to be drawn back into the funnel. In order to saturate the plasma the separatory funnel is turned end over

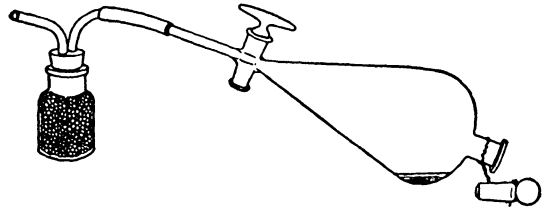


FIG. 9.—Separatory Funnel Used in Saturating Blood Plasma with Carbon Dioxide.

end for 2 minutes, the plasma being distributed in a thin layer as completely over the surface of the funnel's interior as is possible.[93] After saturation has been completed, the funnel is placed upright and allowed to stand for a few minutes until the fluid has drained from the walls and gathered in the contracted space at the bottom of the funnel.

Determination of Carbon Dioxide. A 1-cc. sample, accurately pipetted, is allowed to run into the cup *b* in the apparatus represented in Fig. 8, the tip of the pipette remaining below the surface of the plasma as it is added. The cup should have been previously washed out with distilled water and, together with the entire apparatus, should have been filled with mercury to the top of the capillary tube by placing the leveling bulb of the mercury in position 1.

of the carbon dioxide content of blood is not indispensable in the determination of the carbon dioxide combining capacity, provided the blood is centrifuged immediately after it is collected and the plasma separated without delay.

[93] A suitable shaking device may be used for this purpose. See W. C. Stadie, *J. Biol. Chem.*, **49**, 43 (1921).

With the mercury bulb in position 2 and the cock *f* in the position shown in the figure, the plasma is admitted from the cup into the 50-cc. chamber, just enough being left above the cock *e* to fill the capillary so that no air is introduced when the next solution is added. The cup is washed with two portions of about 0.5 cc. of water, each portion being added to the pipette in turn. A *small* drop of caprylic alcohol is then added to the cup and is permitted to flow entirely into the capillary above *e*. Finally 0.5 cc. of *N* lactic (or 0.5 cc. of 20 per cent tartaric acid) is run in.

After the acid has been added, a drop of mercury is placed in *b* and allowed to run down the capillary as far as the cock in order to seal the latter. Whatever excess of acid remains in the cup is washed out with a little water.

The mercury bulb is now lowered and hung in position 3, and the mercury in the pipette is allowed to run down to the 50-cc. mark, producing a Torricellian vacuum in the apparatus. When the mercury (not the water) meniscus has fallen to the 50-cc. mark the lower cock is closed and the pipette is removed from the clamp. Equilibrium of the carbon dioxide between the 2.5 cc. of water solution and the 47.5 cc. of free space in the apparatus is obtained by shaking the pipette for 2 or 3 minutes in such a way as to give the liquid in the pipette a rotary motion. The pipette is then replaced in the clamp.

By turning the cock, *f*, the water solution is now allowed to flow from the pipette completely into *d*, none of the gas, however, being allowed to follow it. The leveling bulb is then raised in the left hand, while with the right the cock is turned so as to connect the pipette with *c*. The mercury, flowing in from *c*, fills the body of the pipette and as much of the calibrated stem at the top as is not occupied by the gas extracted from the solution. A few hundredths of a cubic centimeter of water, which could not be completely drained into *d*, float on top of the mercury in the pipette, but the error caused by reabsorption of carbon dioxide into this small volume of water is negligible if the reading is made at once. The mercury bulb is placed at such a level that the gas in the pipette is under atmospheric pressure and the volume of the gas is read on the scale. The barometric pressure and temperature are also noted at this time.[94]

[94] Whole blood may be analyzed in essentially the same way, but as oxygen is also extracted in the process, the carbon dioxide is determined by difference, after absorption by alkali. After the volume of gas liberated in the apparatus has been measured, a slight negative pressure is created in the pipette by bringing the level of the solution to the 2-cc. graduation. An excess of 10 per cent sodium

Calculation. To convert the observed gas volume into volumes per cent of carbon dioxide bound as bicarbonate correct for the barometric pressure and refer to the table on page 274.[95]

For further details the reader is referred to Peters and Van Slyke, "Quantitative Clinical Chemistry," Williams and Wilkins Company, Baltimore, 1932, Volume II, Chapter VII. In this chapter are included methods for the determination of the carbon dioxide content of blood and plasma, the determination of hemoglobin by the estimation of oxygen capacity, and the estimation of carbon monoxide and other gases of the blood. Here also is a description of the construction and manipulation of the manometric apparatus of Van Slyke.

hydroxide solution is then introduced into cup *b*. By means of the negative pressure within the pipette, this solution is admitted slowly until all the carbon dioxide is absorbed leaving an excess of alkali in cup *b*. The apparatus may be tilted slightly to allow the residual gas to rise to the top of the capillary of the pipette. After a minute or more has been allowed for absorption of the gas and for draining, the residual gas is measured at atmospheric pressure, by leveling the mercury bulb in the usual way. The difference between the residual gas and the original gas volumes represents the carbon dioxide.

Even in the case of plasma some workers prefer to absorb the carbon dioxide with alkali in order to check up on any possible leaks in the apparatus. A small amount of residual air (about 0.04 to 0.05 cc.) is always found. A correction for this has, however, been included in the calculations upon which the data on page 274 are based.

[95] Example: The observed gas volume is 0.69 cc. at 26° C. and 764 mm. Hg pressure. Multiplying by 764/760 increases the gas volume to 0.694 cc. Referring to the table, it is seen that at 26° (nearest value given is for 25°) this corresponds to 57.4 volumes per cent of carbon dioxide combining capacity.

TABLE FOR CALCULATION OF CARBON DIOXIDE COMBINING POWER OF PLASMA

Observed Vol. Gas $\times\frac{B}{760}$	Cubic Centimeters of CO_2, Reduced to 0°, 760 mm., Bound as Bicarbonate by 100 cc. of Plasma				Observed Vol. Gas $\times\frac{B}{760}$	Cubic Centimeters of CO_2, Reduced to 0°, 760 mm., Bound as Bicarbonate by 100 cc. of Plasma			
	15°	20°	25°	30°		15°	20°	25°	30°
0.20	9.1	9.9	10.7	11 8	0.60	47.7	48 1	48.5	48.6
1	10 1	10.9	11.7	12 6	1	48.7	49.0	49.4	49.5
2	11.0	11.8	12.6	13 5	2	49.7	50.0	50.4	50.4
3	12.0	12.8	13.6	14.3	3	50.7	51.0	51.3	51.4
4	13 0	13.7	14 5	15.2	4	51.6	51.9	52.2	52.3
5	13 9	14.7	15 5	16.1	5	52.6	52.8	53.2	53.2
6	14.9	15.7	16.4	17.0	6	53.6	53.8	54.1	54.1
7	15.9	16.6	17.4	18.0	7	54.5	54.8	55.1	55.1
8	16.8	17.6	18 3	18 9	8	55.5	55.7	56.0	56.0
9	17.8	18.5	19.2	19.8	9	56.5	56.7	57.0	56.9
0.30	18.8	19.5	20 2	20.8	0.70	57.4	57.6	57.9	57.9
1	19.7	20.4	21.1	21.7	1	58.4	58.6	58 9	58.8
2	20.7	21.4	22 1	22.6	2	59.4	59.5	59.8	59.7
3	21.7	22 3	23 0	23 5	3	60 3	60.5	60.7	60.6
4	22.6	23.3	24 0	24.5	4	61 3	61.4	61.7	61.6
5	23.6	24.2	24 9	25.4	5	62.3	62.4	62.6	62.5
6	24.6	25.2	25.8	26.3	6	63 2	63.3	63.6	63.4
7	25.5	26.2	26 8	27.3	7	64.2	64.3	64.5	64.3
8	26.5	27.1	27.7	28.2	8	65 2	65.3	65.5	65.3
9	27.5	28.1	28.7	29.1	9	66.1	66.2	66.4	66.2
0.40	28.4	29.0	29.6	30 0	0.80	67.1	67.2	67 3	67.1
1	29.4	30.0	30 5	31.0	1	68.1	68.1	68.3	68 0
2	30 3	30.9	31 5	31 9	2	69.0	69.1	69 2	69 0
3	31.3	31.9	32.4	32 8	3	70.0	70 0	70 2	69.9
4	32 3	32 8	33.4	33.8	4	71 0	71.0	71.1	70.8
5	33.2	33.8	34.3	34.7	5	71 9	72.0	72.1	71.8
6	34.2	34.7	35.3	35.6	6	72 9	72.9	73.0	72.7
7	35.2	35.7	36.2	36 5	7	73 9	73.9	74.0	73.6
8	36.1	36.6	37.2	37.4	8	74 8	74.8	74.9	74.5
9	37.1	37.6	38.1	38 4	9	75.8	75.8	75.8	75.4
0.50	38.1	38.5	39.0	39.3	0.90	76.8	76.7	76.8	76.4
1	39.1	39.5	40 0	40.3	1	77.8	77.7	77.7	77.3
2	40.0	40.4	40 9	41.2	2	78.7	78.6	78.7	78.2
3	41.0	41.4	41 9	42.1	3	79.7	79.6	79.6	79.2
4	42.0	42.4	42.8	43.0	4	80.7	80.5	80.6	80.1
5	42.9	43.3	43.8	43.9	5	81.6	81.5	81.5	81.0
6	43.9	44.3	44.7	44.9	6	82.6	82.5	82.4	82.0
7	44.9	45.3	45.7	45.8	7	83.6	83.4	83.4	82.9
8	45.8	46.2	46.6	46.7	8	84.5	84.4	84.3	83.8
9	46 8	47.1	47.5	47.6	9	85.5	85.3	85.2	84.8
0.60	47.7	48.1	48.5	48.6	1.00	86.5	86.2	86.2	85.7

APPENDIX

LOGARITHMS.

Natural Numbers	0	1	2	3	4	5	6	7	8	9	Proportional Parts. 1	2	3	4	5	6	7	8	9
10	0000	0043	0086	0128	0170	0212	0253	0294	0334	0374	4	8	12	17	21	25	29	33	37
11	0414	0453	0492	0531	0569	0607	0645	0682	0719	0755	4	8	11	15	19	23	26	30	34
12	0792	0828	0864	0899	0934	0969	1004	1038	1072	1106	3	7	10	14	17	21	24	28	31
13	1139	1173	1206	1239	1271	1303	1335	1367	1399	1430	3	6	10	13	16	19	23	26	29
14	1461	1492	1523	1553	1584	1614	1644	1673	1703	1732	3	6	9	12	15	18	21	24	27
15	1761	1790	1818	1847	1875	1903	1931	1959	1987	2014	3	6	8	11	14	17	20	22	25
16	2041	2068	2095	2122	2148	2175	2201	2227	2253	2279	3	5	8	11	13	16	18	21	24
17	2304	2330	2355	2380	2405	2430	2455	2480	2504	2529	2	5	7	10	12	15	17	20	22
18	2553	2577	2601	2625	2648	2672	2695	2718	2742	2765	2	5	7	9	12	14	16	19	21
19	2788	2810	2833	2856	2878	2900	2923	2945	2967	2989	2	4	7	9	11	13	16	18	20
20	3010	3032	3054	3075	3096	3118	3139	3160	3181	3201	2	4	6	8	11	13	15	17	19
21	3222	3243	3263	3284	3304	3324	3345	3365	3385	3404	2	4	6	8	10	12	14	16	18
22	3424	3444	3464	3483	3502	3522	3541	3560	3579	3598	2	4	6	8	10	12	14	15	17
23	3617	3636	3655	3674	3692	3711	3729	3747	3766	3784	2	4	6	7	9	11	13	15	17
24	3802	3820	3838	3856	3874	3892	3909	3927	3945	3962	2	4	5	7	9	11	12	14	16
25	3979	3997	4014	4031	4048	4065	4082	4099	4116	4133	2	3	5	7	9	10	12	14	15
26	4150	4166	4183	4200	4216	4232	4249	4265	4281	4298	2	3	5	7	8	10	11	13	15
27	4314	4330	4346	4362	4378	4393	4409	4425	4440	4456	2	3	5	6	8	9	11	13	14
28	4472	4487	4502	4518	4533	4548	4564	4579	4594	4609	2	3	5	6	8	9	11	12	14
29	4624	4639	4654	4669	4683	4698	4713	4728	4742	4757	1	3	4	6	7	9	10	12	13
30	4771	4786	4800	4814	4829	4843	4857	4871	4886	4900	1	3	4	6	7	9	10	11	13
31	4914	4928	4942	4955	4969	4983	4997	5011	5024	5038	1	3	4	6	7	8	10	11	12
32	5051	5065	5079	5092	5105	5119	5132	5145	5159	5172	1	3	4	5	7	8	9	11	12
33	5185	5198	5211	5224	5237	5250	5263	5276	5289	5302	1	3	4	5	6	8	9	10	12
34	5315	5328	5340	5353	5366	5378	5391	5403	5416	5428	1	3	4	5	6	8	9	10	11
35	5441	5453	5465	5478	5490	5502	5514	5527	5539	5551	1	2	4	5	6	7	9	10	11
36	5563	5575	5587	5599	5611	5623	5635	5647	5658	5670	1	2	4	5	6	7	8	10	11
37	5682	5694	5705	5717	5729	5740	5752	5763	5775	5786	1	2	3	5	6	7	8	9	10
38	5798	5809	5821	5832	5843	5855	5866	5877	5888	5899	1	2	3	5	6	7	8	9	10
39	5911	5922	5933	5944	5955	5966	5977	5988	5999	6010	1	2	3	4	5	7	8	9	10
40	6021	6031	6042	6053	6064	6075	6085	6096	6107	6117	1	2	3	4	5	6	8	9	10
41	6128	6138	6149	6160	6170	6180	6191	6201	6212	6222	1	2	3	4	5	6	7	8	9
42	6232	6243	6253	6263	6274	6284	6294	6304	6314	6325	1	2	3	4	5	6	7	8	9
43	6335	6345	6355	6365	6375	6385	6395	6405	6415	6425	1	2	3	4	5	6	7	8	9
44	6435	6444	6454	6464	6474	6484	6493	6503	6513	6522	1	2	3	4	5	6	7	8	9
45	6532	6542	6551	6561	6571	6580	6590	6599	6609	6618	1	2	3	4	5	6	7	8	9
46	6628	6637	6646	6656	6665	6675	6684	6693	6702	6712	1	2	3	4	5	6	7	7	8
47	6721	6730	6739	6749	6758	6767	6776	6785	6794	6803	1	2	3	4	5	5	6	7	8
48	6812	6821	6830	6839	6848	6857	6866	6875	6884	6893	1	2	3	4	4	5	6	7	8
49	6902	6911	6920	6928	6937	6946	6955	6964	6972	6981	1	2	3	4	4	5	6	7	8
50	6990	6998	7007	7016	7024	7033	7042	7050	7059	7067	1	2	3	3	4	5	6	7	8
51	7076	7084	7093	7101	7110	7118	7126	7135	7143	7152	1	2	3	3	4	5	6	7	8
52	7160	7168	7177	7185	7193	7202	7210	7218	7226	7235	1	2	2	3	4	5	6	7	7
53	7243	7251	7259	7267	7275	7284	7292	7300	7308	7316	1	2	2	3	4	5	6	6	7
54	7324	7332	7340	7348	7356	7364	7372	7380	7388	7396	1	2	2	3	4	5	6	6	7

LOGARITHMS.

Natural Numbers.	0	1	2	3	4	5	6	7	8	9	Proportional Parts.								
											1	2	3	4	5	6	7	8	9
55	7404	7412	7419	7427	7435	7443	7451	7459	7466	7474	1	2	2	3	4	5	5	6	7
56	7482	7490	7497	7505	7513	7520	7528	7536	7543	7551	1	2	2	3	4	5	5	6	7
57	7559	7566	7574	7582	7589	7597	7604	7612	7619	7627	1	2	2	3	4	5	5	6	7
58	7634	7642	7649	7657	7664	7672	7679	7686	7694	7701	1	1	2	3	4	4	5	6	7
59	7709	7716	7723	7731	7738	7745	7752	7760	7767	7774	1	1	2	3	4	4	5	6	7
60	7782	7789	7796	7803	7810	7818	7825	7832	7839	7846	1	1	2	3	4	4	5	6	6
61	7853	7860	7868	7875	7882	7889	7896	7903	7910	7917	1	1	2	3	4	4	5	6	6
62	7924	7931	7938	7945	7952	7959	7966	7973	7980	7987	1	1	2	3	3	4	5	6	6
63	7993	8000	8007	8014	8021	8028	8035	8041	8048	8055	1	1	2	3	3	4	5	5	6
64	8062	8069	8075	8082	8089	8096	8102	8109	8116	8122	1	1	2	3	3	4	5	5	6
65	8129	8136	8142	8149	8156	8162	8169	8176	8182	8189	1	1	2	3	3	4	5	5	6
66	8195	8202	8209	8215	8222	8228	8235	8241	8248	8254	1	1	2	3	3	4	5	5	6
67	8261	8267	8274	8280	8287	8293	8299	8306	8312	8319	1	1	2	3	3	4	5	5	6
68	8325	8331	8338	8344	8351	8357	8363	8370	8376	8382	1	1	2	3	3	4	4	5	6
69	8388	8395	8401	8407	8414	8420	8426	8432	8439	8445	1	1	2	2	3	4	4	5	6
70	8451	8457	8463	8470	8476	8482	8488	8494	8500	8506	1	1	2	2	3	4	4	5	6
71	8513	8519	8525	8531	8537	8543	8549	8555	8561	8567	1	1	2	2	3	4	4	5	5
72	8573	8579	8585	8591	8597	8603	8609	8615	8621	8627	1	1	2	2	3	4	4	5	5
73	8633	8639	8645	8651	8657	8663	8669	8675	8681	8686	1	1	2	2	3	4	4	5	5
74	8692	8698	8704	8710	8716	8722	8727	8733	8739	8745	1	1	2	2	3	4	4	5	5
75	8751	8756	8762	8768	8774	8779	8785	8791	8797	8802	1	1	2	2	3	3	4	5	5
76	8808	8814	8820	8825	8831	8837	8842	8848	8854	8859	1	1	2	2	3	3	4	5	5
77	8865	8871	8876	8882	8887	8893	8899	8904	8910	8915	1	1	2	2	3	3	4	4	5
78	8921	8927	8932	8938	8943	8949	8954	8960	8965	8971	1	1	2	2	3	3	4	4	5
79	8976	8982	8987	8993	8998	9004	9009	9015	9020	9025	1	1	2	2	3	3	4	4	5
80	9031	9036	9042	9047	9053	9058	9063	9069	9074	9079	1	1	2	2	3	3	4	4	5
81	9085	9090	9096	9101	9106	9112	9117	9122	9128	9133	1	1	2	2	3	3	4	4	5
82	9138	9143	9149	9154	9159	9165	9170	9175	9180	9186	1	1	2	2	3	3	4	4	5
83	9191	9196	9201	9206	9212	9217	9222	9227	9232	9238	1	1	2	2	3	3	4	4	5
84	9243	9248	9253	9258	9263	9269	9274	9279	9284	9289	1	1	2	2	3	3	4	4	5
85	9294	9299	9304	9309	9315	9320	9325	9330	9335	9340	1	1	2	2	3	3	4	4	5
86	9345	9350	9355	9360	9365	9370	9375	9380	9385	9390	1	1	2	2	3	3	4	4	5
87	9395	9400	9405	9410	9415	9420	9425	9430	9435	9440	0	1	1	2	2	3	3	4	4
88	9445	9450	9455	9460	9465	9469	9474	9479	9484	9489	0	1	1	2	2	3	3	4	4
89	9494	9499	9504	9509	9513	9518	9523	9528	9533	9538	0	1	1	2	2	3	3	4	4
90	9542	9547	9552	9557	9562	9566	9571	9576	9581	9586	0	1	1	2	2	3	3	4	4
91	9590	9595	9600	9605	9609	9614	9619	9624	9628	9633	0	1	1	2	2	3	3	4	4
92	9638	9643	9647	9652	9657	9661	9666	9671	9675	9680	0	1	1	2	2	3	3	4	4
93	9685	9689	9694	9699	9703	9708	9713	9717	9722	9727	0	1	1	2	2	3	3	4	4
94	9731	9736	9741	9745	9750	9754	9759	9763	9768	9773	0	1	1	2	2	3	3	4	4
95	9777	9782	9786	9791	9795	9800	9805	9809	9814	9818	0	1	1	2	2	3	3	4	4
96	9823	9827	9832	9836	9841	9845	9850	9854	9859	9863	0	1	1	2	2	3	3	4	4
97	9868	9872	9877	9881	9886	9890	9894	9899	9903	9908	0	1	1	2	2	3	3	4	4
98	9912	9917	9921	9926	9930	9934	9939	9943	9948	9952	0	1	1	2	2	3	3	4	4
99	9956	9961	9965	9969	9974	9978	9983	9987	9991	9996	0	1	1	2	2	3	3	3	4

TABLE I

COMPOSITION OF MIXTURES GIVING *p*H VALUES AT 20° C. AT INTERVALS OF 0.2

KCl–HCl Mixtures*

*p*H			
1.2	24.9 cc. 0.2 *M* KCl	75.1 cc. 0.2 *M* HCl	Dilute to 200 cc.
1.4	52.6 cc. 0.2 *M* KCl	47.4 cc. 0.2 *M* HCl	Dilute to 200 cc.
1.6	70.1 cc. 0.2 *M* KCl	29.9 cc. 0.2 *M* HCl	Dilute to 200 cc.
1.8	81 1 cc. 0.2 *M* KCl	18.9 cc. 0.2 M HCl	Dilute to 200 cc.
2.0	88.1 cc. 0.2 *M* KCl	11.9 cc. 0.2 *M* HCl	Dilute to 200 cc.
2.2	92.5 cc. 0.2 *M* KCl	7.5 cc. 0.2 *M* HCl	Dilute to 200 cc.

* The KCl-HCl mixtures are based on data given by Clark on p. 201.

Phthalate–HCl Mixtures

2.2	50 cc. 0.2 *M* KHPhthalate	46.70 cc. 0.2 *M* HCl	Dilute to 200 cc.
2.4	50 cc. 0.2 *M* KHPhthalate	39.60 cc. 0.2 *M* HCl	Dilute to 200 cc.
2.6	50 cc. 0.2 *M* KHPhthalate	32.95 cc. 0.2 *M* HCl	Dilute to 200 cc.
2.8	50 cc. 0.2 *M* KHPhthalate	26.42 cc. 0.2 *M* HCl	Dilute to 200 cc.
3.0	50 cc. 0.2 *M* KHPhthalate	20.32 cc. 0.2 *M* HCl	Dilute to 200 cc.
3.2	50 cc. 0.2 *M* KHPhthalate	14.70 cc. 0.2 *M* HCl	Dilute to 200 cc.
3.4	50 cc. 0.2 *M* KHPhthalate	9.90 cc. 0.2 *M* HCl	Dilute to 200 cc.
3.6	50 cc. 0.2 *M* KHPhthalate	5.97 cc. 0.2 *M* HCl	Dilute to 200 cc.
3.8	50 oc. 0.2 *M* KHPhthalate	2.63 cc. 0.2 *M* HCl	Dilute to 200 cc.

Phthalate–NaOH Mixtures

4.0	50 cc. 0.2 *M* KHPhthalate	0.40 cc. 0.2 *M* NaOH	Dilute to 200 cc.
4.2	50 cc. 0.2 *M* KHPhthalate	3.70 cc. 0.2 *M* NaOH	Dilute to 200 cc.
4.4	50 cc. 0.2 *M* KHPhthalate	7.50 cc. 0.2 *M* NaOH	Dilute to 200 cc.
4.6	50 cc. 0.2 *M* KHPhthalate	12.15 cc. 0.2 *M* NaOH	Dilute to 200 cc.
4.8	50 cc. 0.2 *M* KHPhthalate	17.70 cc. 0.2 *M* NaOH	Dilute to 200 cc.
5.0	50 cc. 0.2 *M* KHPhthalate	23.85 cc. 0.2 *M* NaOH	Dilute to 200 cc.
5.2	50 cc. 0.2 *M* KHPhthalate	29.95 cc. 0.2 *M* NaOH	Dilute to 200 cc.
5.4	50 cc. 0.2 *M* KHPhthalate	35.45 cc. 0.2 *M* NaOH	Dilute to 200 cc.
5.6	50 cc. 0.2 *M* KHPhthalate	39.85 cc. 0.2 *M* NaOH	Dilute to 200 cc.
5.8	50 cc. 0.2 *M* KHPhthalate	43.00 cc. 0.2 *M* NaOH	Dilute to 200 cc.
6.0	50 cc. 0.2 *M* KHPhthalate	45.45 cc. 0.2 *M* NaOH	Dilute to 200 cc.
6.2	50 cc. 0.2 *M* KHPhthalate	47.00 cc. 0.2 *M* NaOH	Dilute to 200 cc.

TABLE I—*Continued*

KH_2PO_4–NaOH Mixtures

*p*H			
5.8	50 cc. 0.2 *M* KH_2PO_4	3.72 cc. 0.2 *M* NaOH	Dilute to 200 cc.
6.0	50 cc. 0.2 *M* KH_2PO_4	5.70 cc. 0.2 *M* NaOH	Dilute to 200 cc.
6.2	50 cc. 0.2 *M* KH_2PO_4	8.60 cc. 0.2 *M* NaOH	Dilute to 200 cc.
6.4	50 cc. 0.2 *M* KH_2PO_4	12.60 cc. 0.2 *M* NaOH	Dilute to 200 cc.
6.6	50 cc. 0.2 *M* KH_2PO_4	17.80 cc. 0.2 *M* NaOH	Dilute to 200 cc.
6 8	50 cc. 0.2 *M* KH_2PO_4	23.65 cc. 0.2 *M* NaOH	Dilute to 200 cc.
7.0	50 cc. 0.2 *M* KH_2PO_4	29.63 cc. 0.2 *M* NaOH	Dilute to 200 cc.
7.2	50 cc. 0.2 *M* KH_2PO_4	35.00 cc. 0.2 *M* NaOH	Dilute to 200 cc.
7.4	50 cc. 0.2 *M* KH_2PO_4	39.50 cc. 0.2 *M* NaOH	Dilute to 200 cc.
7.6	50 cc. 0.2 *M* KH_2PO_4	42.80 cc. 0.2 *M* NaOH	Dilute to 200 cc.
7.8	50 cc. 0.2 *M* KH_2PO_4	45.20 cc. 0.2 *M* NaOH	Dilute to 200 cc.
8.0	50 cc. 0.2 *M* KH_2PO_4	46.80 cc. 0.2 *M* NaOH	Dilute to 200 cc.

Boric Acid, KCl–NaOH Mixtures.

7.8	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	2 61 cc. 0.2 *M* NaOH	Dilute to 200 cc.
8.0	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	3 97 cc. 0 2 *M* NaOH	Dilute to 200 cc.
8.2	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	5.90 cc. 0.2 *M* NaOH	Dilute to 200 cc.
8.4	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	8 50 cc. 0.2 *M* NaOH	Dilute to 200 cc.
8.6	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	12.00 cc. 0.2 *M* NaOH	Dilute to 200 cc.
8.8	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	16.30 cc. 0.2 *M* NaOH	Dilute to 200 cc.
9.0	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	21.30 cc. 0.2 *M* NaOH	Dilute to 200 cc.
9.2	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	26.70 cc. 0.2 *M* NaOH	Dilute to 200 cc.
9.4	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	32.00 cc. 0.2 *M* NaOH	Dilute to 200 cc.
9.6	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	36.85 cc. 0 2 *M* NaOH	Dilute to 200 cc.
9.8	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	40.80 cc. 0.2 *M* NaOH	Dilute to 200 cc.
10.0	50 cc. 0.2 *M* H_3BO_3, 0.2 *M* KCl	43.90 cc. 0.2 *M* NaOH	Dilute to 200 cc.

From W. M. Clark, The Determination of Hydrogen Ions. Williams & Wilkins Company, Baltimore, 1928. By Permission. For details in the preparation of these and other buffer mixtures consult Clark, Chapter IX.

TABLE II

Indicators selected by Clark and Lubs, supplemented by Cohen*

Common Name	NaOH 0.01N, to be used for 0.1 g. of dry indicator	Range	Color Change Acid → Alkaline
	cc.	*p*H	
Meta cresol purple	26.2	1.2–2.8	red-yellow
Thymol blue	21.5	1.2–2.8	red-yellow
Brom phenol blue	14.9	3.0–4.6	yellow-blue
Brom cresol green	14.3	3.8–5.4	yellow-blue
Chlor phenol red	23.6	4.8–6.4	yellow-red
Brom phenol red	19.5	5.2–6.8	yellow-red
Brom cresol purple	18.5	5.2–6.8	yellow-purple
Brom thymol blue	16.0	6.0–7.6	yellow-blue
Phenol red	28.2	6.8–8.4	yellow-red
Cresol red	26.2	7.2–8.8	yellow-red
Meta cresol purple	26.2	7.4–9.0	yellow-purple
Thymol blue	21.5	8.0–9.6	yellow-blue
Cresol phthalein	....	8.2–9.8	colorless-red

* *Preparation of Indicator Solutions* —Clark recommends the following procedure: Grind 0.1 g. of the dry powder in an agate mortar with the quantity of 0.01N NaOH shown in the table. Dilute to 250 cc with distilled water. This gives a 0.04 per cent solution of the indicator. For ordinary purposes this solution will be satisfactory in testing 10 cc. of a solution with about 5 drops of the indicator.

For a detailed discussion of indicators, consult Clark, Chapter III.

TABLE III

Indicators Commonly Used in Acid–Base Titrations

Common Name	Range, *p*H	Color Change	
		Acid	Alkaline
Dimethylaminoazobenzene (Topfer's reagent)	2.9–4.0	red	yellow
Methyl orange	3.1–4.4	red	yellow
Congo red	3.0–5.0	blue	red
Methyl red	4.2–6.3	red	yellow
Alizarin	5.5–6.8	yellow	red
Phenolphthalein	8.2–10.0	colorless	red
Alizarin	10.1–12.1	violet	purple

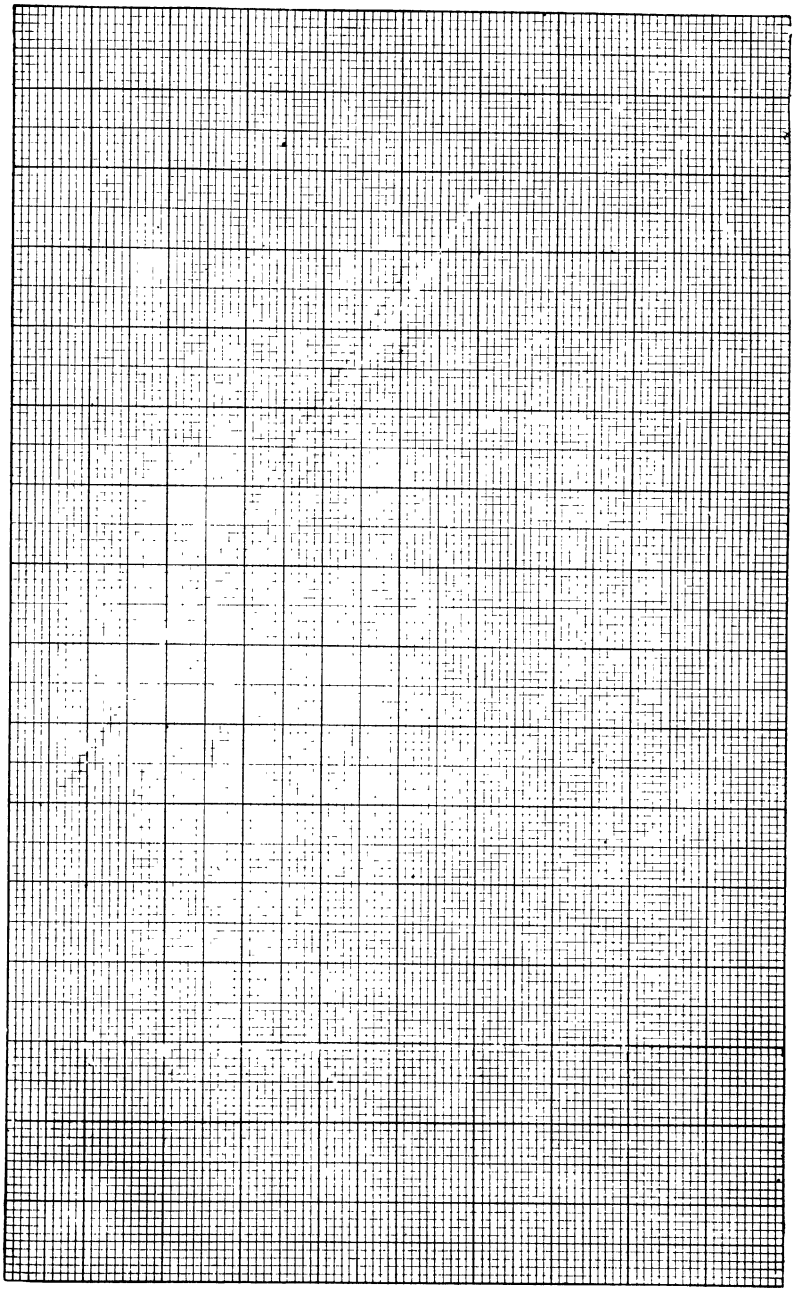
TABLE IV

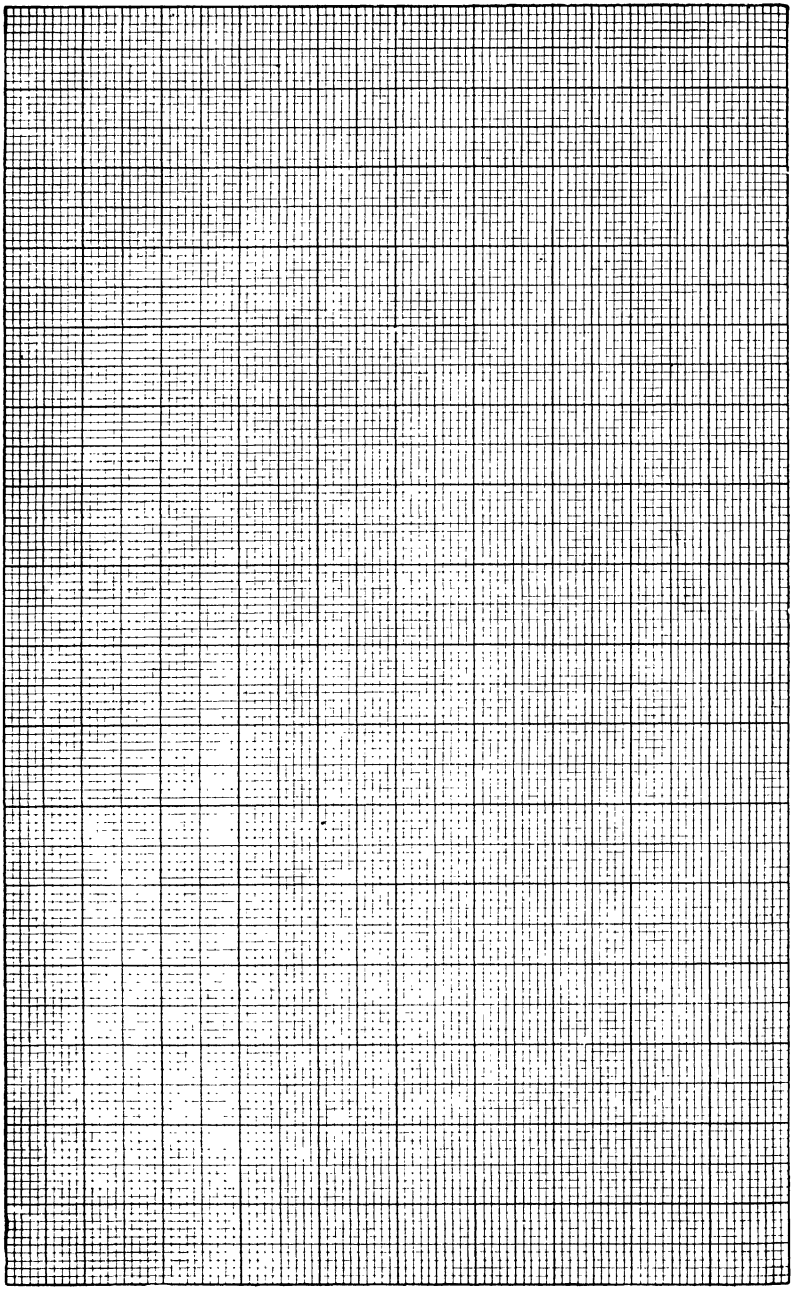
Energy Expenditure per Hour Under Different Conditions of Muscular Activity

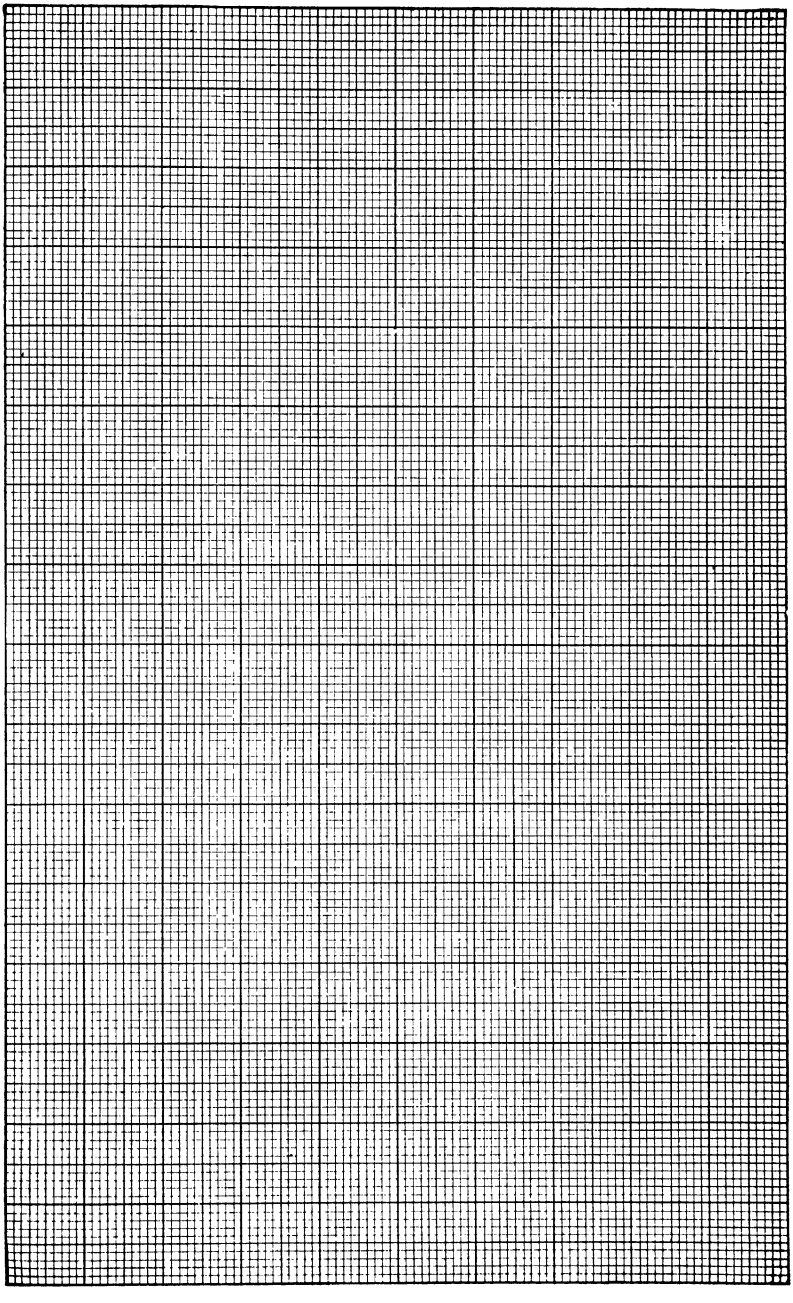
Form of Activity	Calories per Hour			
	Man		*Woman*	
	Per Kilogram	Per Pound	Per Kilogram	Per Pound
Sleeping	0 93	0 43	0 87	0 40
Awake lying still	1 10	0 50	1 02	0 47
Sitting quietly	1 43	0 65	1 33	0 60
Reading aloud	1 50	0 69	1 39	0 63
Standing relaxed	1.50	0 69	1.39	0 63
Hand sewing	1 59	0 72	1 47	0 67
Standing at attention	1 63	0 74	1.53	0 69
Knitting (23 stitches per minute on sweater)	1 66	0 75	1.54	0 70
Dressing and undressing	1 69	0 77	1 57	0 71
Singing	1 74	0 79	1 62	0 74
Tailoring	1 93	0 88	1 79	0 81
Typewriting rapidly	2 00	0 91	1 86	0 85
Ironing (with five-pound iron)	2 06	0 93	1 91	0 87
Dishwashing (plates, bowls, cups, and saucers)	2 06	0 93	1 91	0 87
Sweeping bare floor (38 strokes per minute)	2 41	1 09	2 24	1 02
Bookbinding	2 43	1 10	2 26	1.02
"Light exercise"	2 43	1 10	2 26	1 02
Shoemaking	2 57	1.17	2.41	1.10
Laundry work (towels rubbed on a board without water, 35 times per minute)	2 60	1 18	2 42	1 10
Walking slowly (2.6 miles per hour)	2 86	1 30	2 66	1 21
Carpentry, metal working, industrial printing	3 43	1 56	3 19	1 45
"Active exercise"	4 14	1 88	3 85	1 75
Walking moderately fast (3.75 miles per hour)	4 28	1 95	3 99	1 81
Stoneworking	5 71	2 60	5 31	2 41
"Severe exercise"	6 43	2 92	5 98	2 72
Sawing wood	6 86	3 12	6 39	2 90
Swimming	7 14	3 25	6 64	3 02
Running (5.3 miles per hour)	8 14	3 70	7 57	3.44
"Very severe exercise"	8 57	3 90	7 97	3 62
Walking very fast (5.3 miles per hour)	9.28	4 22	8 63	3.92

Reproduced from "A Laboratory Handbook for Dietetics," by Mary Swartz Rose, The Macmillan Co., with the permission of the author and of the publisher.

The data in this table represent the total caloric output, including the basal metabolism, the influence of food, as well as the effect of activity.







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	Symbol	*Atomic Number*	*Atomic Weight*		*Symbol*	*Atomic Number*	*Atomic Weight*
Aluminum	Al	13	26.97	Molybdenum	Mo	42	95.95
Antimony	Sb	51	121.76	Neodymium	Nd	60	144.27
Argon	A	18	39.944	Neon	Ne	10	20.183
Arsenic	As	33	74.91	Nickel	Ni	28	58.69
Barium	Ba	56	137.36	Nitrogen	N	7	14.008
Beryllium	Be	4	9.02	Osmium	Os	76	190.02
Bismuth	Bi	83	209.00	Oxygen	O	8	16.0000
Boron	B	5	10.82	Palladium	Pd	46	106.7
Bromine	Br	35	79.916	Phosphorus	P	15	31.02
Cadmium	Cd	48	112.41	Platinum	Pt	78	195.23
Calcium	Ca	20	40.08	Potassium	K	19	39.096
Carbon	C	6	12.01	Praseodymium	Pr	59	140.92
Cerium	Ce	58	140.13	Protactinium	Pa	91	231
Cesium	Cs	55	132.91	Radium	Ra	88	226.05
Chlorine	Cl	17	35.457	Radon	Rn	86	222
Chromium	Cr	24	52.01	Rhenium	Re	75	186.31
Cobalt	Co	27	58.94	Rhodium	Rh	45	102.91
Columbium	Cb	41	92.91	Rubidium	Rb	37	85.48
Copper	Cu	29	63.57	Ruthenium	Ru	44	101.7
Dysprosium	Dy	66	162.46	Samarium	Sm	62	150.43
Erbium	Er	68	167.20	Scandium	Sc	21	45.10
Europium	Eu	63	152.0	Selenium	Se	34	78.96
Fluorine	F	9	19.00	Silicon	Si	14	28.06
Gadolinium	Gd	64	156.9	Silver	Ag	47	107.880
Gallium	Ga	31	69.72	Sodium	Na	11	22.997
Germanium	Ge	32	72.60	Strontium	Sr	38	87.63
Gold	Au	79	197.2	Sulfur	S	16	32.06
Hafnium	Hf	72	178.6	Tantalum	Ta	73	180.88
Helium	He	2	4.003	Tellurium	Te	52	127.61
Holmium	Ho	67	163.5	Terbium	Tb	65	159.2
Hydrogen	H	1	1.081	Thallium	Tl	81	204.39
Indium	In	49	114.76	Thorium	Th	90	232.12
Iodine	I	53	126.92	Thulium	Tm	69	169.4
Iridium	Ir	77	193.1	Tin	Sn	50	118.70
Iron	Fe	26	55.84	Titanium	Ti	22	47.90
Krypton	Kr	36	83.7	Tungsten	W	74	183.92
Lanthanum	La	57	138.92	Uranium	U	92	238.07
Lead	Pb	82	207.21	Vanadium	V	23	50.95
Lithium	Li	3	6.940	Xenon	Xe	54	131.3
Lutecium	Lu	71	175 0	Ytterbium	Yb	70	173.04
Magnesium	Mg	12	24.32	Yttrium	Y	39	88.92
Manganese	Mn	25	54.93	Zinc	Zn	30	65.38
Mercury	Hg	80	200.61	Zirconium	Zr	40	91.22