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DIRECTIONS
FOR
LABORATORY WORK
IN
PHYSIOLOGICAL CHEMISTRY.

BY
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PREFACE.

THE second edition of this Manual appears as the result of a thorough revision of the original edition, which was issued last year; numerous additions to the subject-matter have also been inserted.

In its preparation the author has had in mind to provide a guide for systematic work in a course in Physiological Chemistry such as is given in the majority of the American Medical Schools. An attempt has been made to present, in experimental form, all of the essentials of the subject. At the same time, the experiments are simple and of a form which would not overstep the possibilities of equipment and time available in the average laboratory in physiological chemistry. Those facts which do not allow of simple experimental study have been ignored. The text preceding the experiments has been limited to an explanation merely sufficient for a correct understanding and performance of the work indicated, with the expectation that the laboratory course will be supplemented by lectures showing the important relationship of the pure chemistry of the subject to physiological and pathological processes.

The description of the experiments has been set down with considerable detail and, it is hoped, clearness, since the suc-

cessful termination of tests and experiments depends to such a large extent upon the exact manner in which they are conducted.

Direct explanation of the results of the experiments is purposely omitted, with the end in view that the student might be led to reason these out for himself.

Further, it has been presumed that the student taking this work does it in preparation for the practice of medicine. Particular emphasis has therefore been laid on that point, especially in the part devoted to urine analysis, where those methods have been presented which adapt themselves particularly to clinical work.

In conclusion the author claims little originality in the experiments, and acknowledges the use of all text-books and manuals which were available.

H. C. J.

TABLE OF CONTENTS.

	PAGE
CARBOHYDRATES.....	1
Pentoses.....	2
Monosaccharides.....	3
Disaccharides.....	5
Polysaccharides.....	7
FATS.....	9
PROTEINS.....	15
Simple proteins.....	20
Conjugate proteins.....	25
Derived proteins.....	27
MUSCULAR TISSUE.....	29
Proteins.....	29
Nitrogenous extractives.....	30
Non-nitrogenous extractives.....	34
BONE.....	36
NERVOUS TISSUE.....	38
Lipoids.....	38
Cerebrosides.....	40
Cholesterols.....	41
SALIVARY DIGESTION.....	43
GASTRIC DIGESTION.....	46
Peptic proteolysis.....	51
PANCREATIC DIGESTION.....	56
Tryptic proteolysis.....	57
Amylolysis.....	60
Lipolysis.....	60
Pancreatic rennin.....	61
INTESTINAL PUTREFACTION.....	62
BILE.....	67
Conjugate acids.....	68
Pigments.....	69

	PAGE
BLOOD.....	72
Blood serum.....	73
Blood plasma.....	74
Form elements.....	75
Spectroscopic examination.....	77
MILK.....	80
Quantitative separation.....	81
URINE.....	85
Normal constituents.....	88
Quantitative determinations.....	103
Pathological constituents.....	121
SEDIMENTS.....	134
APPENDIX.....	137
INDEX.....	145

LABORATORY WORK

IN

PHYSIOLOGICAL CHEMISTRY.

THE CARBOHYDRATES.

The term carbohydrate is usually considered as embracing those compounds which contain the elements C, H, and O, the H and O being present in the same ratio as they exist in water. This definition, strictly followed, would include in the group substances such as lactic acid, acetic acid, and inosit, which are obviously not carbohydrates. The carbohydrates may be conveniently divided into three groups; namely, Monosaccharides or Glucoses, Disaccharides or Saccharoses, and Polysaccharides or Amyloses; but a more strictly chemical classification would designate them according to the number of carbon atoms present in the molecule, the prefix *aldo-* or *keto-* denoting to what general class of compounds (aldehydes or ketones) they belong: thus trioses, tetroses, pentoses, hexoses, heptoses, etc., and aldohexose, ketopentose, etc. Of the greatest physiological importance are the pentoses, hexoses, hexobioses (Disaccharides), and polyoses (Amyloses).

Note the general character and appearance of the vari-

2 LABORATORY WORK IN PHYSIOLOGICAL CHEMISTRY

ous specimens of carbohydrates which are presented for study.

Test for the constituent elements as follows:

(a) *Carbon*.—1. Heat cautiously some of the substance on a platinum foil. The piece will char, owing to the separation of the carbon in the substance. Further heating renders the carbon capable of combining with the oxygen of the air, with the result that the former passes off as CO_2 , and in the case of carbohydrates, where combustion is complete, no residue is obtained. A substance containing oxygen in sufficient quantities to form CO_2 with all the carbon present will not carbonize.

2. Mix thoroughly some of the dried substance with powdered CuO and place the mixture in the bottom of a dry test-tube. Upon warming, the carbon of the substance is oxidized by the oxygen of the CuO and escapes as CO_2 . This CO_2 may be detected by holding a glass rod moistened with lead acetate at the mouth of the test-tube.

(b) *Hydrogen*.—1. In the latter experiment, moisture will have collected on the cold part of the test-tube. The hydrogen of the substance, in the presence of heat, has combined with the oxygen supplied by the CuO , forming H_2O .

PENTOSES, $\text{C}_5\text{H}_{10}\text{O}_5$.

ARABINOSE. XYLOSE.

The pentoses do not occur free in nature, but exist in the form of pentosanes—bodies which are found in the fruits and polysaccharide gums (e.g., gum arabic, cherry gum) and from which the pentoses may be obtained by hydrolysis with weak acids. The ingestion of pentosanes also causes pentoses to appear in the urine. Chemically they are aldehydes and as such reduce Fehling's solution. With regard to their

action on polarized light, they exist in three modifications, but those derived from the pentosanes are dextrogyrate. Pentose solutions when heated with phloroglucinol or orcinol and HCl (sp. gr. 1.09) acquire respectively a cherry-red or green color and possess characteristic absorption spectra. The pentoses are non-fermentable and yield with phenylhydrazin osazones having characteristic properties. Boil some cherry gum for some hours with 1 per cent H_2SO_4 . Test the solution for pentose as follows:

(a) Saturate 5 c.c. of HCl (sp. gr. 1.09) with phloroglucinol by warming on the boiling-water bath. A slight excess of the phloroglucinol is advantageous. Add to this a few c.c. of the pentose solution and continue the heating. Gradually a cherry-red color develops in the solution and the latter shows characteristic absorption bands between D and E.

(b) Try the same test using orcinol instead of phloroglucinol. The solution turns reddish, then reddish blue, and finally green, with a precipitate of that color settling out of the solution. This precipitate is soluble in amyl alcohol, in which solvent it also has characteristic absorption bands between C and D.

(c) Prepare pentosazones after the method employed on p. 5.

HEXOSES OR MONOSACCHARIDES, $\text{C}_6\text{H}_{12}\text{O}_6$.

DEXTROSE. LEVULOSE. GALACTOSE.

The monosaccharides, in general, are soluble, crystalline, and optically active bodies which yield, upon boiling with alkalis, oxidation products and caramel; they reduce metallic oxides in alkaline solution; ferment with yeast and with bacterium lactis; and give, with phenylhydrazin, osazones

4 LABORATORY WORK IN PHYSIOLOGICAL CHEMISTRY.

with characteristic crystalline forms, solubilities, and melting-points.

GLUCOSE = DEXTROSE = GRAPE-SUGAR.

Of the monosaccharides, dextrose holds first place in importance in the animal economy. Examine and taste the dry substance. Test its solubility in water and in hot and cold alcohol. In the following tests make use of a 1 per cent solution:

(a) *Moore's Test*.—To 5 c.c. of the dextrose solution add an equal volume of NaOH, and heat. The mixture becomes yellow and finally brown, due to the formation of caramel. This test lacks delicacy and reliability in examining urine.

(b) *Trommer's Test*.—To 5 c.c. of the dextrose solution add an equal volume of NaOH. Then add, drop by drop, a dilute solution of CuSO_4 (so dilute that the green color is just visible) until a trace of permanent precipitate remains. The solution should be deep blue in color. Warm the upper part of the solution and note result. Write all the equations for the reactions taking place in this experiment.

(c) *Fehling's Test*.—Heat 5 c.c. of Fehling's solution just to boiling and add a few drops of the dextrose solution. Continue the boiling until the solution commences to respond as with Trommer's test. Compare the reactions of this test with those of the previous ones.

(d) *Barfoed's Test*.—To 5 c.c. of Barfoed's reagent add a few drops of the dextrose solution and boil. Note the result and write equations.

(e) *Nylander's Test*.—To 5 c.c. of the dextrose solution add 10 drops of Nylander's reagent and boil. The solution gradually turns yellow and finally black, bismuth being precipitated.

(f) *Silver Nitrate*.—To 5 c.c. of AgNO_3 solution in a clean test-tube add dilute NH_4OH , drop by drop, until the precipitate, which is at first formed, dissolves. Then add a few drops of the dextrose solution and warm on the water-bath. Note the formation of a metallic mirror on the side of the test-tube. Explain the chemical changes.

(g) *Phenylhydrazin*.—In a test-tube prepare a mixture of 5 drops of phenylhydrazin, 10 drops of glacial acetic acid, and 1 c.c. of a saturated solution of NaCl ; then add 5 c.c. of the dextrose solution and boil for a few minutes. Yellow phenylglucosazone crystals will appear on cooling.

Under the microscope these appear as fine yellow needles, usually arranged in sheath-shaped bundles. If determinations are made of the composition and melting-point of these crystals, this test furnishes most characteristic and conclusive evidence for dextrose. Write the equations for the formation of the osazones.

(h) *Fermentation*.—In a test-tube shake 20 c.c. of the dextrose solution with a small piece of compressed yeast. Place the mixture in a saccharometer and allow it to stand in a thermostat at 40°C . A gas collects at the top of the tube and its volume is in a direct ratio to the amount of dextrose in the solution. What is the character of the chemical changes which have taken place?

(i) *Polarization*.—See demonstration and make a reading on the instrument of the degree of right-handed rotation.

HEXABIOSES OR DISACCHARIDES, $\text{C}_{12}\text{H}_{22}\text{O}_{11}$.

SACCHAROSE. LACTOSE. MALTOSE.

The disaccharides possess, with the exception of saccharose, the same general properties as the simple sugars. As their name implies, upon hydrolysis they take up a molecule

6 LABORATORY WORK IN PHYSIOLOGICAL CHEMISTRY.

of water and break down into two molecules of a monosaccharide.

This decomposition in the case of saccharose has assumed the name of *inversion* from the fact that when a dextro-rotatory solution of cane-sugar suffers hydrolysis, the resultant mixture is lævogyrate. This is caused by the strong lævo-rotation of the lævulose more than overcoming the dextrogyrate polarization of the dextrose.

Saccharose = dextrose + lævulose.

SACCHAROSE = CANE-SUGAR = SUCROSE.

Saccharose differs from the other members of this group in not reducing metallic oxides in alkaline solution and in not forming osazones with phénylhydrazin. It is not directly fermentable with yeast, but only after previous inversion by the ferment *invertin*, secreted by the yeast-cell. A slight reduction which is sometimes obtainable with Fehling's test may be explained by the inverting action of the strong alkali.

Use a 1 per cent solution of cane-sugar.

Tests: (a) Fehling's; Moore's; Nylander's; Barfoed's; using in each case 5 c.c. of the saccharose solution. Compare these results with those obtained for dextrose.

(b) To 10 c.c. of the saccharose solution add 1 c.c. concentrated HCl and boil several minutes. Allow this to cool and then neutralize with NaOH. Use this solution in making Fehling's, Nylander's, and Barfoed's tests. Write the equations for the chemical changes which have taken place and determine the character of the carbohydrate formed.

Inversion and Fermentation.

1. Examine the saccharose solution in the polariscope and determine the degree of rotation.

2. Place 10 c.c. of the saccharose solution in a test-tube with 5 c.c. of 1 per cent HCl. Allow the mixture to remain two hours at 37° C. Finally examine this in the polariscope and test its reducing power. To what condition in the body is this comparable?

3. To 10 c.c. of the saccharose solution add some invertin and allow the test-tube to remain at 37° C. Test for invert action.

4. See demonstration of the formation of alcohol and CO₂ from a fermentation of cane-sugar by yeast. The CO₂ as it forms is caught in passing out of the flask by a valve of Ba(OH)₂. The alcohol is distilled off from the mixture in the flask.

MALTOSE will be studied under Salivary Digestion and LACTOSE under Milk.

POLYPOSES OR POLYSACCHARIDES, (C₆H₁₀O₅)_n.

STARCHES. DEXTRINS. GLYCOGEN. CELLULOSES.
VEGETABLE GUMS.

The polysaccharides, as a class, are amorphous, more or less insoluble substances which do not diffuse through animal membranes. They are optically active, and with the exception of the dextrins do not reduce Fehling's solution. By hydration with weak acids or with enzymes the polysaccharides are converted into monosaccharides; dextrins and disaccharides being the intermediate products. The polysaccharides are incapable of undergoing fermentation unless previously inverted.

STARCHES.

The starches are insoluble bodies which, with hot water, form opalescent colloidal solutions or pastes. These, if suffi-

8 LABORATORY WORK IN PHYSIOLOGICAL CHEMISTRY.

ciently concentrated, gelatinize upon cooling. The most characteristic reaction of the starches is their behavior toward iodine, with which they form blue compounds whose color disappears upon heating, but returns as the solution cools.

(a) Examine under the microscope and sketch the following starch granules: potato, corn, wheat, rice, and arrowroot.

(b) Place some starch in a test-tube half full of water and shake thoroughly until the starch is finely divided. Heat water to boiling in another test-tube and to this add enough of the cold starch mixture to make a translucent solution (about 2 per cent). What takes place upon pouring the suspended starch into the hot water? The solution of starch thus obtained is called a paste, and in making experiments with this substance such a *paste* must always be prepared.

(c) To 5 c.c. of starch paste add a drop of iodine solution. Warm gradually and then allow to cool. Note changes.

(d) Boil 10 c.c. of starch paste with 1 c.c. of concentrated HCl for 5 minutes. Observe the change which the solution undergoes. Neutralize part of this *cold* solution with NaOH, and apply Fehling's and Barfoed's tests. (If no reduction appears, continue the boiling of the original acid solution for some minutes longer and repeat the neutralization and tests.) Determine the character of the sugar causing this reduction.

For the DEXTRINS see Salivary Digestion; for GLYCOGEN see Muscular Tissue.

THE FATS.

The neutral fats are glycerol esters of fatty acids, in that three hydrogens of the carboxyl groups of three fatty acids are replaced by the glycerol radical. A general formula for a neutral fat is represented thus:

in which R, R', and R'' stand for three of the same or different hydrocarbon radicals. In the molecule of the ordinary animal fats R, R', and R'' may be represented by three hydrocarbon residues of palmitic, of stearic, of oleic, or of butyric acid, the compounds being designated respectively as tripalmitin, tristearin, etc.

The specific character of a fat is dependent upon the nature of the fatty acid in the molecule; and since, in general, the melting-points of the saturated fatty acids increase with their carbon content, the lower compounds remain liquid at ordinary temperature and the character of tributyrin (R, R', and R'' = C₃H₇) is that of a soft fat, while tristearin (R, R', and R'' = C₁₇H₃₅) possesses considerable firmness.

Observe the olive oil, butter, mutton and beef fats presented for study. Pure neutral fats are devoid of color, odor, and taste, and their low specific gravity and insolubility

10 LABORATORY WORK IN PHYSIOLOGICAL CHEMISTRY.

allow them to float on the surface of water. They are soluble in ether, chloroform, and benzol; hot alcohol also dissolves them, but upon cooling they separate out usually in crystalline form. Under certain conditions fats undergo a peculiar physical change called emulsification, which is beautifully illustrated in the natural state of the fat in milk. Under the influence of superheated steam, acids, or ferments, fats are broken down into their component parts—fatty acids and glycerol. When an alkali or sodium alcoholate is employed, soaps and glycerol are formed and the process is called *saponification*. Another form of decomposition is effected when fats remain for any length of time in contact with the oxygen of the air. They then become rancid by the liberation and subsequent oxidation of the fatty acid from the molecule and the formation of lower volatile acids which cause unpleasant odors and tastes.

Try the following tests, making use of olive oil:

(a) Test its solubility in water; ether; chloroform; alcohol.

(b) Test its reaction. What is the normal reaction of a fat? Express by equations what occurs when butter becomes rancid.

(c) In a test-tube warm a few drops with potassium bisulphate. Notice odor. What is the reaction taking place?

(d) Let a drop of an ether solution of a fat fall upon paper.

(e) Dissolve a little lard in 10 c.c. of a mixture of equal parts of alcohol and ether. Allow this to remain uncovered until crystals begin to form.

(f) *Saponification (Bayberry Wax—Tripalmitin).*

Place a piece of the wax, half the size of a walnut, in an evaporating-dish which is half full of water. Then add about

10 c.c. NaOH and boil until the substance is dissolved, adding water from time to time as the solution evaporates, so that the *original volume is constantly maintained*. Write the reaction which is taking place. After complete solution, add carefully *dilute* H_2SO_4 until the reaction just becomes acid and then cool. A greenish crust of free fatty acid will form on the surface of the liquid, so that the solution may be poured off. (Keep this solution.) Break the fatty acid crust into small pieces and wash with tap water; finally dry between filter-paper. Dissolve part of the fatty acid in 50 c.c. of hot 95 per cent alcohol, filter hot through a dry funnel and allow the filtrate to cool slowly.

Palmitic acid separates out in snowy-white crystalline form. Examine some under the microscope and sketch the crystals.

(g) *Saponification (Lard)*.

Place about 100 c.c. of alcoholic potash in a flask containing 25 grams of lard. Warm the mixture upon the water-bath until a drop let fall into water is perfectly soluble. Then pour the solution into an evaporating-dish containing 100 c.c. water and evaporate until the alcohol has been driven off. While still hot acidify with dilute H_2SO_4 and then cool. Write the chemical equations for the various steps in the procedure. The fatty acid rises to the surface and may be easily removed from the liquid beneath. Unite this solution with the similar one in Experiment 1 and, after *neutralization* with Na_2CO_3 , evaporate it down to about 5 c.c. What substances are present in this solution? Retain this.

FATTY ACIDS AND SOAPS.

Since the character of the fatty acid determines the character of the fat, the properties and solubilities of the fatty acids and fats must be similar, as the tests will prove.

The soaps are salts of the fatty acids; the sodium salts being solid and hard, while those of potassium are soft. Both are soluble in water. The remaining metallic salts are insoluble in water, but generally dissolve in alcohol. The lead combination with oleic acid (lead plaster) differs from the corresponding one with palmitic and stearic acids in being soluble in ether.

Perform the following reactions with the *fatty (oleic) acid*:

(a) Test its solubility in ether; chloroform; water; alcohol.

(b) Warm some in a dry test-tube with potassium bisulphate.

(c) Shake up a few pieces of the fatty acid with 25 c.c. water and add NaOH, drop by drop, until the acid is all dissolved. Write the reaction. What is formed?

Use this *soap* solution for the following reactions:

(a) Add 5 drops to a saturated solution of NaCl.

(b) To 5 c.c. of lead acetate add a few c.c. of the soap solution. A sticky precipitate results (lead oleate), which is soluble in ether. Write the reaction. What is the importance of this compound?

(c) To 50 c.c. of tap water in a flask add the soap solution, drop by drop, alternately shaking until a *permanent* lather is obtained. The amount of soap solution required to obtain the lather is in direct proportion to the hardness of the water. Explain the chemistry of this experiment.

Emulsification of Fats.

Certain poorly understood factors enter into the formation and preservation of an emulsion. Apparently the viscosity of the menstruum and the condition of the surface tension existing between that menstruum and the globules form the fundamental physical requisites for the obtaining of a *permanent* emulsion. Solutions of gums, proteins, and soaps emulsify fats with varying degrees of permanency. The mere *presence* of a soap in the solution does not seem to render the fat particularly prone to emulsify; the necessary condition is apparently the *intermolecular formation* of a soap *in* the solution as exemplified upon the addition of an alkali to fat containing some free fatty acid. An emulsion formed in this manner is the most permanent of all those induced by artificial means; a similar procedure takes place in the emulsification of fat in the intestine.

Make up the following mixtures in test-tubes:

(a) 2 drops of neutral olive oil + 10 c.c. water.

(b) 2 drops of neutral olive oil + 10 c.c. water + a few drops of Na_2CO_3 .

(c) 2 drops of neutral olive oil + 10 c.c. of the soap solution.

(d) 2 drops of neutral olive oil + 10 c.c. of an egg-albumin solution.

(e) 2 drops of rancid olive oil + 10 c.c. of water + a few drops of Na_2CO_3 .

Shake each tube approximately the same length of time and compare the relative permanence of the various emulsions. Which conditions are the most favorable, and why?

14 LABORATORY WORK IN PHYSIOLOGICAL CHEMISTRY.

Make use of the solution obtained from the two saponifications for the following tests:

(a) Note the taste and try its solubility in alcohol; ether; water.

(b) Add some dry potassium bisulphate and warm. Compare this with the same reaction under Fats. Write the equation.

(c) Test with Fehling's solution.

(d) To some dilute CuSO_4 add the remainder of the glycerol solution; then drop by drop add dilute NaOH . What causes the blue color of the solution?

THE PROTEINS.

The proteins are complex compounds of C, H, O, N, and S (in some cases P and Fe) occurring widely distributed in the plant and animal kingdoms. The members of this class of bodies, although differing greatly in chemical and physical characteristics, possess in common certain definite properties and chemical reactions which allow of classification and subdivision. The proteins form the chief type of the food-stuffs, since the nitrogen present in them is absolutely essential in order to sustain life.

They may be divided as follows:

The *simple proteins*, those giving upon decomposition indole, skatole, tyrosine, cystine, amino acids, pyrrol derivatives, etc.

The *conjugate proteins*, composed of a simple protein and a complex non-protein component.

The *derived proteins*, derivatives of the proteins and formed through hydrolytic changes.

Reactions General to all Proteins.

For the following tests make use of dried egg-white or casein:

Test the substance for *carbon* and *hydrogen*.

Nitrogen.—(a) Make an intimate mixture of the substance with soda-lime and place it in a dry test-tube. Warm gently. Hold a piece of moistened red litmus paper or a

glass rod moistened with HCl at the mouth of the test-tube. What is the reaction which takes place?

(b) Warm together *carefully* in a *dry* test-tube a few particles of the dry substance and a 3-4 mm. cube of metallic sodium. (Caution: Do not place the tube in the flame. Gases are evolved which are explosive unless the temperature is kept moderate.) When the fusion is complete and the tube has cooled somewhat plunge the end into a small amount of water placed in a suitable vessel, preferably a conical glass. The glass of the test-tube will probably break, and unless the sodium has been completely fused a slight explosion will result. When the water has thoroughly impregnated the fused mass, filter and to the filtrate add a few drops of ferric chloride and of ferrous sulphate solution. Upon acidifying with HCl a blue precipitate of Prussian blue is obtained. Write all the reactions which take place in this manipulation.

Sulphur.—The sulphur in the protein molecule exists in part in such a form that upon boiling with caustic soda it is easily split off as sodium sulphide. This in the presence of a lead salt forms black lead sulphide. On this account such sulphur has assumed the name of loosely combined or *lead-blackening sulphur*. If the quantity of sulphur obtained in the above manner be multiplied by $\frac{4}{3}$, the product is the amount of sulphur considered to be present in the cystine nuclei of the given protein. In some proteins—keratin, serum albumin, serum globulin—the total sulphur and cystine sulphur content are the same and we are justified in assuming that all the sulphur in the molecule exists in the form of cystine. In other proteins only $\frac{1}{3}$ — $\frac{1}{2}$ of the total sulphur is to be accounted for as cystine S; hence it must be concluded that sulphur exists in some atomic complex which yields, upon the decomposition of the molecule, substances like α -thio-lactic acid, mercaptans, ethyl sulphide, etc. Sulphur of this type may be styled, in contradistinction, *firmly combined sulphur*. The sum of the loosely and firmly combined sulphur equals the total sulphur of the substance.

Test for *lead-blackening* sulphur as follows:

Place about 5 c.c. of dilute NaOH in a test-tube with a small quantity of the substance, and add two drops of lead acetate. Boil the mixture for a few minutes and note the changes. The depth of color may be said to correspond roughly to the amount of neutral sulphur present in the substance.

Test for *total* sulphur as follows:

Mix some of the substance with double its quantity of the fusion mixture ($\text{Na}_2\text{CO}_3 + \text{KNO}_3$). Place the whole in a small porcelain crucible and warm *cautiously* until the mixture becomes colorless. (If the fusion begins to sputter, remove the flame.) The residue (fused mass) should be nearly white. It is then dissolved in a small quantity of water, filtered, and the filtrate, after the addition of a few drops of HCl, is brought to boiling and treated with BaCl_2 solution. What is this white precipitate? Write the equation.

Phosphorus.—Heat some casein with the fusion mixture as in the previous experiment. Dissolve the fused mass in 10 c.c. of water acidified with HNO_3 . Filter and add 5 c.c. of ammonium molybdate solution. Warm some minutes at about 80°C . What is the yellow precipitate?

Iron.—Incinerate a small quantity of hæmoglobin in a porcelain crucible. The ash should be red. Dissolve out the ash with 10 c.c. of dilute HCl. Filter. Test this solution for Fe with potassium sulphocyanide or potassium ferrocyanide.

Color Reactions.

The following tests are based upon reactions taking place between the reagent employed and certain more or less well-defined groups or atomic complexes of the protein molecule. These groups are present in varying proportions in the different proteins and in some cases even certain complexes

may be lacking altogether, facts which explain the individual differences in the strengths of the reactions obtained.

For the following tests make use of the egg-white solution as an example of a typical protein:

(a) *Xanthoproteic Test*.—To 5 c.c. of the protein solution (or dry substance) add 5 c.c. of HNO_3 . Note the white precipitate. Boil until the precipitate is dissolved and the solution becomes light yellow. Cool, and add an excess of NH_4OH . The color changes to orange. Other substances may give a yellow solution with HNO_3 , but their solutions do not respond orange upon neutralization with NH_4OH . What are the chemical changes of this reaction and the atomic complexes involved?

(b) *Millon's Test*.—Add a few c.c. of Millon's reagent to 5 c.c. of the protein solution. The precipitate which forms turns red slowly upon heating. This reaction is suitable for solids, or liquids when the quantity of salts in the solution is not excessive. What atomic complex in the protein molecule causes this reaction? What simple substance also responds to this test?

(c) *Biuret Test*.—Suited for testing solutions only.—Place 5 c.c. of the protein solution in a test-tube and add an equal volume of NaOH . Then add, drop by drop, a CuSO_4 solution so dilute that the color is hardly visible. The color obtained will vary from blue violet to reddish violet, according to the nature of the protein in solution. If too much CuSO_4 is added the solution becomes green. A specific atomic complex in the protein molecule is also accountable for this reaction. What simple substance reacts positively to this test?

(d) *Adamkiewicz Test*.—To 3 c.c. of a glyoxylic acid solution (Hopkins-Cole reagent) add 1-2 c.c. of the protein solution. Stratify this mixture upon 5 c.c. of conc. H_2SO_4 .

A red to reddish-violet color develops at the junction of the two liquids. Of what important complex does this reaction show the presence?

Precipitation Reactions.

The precipitation reactions may be divided into three general classes according as the protein acts as a base, as an acid, or as a neutral body. To the first class belong those reactions in which the weak organic acids combine with the protein as a base and an insoluble salt results. The second class of reactions embrace those between neutral salts and the protein in which an albuminate of the base (metal) is formed. In the third class the protein does not take part in the reaction. Precipitation occurs when the reagent which is added (in this case a salt such as MgSO_4 , Na_2SO_4 , $(\text{NH}_4)_2\text{SO}_4$) has appropriated to itself sufficient of the solvent to throw the protein out of its solution (salted out).

(a) *Heller's Test*.—If 5 c.c. of HNO_3 is placed in a test-tube and a few c.c. of the albumin solution allowed to flow gently down the side of the test-tube and stratify itself on top of the HNO_3 , a white ring of precipitated albumin will form where the two liquids meet. HCl and H_2SO_4 will also give the same reaction, but the precipitates are soluble in an excess of the reagents.

(b) *Acetic acid and potassium ferrocyanide*.—Make 5 c.c. of the protein solution acid with acetic acid and add, drop by drop, a *dilute* solution of potassium ferrocyanide. Note result.

(c) Make 25 c.c. of the protein solution slightly acid, using dilute acetic acid.

1. To 5 c.c. of this solution add 2 drops of tannic acid solution.

2. To 5 c.c. of this solution add 2 drops of picric acid solution.

3. To 5 c.c. of this solution add 3 volumes of 95 per cent alcohol.

4. To 5 c.c. of this solution add MgSO_4 (in substance) to saturation.

5. To 5 c.c. of this solution add $(\text{NH}_4)_2\text{SO}_4$ (in substance) to saturation.

Note results and see if the precipitation is complete in each case.

(d) Acidify 10 c.c. of the protein solution with dilute HCl.

1. To 5 c.c. of this solution add 2 drops of phosphotungstic acid.

2. To 5 c.c. of this solution add 2 drops of potassium-mercuric-iodide.

(e) To successive portions of 5 c.c. of the protein solution add a few drops of CuSO_4 ; neutral and basic lead acetate; HgCl_2 ; trichloroacetic acid (2-5 per cent solution); Fe_2Cl_6 .

Make careful notes of the results of the above reactions and where possible write equations. Decide as to which class of reaction each belongs.

The use of metallic salts as antidotes in cases of poisoning is based upon these precipitation reactions.

SIMPLE PROTEINS.

The group of simple proteins allows of a further subdivision into the simple native proteins and the albuminoids.

The native proteins, as far as it can be ascertained, exist in the fluids and tissues of the body in the same or at least a similar form in which they appear in the laboratory. The albuminoids are chemically closely related but are characterized by great insolubility in all neutral solvents. To

the former sub-group belong the albumins, the globulins, histones, and protamines; to the latter the keratins, elastins, collagen, recticulin, and the skeletins.

ALBUMINS.

The albumins present the most characteristic type of the proteins since they give a positive response to all the typical protein reactions.

Remember that an albumin (egg-white) solution was employed for all the protein tests.

(a) To 10 c.c. of the albumin solution add $(\text{NH}_4)_2\text{SO}_4$ to saturation. Note result and compare with experiment (c) 5, p. 20. Filter off the precipitate and test the filtrate with the biuret test.

(b) To 10 c.c. of the albumin solution add MgSO_4 to saturation. Compare this result with that of experiment (c) 4, p. 20. Now add 2 drops of acetic acid. What is the precipitate?

(c) Test the albumin solution for lead-blackening sulphur.

Coagulation.

Certain of the proteins in the presence of water and heat undergo a change which is confined to an intramolecular rearrangement of the atoms in the molecule. This transformation, which is termed coagulation, brings about physical differences in the behavior of the proteins. They become insoluble in all of the ordinary protein solvents. *Under similar conditions* the degree of temperature at which a definite protein will coagulate is fairly constant, a fact which is employed to ascertain not only the character but also the purity of an unknown protein.

22 LABORATORY WORK IN PHYSIOLOGICAL CHEMISTRY.

(d) Heat 5 c.c. of the albumin solution to boiling and then add one or two drops of very dilute acetic acid. The albumin separates out in an insoluble, flocky (coagulated) form. Why is the addition of the acetic acid necessary and why is it added *after* boiling? Try to dissolve the coagulum in some of the ordinary protein solvents. Make 5 c.c. of the albumin solution faintly alkaline, and heat. Note differences.

(e) The temperature of coagulation is determined as follows:

Fill a test-tube (one-third of its capacity) with the clear, very slightly acidulated solution of the protein. By means of a bored cork fasten into the test-tube a thermometer in such a manner that its bulb is entirely immersed in the solution. Suspend the test-tube in a large beaker of water which rests upon a wire gauze. By cautious heating, the temperature of the water in the beaker may be raised slowly and the point on the thermometer noted at which the first flocks of coagulated protein appear in the solution. This point is considered as the coagulation temperature of the substance under these conditions, but it varies with the reaction of the solution and the nature and quantity of the salts present.

Another form of coagulation is induced by the action of certain ferments, such as the fibrin ferment of the blood, by the action of which the soluble fibrinogen is transformed into insoluble fibrin.

(f) Heat some dried albumin in a *dry* test-tube to about 100° C. After cooling the tube try to dissolve the substance in water. Why does not the albumin coagulate by the heat?

GLOBULINS.

As a class the globulins are characterized by their insolubility in water and solubility in weak salt solutions (5-10 per cent). A suitable solution of a typical globulin (edestin) is easily prepared by extracting finely ground hemp-seed for an hour with a 10 per cent solution of NaCl, and finally filtering the mixture through paper. The edestin may be obtained in crystalline form if the extraction of the seed is made with a 5 per cent solution of NaCl at 55° C. and the extract filtered through a hot-water funnel. Upon cooling the protein separates out of the solution in well-formed and characteristic crystals, hexagonal in shape.

(a) Try two protein color reactions and three precipitation reactions. Test the coagulability of the solution.

(b) Pour some of the solution, drop by drop, into a beaker of water. A precipitation of the globulin occurs owing to the decrease in the percentage of salt in the solution by dilution. A similar precipitation may also be accomplished by removing the salts by dialysis.

(c) Start dialysis experiment. A very simple form of dialyzer can be arranged as follows:

Select a beaker and a funnel whose stem has been removed at the neck and whose diameter is somewhat greater than that of the beaker. Allow the funnel to hang in the beaker resting upon its rim. Cut and fold some well-moistened parchment paper in the manner of a filter and place it in the funnel. Fill the beaker nearly to the top with water and pour into the parchment filter enough of the solution to be dialyzed so that it about half fills it. The levels of the inner and outer fluids should correspond.

In 24 hours note carefully any changes which may have occurred. What has caused them?

(d) Saturate 10 c.c. of the globulin solution with MgSO_4 .

24 LABORATORY WORK IN PHYSIOLOGICAL CHEMISTRY.

Filter off the precipitate and test the filtrate for protein. Compare this reaction with the similar one for albumin.

(e) Cool 10 c.c. of the globulin solution to about 0° C. and let CO₂ gas bubble through it. Note result.

(f) Take 10 c.c. of blood-serum and saturate it with MgSO₄. What is this precipitate? Filter, and to the filtrate add two drops of acetic acid. What is this second precipitate? Test both precipitates for protein.

ALBUMINOIDS.

The albuminoids form a class of substances of heterogeneous nature closely allied to the proteins, but exhibiting also marked differences. They form the chemical basis of the skeletal and epidermal structures and the variations in the properties, and characteristics which are observed in them are probably dependent upon morphological changes. In general they occur in an insoluble form and are characterized by their resistance to reagents which tend to dissolve them.

COLLAGEN.

Collagen occurs as the chief constituent of connective tissue (e.g., cartilage) and also as the organic matrix of bone (ossein). It is easily hydrated by boiling with water or weak acids and is then converted into gelatin, which on this account may be considered as the hydrate of collagen.

GELATIN.

Gelatin possesses the characteristic of forming, with hot water, solutions which set in a jelly upon cooling, if the concentration is greater than 1 per cent.

Finely cut tendons or bones from which the salts have

been previously removed by allowing them to remain in weak HCl for several days, serve as excellent material for the preparation of gelatin.

Place the substance in an evaporating-dish half full of slightly acidulated water and continue to boil until the material is dissolved. Do not allow the solution to become concentrated. Prolonged boiling will also cause the gelatin to be converted into gelatoses which do not possess the power of gelatinization.

Make the following tests, dissolving the jelly in hot water as it is needed:

(a) Biuret; (b) Millon's; (c) Acetic acid and potassium ferrocyanide; (d) Tannic acid; (e) HCl or H_2SO_4 ; (f) Saturation with $(\text{NH}_4)_2\text{SO}_4$; (g) Lead-blackening sulphur; (h) Adamkiewicz. Note and compare the results carefully with those obtained with proteins.

KERATINS.

These form the chief constituent of hair, nails, hoofs, horns, feathers, etc. Their main characteristic is insolubility, and the relatively large percentage of sulphur which they contain. This is probably present in a cystine nucleus in the molecule, and the largest part is given off in the lead-blackening form.

Use horn shavings for the following reactions:

(a) Lead-blackening sulphur; (b) Millon's; (c) Xanthoproteic; (d) Adamkiewicz; (e) Try its solubility in water, dilute acids, and alkalies.

CONJUGATE PROTEINS.

These substances are much more complex in their chemical constitution than the simple proteins. They may be subdivided into—

1. The *Glycoproteins*—compounds of a protein component and a carbohydrate complex.

2. The *Nucleoproteins*—compounds of a protein component and nucleins or nucleic acids.

3. The *Hæmoglobins* and related bodies—compounds of a protein component and a pigment which is chiefly respiratory in character.

4. The *Lecithoproteins*—compounds of a protein component with lecithins.

5. The *Phosphoproteins*—compounds of a protein component and a phosphorus containing substance other than nucleic acid or lecithins.

GLYCOPROTEINS.

In a certain sense some of the simple proteins may be considered as glycoproteins, in that they yield, upon decomposition, reducing substances of undoubted carbohydrate nature; in the case of the true glycoproteins, however, the carbohydrate nucleus is obtained without interference with the integrity of the component protein molecule. The mucins, mucoids, and chondroproteins form the chief classes of the glycoproteins.

Of the *mucins*, that obtained from the saliva is selected as a typical example for study. They do not contain phosphorus, are acid in nature, and are mainly characterized by forming viscous colloidal solutions. The mucins dissolve in weak alkali solutions and may be reprecipitated on the addition of a weak acid. Upon boiling with a dilute acid they split off a carbohydrate nucleus which reduces Fehling's solution.

Add 100 c.c. of saliva to 200 c.c. of 95 per cent alcohol.

Filter off the precipitated mucin and make the following tests:

(a) Try some color-reactions for proteins.

(b) Dissolve some of the precipitate in weak NaOH and then add dilute acetic acid, drop by drop. Note results.

(c) Boil the remaining precipitate for 10 minutes in a small flask with 50 c.c. of 5 per cent HCl, cool, neutralize with NaOH, and test for a reducing body with Fehling's solution.

THE NUCLEOPROTEINS AND NUCLEINS.

As the name indicates, the nucleoproteins originate in and are derived from the so-called mitoplasm or chromatin of the cell nucleus and consequently exist in greater quantities in the glandular organs than elsewhere in the body. Their individual nomenclature is founded upon their place of origin; thus the pancreas, spleen, and yeast nucleoprotein. By boiling with a weak acid they are decomposed into a protein component and the nucleins which, in turn, upon further boiling, split up into another protein part and the nucleic acids. The nucleoproteins and nucleins only differ, therefore, in the quantity of the protein component which they contain, and since the nucleic acid radical is the carrier of all the phosphorus which is present in the compound, it follows that the nucleins are chemically distinguishable from the nucleoproteins by their larger phosphorus content. The two terms are sometimes used interchangeably. The nucleins are acidic in character, soluble in alkalies, but are precipitated by acids and alcohol. Upon treatment with caustic alkalies, nucleic acids result,—substances which contain phosphorus in the form of phosphoric acid. The

nucleic acids vary according to their content of carbohydrate nucleus, purine and pyrimidine bases.

Impure nucleins may be prepared for study by thorough digestion of a nucleoprotein-containing tissue with pepsin-hydrochloric acid and extraction of the residue with NH_4OH . The nuclein is then precipitated from the solution with dilute HCl .

(a) Try the solubility of the substance in water, dilute alkali and alcohol.

(b) Try three color protein tests.

(c) Boil some with 10 per cent H_2SO_4 for 5 minutes, cool, add an excess of NH_4OH , and finally a few drops of AgNO_3 solution. Of what is the precipitate indicative?

(d) Treat a little of the substance in a crucible with some fusion mixture. Test the fused mass for phosphorus.

DERIVED PROTEINS.

These substances may be subdivided into primary and secondary derivatives according as the hydrolytic change is slight or more deep seated.

1. PRIMARY PROTEIN DERIVATIVES.

(A) *Metaproteins*.—Products of the action of acids and alkalis whereby the protein molecule is so far altered as to form products soluble in very weak acids and alkalis but insoluble in neutral fluids.

ACID AND ALKALI ALBUMINATES.

The albuminates are modified proteins derived from either albumins or globulins by the action of acids and alkalis with the aid of heat. They are non-coagulable by heat except when held in suspension in the solutions from which they have been precipitated by neutralization respectively

with acids or alkalies. In the formation of alkali albuminate neutral sulphur is split off from the protein molecule.

To 25 c.c. of the albumin solution add 5 drops of very dilute HCl and warm for 15 minutes at 40° C. Cool.

(a) *Exactly* neutralize one-half of this solution with very dilute NaOH. What is this precipitate? Shake up the precipitate and divide the solution into two parts. To the first add a drop of NaOH and heat. Heat the other part and then add a drop of NaOH. Explain the differences in results.

(b) Try three characteristic protein tests on the remaining half of the solution.

To 25 c.c. of the albumin solution add 10 drops of NaOH and warm for 15 minutes at 40° C. Cool.

(c) Try the effect of heating some of this solution.

(d) Neutralize the solution and repeat the procedure employed under (a), using HCl instead of NaOH.

(e) Try three characteristic protein tests.

Explain the results.

(B) *Coagulated Proteins*.—Insoluble products which result from (1) action of heat on their solutions, (2) action of alcohols on the protein. Heat coagulation has been considered on p. 21.

2. SECONDARY PROTEIN DERIVATIVES.

Products of a more pronounced hydrolytic cleavage of the protein molecule.

(A) *Proteoses*, see p. 52.

(B) *Peptones*, see p. 53.

(C) *Peptides*.—These are definitely characterized combinations of two or more amino-acids, the carboxyl group of one being united with the amino group of the other with the elimination of a molecule of water. Some of the more complex polypeptides give the biuret reaction.

MUSCULAR TISSUE.

The chief chemical constituents of muscular tissue may be divided as follows:

The *proteins* of which the two most important are myogen, an albumin (not typical), and myosin, a globulin.

The *nitrogenous extractives*, comprising creatine, purine bases, uric acid, carnine, and urea.

The *non-nitrogenous extractives*, made up of glycogen, dextrose, lactic acid, inosit, fats, and inorganic salts.

Reaction.—Test the reaction of living and dead muscle to litmus and Congo-red paper. Explain the differences in the results obtained.

PROTEINS.

MYOGEN.

Place 25 grms. of hashed fresh muscle in a beaker with 75 c.c. of water and allow it to stand, with frequent stirring, for one hour. Strain off the muscle through some cheese-cloth (keep the residue), and filter the solution.

Use this solution for the following tests:

- (a) What is the reaction of the solution?
 - (b) Test the coagulability of the protein in the solution.
 - (c) Perform three color protein tests.
 - (d) Is the protein precipitated by MgSO_4 or $(\text{NH}_4)_2\text{SO}_4$?
- Why is myogen not a typical albumin or globulin?

MYOSIN.

Digest the above meat residue with 100 c.c. of 15 per cent NH_4Cl solution for 24 hours in a covered beaker. Then filter and use the filtrate for the following reactions:

(a) Pour a little of the solution, drop by drop, into a beaker filled with water. Compare this reaction with a similar one under Globulins.

(b) Heat a few c.c. of the filtrate. Filter and test the filtrate for Ca. Is myosin a coagulable protein?

(c) Saturate 10 c.c. of the filtrate with MgSO_4 . Filter and test the filtrate for protein.

(d) Try Adamkiewicz, Biuret, and Xanthoproteic tests.

NITROGENOUS EXTRACTIVES.

These substances, together with the non-nitrogenous extractives, form the chief constituents of a hot-water extract of muscular tissue, such as is known commercially as Liebig's Extract. The most important of the nitrogenous extractives are creatine, xanthine, hypoxanthine, guanine, uric acid, and urea. The latter two are only present in traces. The purine bases represent in all probability an intermediate stage in the nuclear metabolism of the muscle-cell between the nucleins on the one hand and uric acid or urea on the other.

Extract 500 grms. chopped beef with 500 c.c. water for half an hour over the water-bath at 50°C . Strain as dry as possible through muslin and make a second extraction in the

same way, with an equal amount of water (save residue). Unite the two extracts, and after concentration to about 200 c.c. acidify the solution with 2 or 3 drops of acetic acid. Remove the coagulated proteids by filtration, and to the filtrate * add basic lead acetate, *carefully avoiding any excess*; the precipitate consists of phosphates, chlorides, sulphates, etc. Allow this to settle and then filter. Warm the filtrate and pass H_2S through it to remove the excess of lead. Filter hot. The filtrate, which should be water-clear, is then concentrated on a water-bath to a thin syrup. Upon standing several days in a cool place crystals of creatine will deposit. Filter off the crystals, and wash them with 88 per cent alcohol. (Keep the filtrate for the separation of the *purine bases*.)

Place some of the crystals in a small flask with 10 c.c. dilute H_2SO_4 , and heat for half an hour on the water-bath, keeping the volume constant. While still warm add $BaCO_3$, in substance, to neutralization. Filter and evaporate the filtrate to 10 c.c. The creatine has been changed to creatinine. Write the equation. Perform the following tests with the solution:

(a) Place 2 drops of the solution upon a watch-glass and add to it a few drops of an alcoholic solution of $ZnCl_2$; allow it to stand for several days and then examine the crystals under the microscope.

(b) *Weyl's Reaction*.—To 2 c.c. of the creatinine solution add three drops of a freshly prepared dilute solution of sodium nitroprusside. Then add, drop by drop, dilute $NaOH$. A ruby-red color is produced which quickly changes to yellow. If the solution is now acidified with acetic acid

* This filtrate corresponds to Liebig's extract, and if the latter is used for study instead of chopped beef, the procedure of separation is taken up at this point.

and heated, a green color is obtained, and upon continued boiling a precipitate of Prussian blue settles out.

(c) *Jaffe's Reaction*.—Treat some of the solution with a dilute solution of picric acid, and make it faintly alkaline with NaOH. The solution immediately becomes a deep red. Acetone responds to this test, but gives merely a yellowish-red color.

PURINE BASES.

This represents structurally the compound, purine, the nucleus from which all the purine bases may be derived. The structure of the bases is easily obtained by substitution in the nucleus at the position indicated by number.

Hypoxanthine = 6-Oxypurine.

Guanine = 2-Amino-6-oxypurine.

Xanthine = 2,6-Dioxypurine.

Adenine = 6-Aminopurine.

The purine bases in greater part exist as constituent groups in the more complex nucleic acid molecule. In some tissues, however, they have been found in the free state. They form crystalline salts with the mineral acids and are all precipitated from acid solutions by means of phosphotungstic acid. Upon the addition of ammoniacal silver nitrate they separate from their solutions as silver combinations. Copper acetate combines with them to form insoluble compounds.

The separation of the bases from the creatine filtrate (see p. 31) takes place as follows:

After removal of the alcohol, the filtrate is made ammoniacal with NH_4OH and ammoniacal AgNO_3 solution added until no further precipitation occurs. This precipitate is composed of the double silver salts of all the purine bases. These silver combinations, after their removal from the solution by filtration, are dissolved in boiling-hot HNO_3 (sp. gr. 1.1) and the mixture filtered hot.

In this reaction the silver nitrate compounds of the bases are formed, all of which are soluble in the hot nitric acid solution. Upon cooling, the guanine, adenine, and hypoxanthine combinations crystallize out, but the xanthine remains in the solution, from which, after filtration and treatment with NH_4OH in excess, it is thrown down as a reddish precipitate (xanthine silver oxide). This precipitate, suspended in its solution, is next treated with hydrogen sulphide and the mixture warmed and filtered. The xanthine will crystallize out of the filtrate upon concentration.

The precipitate of the three other bases is suspended in water and treated with hydrogen sulphide, warmed and filtered hot in the same manner as the xanthine. After concentration the solution is saturated with NH_4OH and digested on the water-bath. The guanine remains insoluble, while the other two bases pass into solution. Filter the mixture hot. From this filtrate when freed from the ammonia and allowed to cool, the adenine separates, leaving the hypoxanthine in the solution.

(a) Try some of the crystals with murexide test.

(b) Examine the various precipitates for crystals. Make sketches of them.

(c) Warm some of the xanthine crystals with bromine-water and evaporate the solution to dryness on the water-bath. Allow ammonia vapors to come in contact with the residue; this becomes red.

NON-NITROGENOUS EXTRACTIVES.

GLYCOGEN ($C_6H_{10}O_5$)_n.

This polysaccharide, sometimes called animal starch on account of its similarity in function to plant starch, exists wherever living protoplasm is present and is therefore classed as one of the primary constituents of the cell. It is especially abundant in the liver, where it apparently represents the carbohydrate surplus of the organism and in the muscle, where it serves as a ready source of energy. Glycogen forms a tasteless white powder, insoluble in alcohol and ether, but forming with water an opalescent solution which is dextrorotatory. It does not reduce metallic oxides in alkaline solution. By the action of hydrolytic agents or enzymes it is converted into maltose and dextrose similarly to starch.

The common scallop serves as the most convenient and prolific source of glycogen from muscular tissue. By the simple extraction of the tissues with boiling water, slightly acidulated, a solution is obtained for the following reactions:

(a) Notice the color of the solution. Add 2 drops of the iodine solution to 5 c.c. of the glycogen solution. Warm gently and allow to cool. Note changes.

(b) Test 5 c.c. of the solution with Fehling's solution.

(c) To 5 c.c. of the solution add three volumes of 95 per cent alcohol.

(d) To 5 c.c. of the solution add 5 drops of concentrated HCl and boil for some minutes. Neutralize cold with NaOH and test with Fehling's solution. What is this reducing substance? How could you prove it?

(e) To 5 c.c. of the solution add a few drops of filtered saliva, and warm at 40° C. for a few minutes. Notice changes in the solution. Determine the character of the final reducing body formed.

BONE.

Bone consists of an organic matrix, ossein, which is identical with collagen obtained from the white fibrous connective tissue. This matrix is impregnated with insoluble inorganic salts, which serve to give strength and stability to the tissue. Ossein when hydrated with weak acids is converted into gelatin.

MINERAL CONSTITUENTS.

Incinerate 10 grms. of bone under the hood. Extract the grayish residue with 25 c.c. of hot dilute HNO_3 , filter, and use the filtrate for the following tests:

(a) *Phosphoric Acid*.—To 10 c.c. of the solution add 10 c.c. of the molybdic solution and warm in the water-bath at 75°C . until a canary-colored precipitate settles out. What is this? Filter it off on a small filter, wash once with very dilute HNO_3 , and then dissolve the precipitate upon the filter by adding dilute NH_4OH , drop by drop. To the filtrate add a few c.c. of magnesium mixture. What is this precipitate? Write the equations for all the reactions in this procedure.

(b) *Chlorides*.—To a few c.c. of the solution add a few drops of AgNO_3 . What results?

(c) *Calcium*.—Make 10 c.c. of the solution alkaline with NH_4OH . What is the precipitate? Filter, and to the filtrate add ammonium oxalate. The white precipitate of calcium

oxalate is proof of the presence of calcium combined otherwise than with phosphoric acid.

(d) *Magnesium*.—To 15 c.c. of the solution add a slight excess of NH_4OH . Then make the mixture acid with acetic acid; this should dissolve the greater part of the precipitate. The slight insoluble substance is to be filtered off, dissolved in HCl and tested for iron by means of potassium ferrocyanide. The acetic acid filtrate is treated with a few c.c. of ammonium oxalate and the resultant precipitate filtered off. This new filtrate is made alkaline and a few c.c. of a disodium phosphate solution added. Why does a precipitate at this point prove the presence of magnesium? Write the equations.

(e) *Carbonates*.—Did you notice an effervescence when you added HNO_3 after incineration? What was the cause?

NERVOUS TISSUES.

Nervous tissue presents for chemical study the following classes of substances:

1. *Proteins*—two ordinary cell globulins coagulating at 45° C. and 75° C., and a nucleoprotein.

2. *Lipoids*—bodies of a fatty nature, including the lecithins, cerebrosides, and protagon. It is somewhat questionable whether the latter substance is a unit or a mixture of lecithins, cerebrin, and proteins.

3. *Cholesterols* and fatty acids.

4. *Neurokeratin*—a substance apparently related to keratin.

5. *Extractives*—the same as those of muscular tissue.

LIPOIDS.

The lipoids, together with the cholesterols, are usually classed as the myelin bodies from the fact that they exist as a mixture in the so-called myelin substance of the medulla. They contain fatty acid radicals in their molecule, and on this account are closely related to the fats and sometimes are classified under them. For the sake of convenience in isolation for study they will be considered here, although it must not be forgotten that the lecithins are widely distributed in *all* forms of living tissues and belong to the primary con-

stituents of cells in general. The cerebrosides are found in plant as well as animal tissues. So little that is positive is known about "protagon" that no notice need be taken of it.

LECITHINS.

R and R' = the same or different fatty acid radicals.

The structure of the lecithin molecule is proved by the decomposition products which are obtained from it, viz., choline, glycerophosphoric acid, and fatty acids. From the fact that various fatty acid radicals enter into the composition of the molecule and that the nitrogenous complex may also differ, it follows that the existence of many lecithins is possible. As a class they are soluble in ether and alcohol and from such solutions may be precipitated by cooling to 0° C. or by the addition of acetone. The lecithins combine with acids and bases as well as with proteins and other bodies to form more or less loose combinations, such as the lecithoproteins. With water they swell up, giving off long filaments (myelin forms), and finally pass into an emulsion.

Various lecithins can be separated from the yolk of the egg, from yeast, or from brain-tissue. The procedure for the isolation of the brain-lecithins is as follows: The finely divided brain-tissue, which may or may not have been dehydrated by boiling with acetone for some hours previously, is

allowed to stand for three days in cold ether. The solution is then filtered, and to the filtrate, which contains the lecithins and cholesterol, acetone is added. The precipitated lecithins may be removed by filtration and the ether-acetone filtrate evaporated to dryness. (Keep this cholesterol.)

Try the following reactions with the lecithin:

(a) Place a particle of the substance on a glass slide and add a drop or two of water. Examine this under the microscope.

(b) Shake some of the substance in water and examine microscopically.

(c) To some of the substance add a drop of osmic acid. What is the effect?

(d) Test the substance for phosphorus by fusion.

(e) Test for the glycerol radical.

CEREBROSIDES.

This class of non-phosphorized nitrogenous substances, whose exact composition is not known, is characterized by a glucoside constitution yielding upon decomposition a sugar which is identical with galactose. Fatty acids in relatively large quantities can also be isolated from the cleavage products.

CEREBRIN.

This is the most important substance in this group. Whether it exists uncombined in the tissue is still an undecided question. It is insoluble in water, dilute alkalis, or baryta-water. It dissolves in boiling alcohol, from which solution it separates as a flaky precipitate upon cooling. This precipitate is made up of microscopic globules.

Cerebrin is usually prepared according to the following procedure: A small amount of the finely divided brain-tissue is boiled for one-half hour in a casserole with twice its weight of baryta-water. The solution is filtered off and the residue extracted with boiling 95 per cent alcohol. From this extract, if filtered while still hot, the cerebrin will separate in small crystals. The cholesterol should stay in the alcoholic solution, but if any becomes insoluble with the cerebrin it is easily removed by washing the precipitate with ether in the filter.

Test the substance as follows:

(a) Try to detect the presence of nitrogen and phosphorus.

(b) Boil some of the substance for one hour with 5 per cent H_2SO_4 . After cooling the mixture, neutralize it and test for a reducing action on Fehling's solution.

(c) Warm some of the substance on a platinum foil. Notice the odor. To what is it similar?

THE CHOLESTEROLS.

These bodies belong to the class of primary cell constituents. They are studied here on account of the existence of suitable material at hand. Cholesterol usually exists free, as in the case of the brain-tissue and gall-stones, but it is also found in combination with fatty acids, as esters in the blood-plasma. The best known cholesterol is a monatomic alcohol with $\text{C}_{27}\text{H}_{45}\text{OH}$ as the empirical formula. At one time it was termed a "non-saponifiable" fat. It has the general solubilities of the fats and crystallizes readily from an alcohol or ether solution in the form of superimposed

rhombic plates. The bile-acids also have a solvent action on cholesterol.

Make use of the cholesterol prepared in the course of the separation of lecithin (p. 39).

(a) Try the solubility of the substance in water.

(b) *Salkowski's Test*.—Dissolve a small quantity of the cholesterol in a few c.c. of chloroform and add an equal volume of concentrated H_2SO_4 . The acid solution takes on a greenish fluorescence and the chloroform becomes red.

(c) *Liebermann's Test*.—Dissolve a crystal of cholesterol in about 2 c.c. of chloroform and add first 10 drops of acetic anhydride and then concentrated H_2SO_4 , drop by drop. The mixture will become red, then blue, and finally green.

SALIVARY DIGESTION.

As usually obtained for study, the saliva forms a mixture of the secretion of the submaxillary, sublingual, parotid, and the mucous glands of the mouth. It presents a viscid, slightly opalescent appearance, possessing either alkaline or acid reaction according to the indicator employed, and containing about 0.5-1 per cent solids. Besides the proteins and the potassium sulphocyanide, its chief and essential organic constituent is the enzyme, ptyalin, which gives to the secretion its importance in the digestion of carbohydrates. A physical function of the saliva consists in rendering the food moist, which action, in conjunction with the presence of the mucin, allows the food-bolus to be more readily swallowed.

Chemical Characteristics.

Collect about 50 c.c. of filtered saliva.

(a) Test its reaction with litmus paper and phenolphthaleïn. What causes this apparent discrepancy?

(b) To a few c.c. of saliva add acetic acid, drop by drop, until a precipitate forms. (See under Mucin.) Filter off the mucin and test the filtrate for protein. What is the result?

(c) Allow a drop of saliva to fall in the centre of a piece of filter-paper. Then add a drop of ferric chloride to the paper where it is moist. Note color. Now add a drop of

mercuric chloride. What is the result? For what substance are these tests?

PTYALIN.

This substance belongs to the class of amylolytic enzymes in that its function consists in the conversion of starch and glycogen into maltose. Its action is maximum at 46° C. and is destroyed at 65–70° C. Alkalies and free acids tend to retard its activity. Apparently it acts best in a neutral solution and in the presence of proteins with which the acids or alkalies may combine; in this way a greater degree of acidity or of alkalinity may be borne without injury than in a pure solvent. This is important to remember when considering the possibility of the continuance of saliva digestion in the stomach.

AMYLOLYSIS.

The amylolytic action of ptyalin on starch is a hydrolytic change in which successive products are evolved until the final stage of maltose or isomaltose is reached. The end product of a similar cleavage by means of weak acids is dextrose. Thus from starch is formed successively soluble starch or amygdulin, then erythrodextrin, the achroodextrins, maltodextrin and maltose, and, in case of acids, dextrose. There are indications that at each step of the cleavage small quantities of maltose are produced. These facts can be shown by the following experiment:

(a) Prepare some starch paste and allow it to cool to 40° C. Dilute the saliva five times and regulate the water-bath for 40° C. Take 15 c.c of the paste; add to it 1 c.c. of the diluted saliva and place the test-tube in the water-bath. Watch the mixture until the turbidity disappears and the solution becomes transparent. Then pour out into a clean

test-tube a few c.c. of the solution and add a drop of iodine solution. What color is obtained? Replace the mixture in the bath and as the amylolysis proceeds remove successive portions (2 c.c.) and test with the iodine solution. What successive variations in color do you obtain? Finally the iodine fails to give a color to the solution. This is called the achromic point; why? Test the remainder of the solution with Fehling's solution. What is the character of the sugar present? What is the cause of these changes indicated by the iodine reaction? Make a scheme of the successive hydrolytic products formed in the digestion.

Influence of Conditions upon the Activity of Ptyalin.

(b) Make up the following mixtures in separate test-tubes and keep them in the water-bath at 40° C., using in each case 5 c.c. of the diluted saliva and 10 c.c. of the starch paste.

- (1) Starch paste + saliva.
- (2) Starch paste + saliva first boiled and then cooled.
- (3) Starch paste + saliva exactly neutralized with 0.2% HCl.
- (4) Starch paste + saliva made acid with 0.2% HCl.
- (5) Starch paste + saliva made alkaline with 0.5% Na_2CO_3 .

In all these test-tubes note carefully the changes which are taking place from time to time, testing the rapidity of digestion by means of the iodine reaction. Determine the varying lengths of time necessary for the achromic point to be reached.

After some time it will be noticed that in experiments (4) and (5) the starch paste has not changed; change the reaction in each to that corresponding to the normal saliva and again allow them to digest. What result?

Make deductions from these test-tube experiments.

GASTRIC DIGESTION.

Pure mixed gastric juice such as is obtained from a gastric fistula forms a clear, colorless, and odorless solution with an acid reaction and low specific gravity (1.004). The total solids amount to only 2 per cent, of which the following are the chief.

Inorganic: hydrochloric acid, the chlorides of sodium, potassium, and calcium, with traces of the phosphates of magnesium and iron.

Organic: pepsin, rennin or chymosin, mucin, traces of other proteins, and sometimes lactic acid.

HYDROCHLORIC ACID.

In all probability the most important function of the hydrochloric acid is that of a germicide preventing putrefaction and the formation of obnoxious gases. Secondly, it gives to the gastric contents a more favorable condition for the action of the pepsin, although the establishment of an acid reaction is not absolutely necessary for peptic digestion. The hydrochloric acid also seems to possess some power of inverting cane-sugar.

The following experiments are intended to simulate possible gastric mixtures which may be encountered in testing stomach contents for HCl. The acid may appear alone or together with lactic acid and digestive products. The reliability and sensitiveness of the various indicators employed under the different conditions will also be shown.

Test each of the following solutions with all the indicators mentioned below, and *tabulate* the results whether positive or negative.

(a) 0.3 per cent HCl; (b) 0.05 per cent HCl; (c) 0.8 per cent lactic acid; (d) a mixture containing equal volumes of "a" and "c"; (e) a mixture containing equal volumes of "b" and a 2 per cent albumose solution. Before using, warm the last mixture for a few minutes at 40° C.

Indicators.

An indicator is a substance which possesses a different color when dissolved in an alkaline solution from what it does in an acid one. They are slightly dissociable acids or bases, and the change in color is due to the appearance or disappearance of colored ions.

1. *Dimethylaminoazobenzene*, $\text{N}(\text{CH}_3)_2 - \text{C}_6\text{H}_4 - \text{N} = \text{N} - \text{C}_6\text{H}_5$.

Add one or two drops *directly* to the solution to be tested. Free mineral acid is indicated by a carmine-red color.

2. *TropæolinOO*, $\text{NH}(\text{C}_6\text{H}_5) - \text{C}_6\text{H}_4 - \text{N} = \text{N} - \text{C}_6\text{H}_4 - \text{SO}_3\text{Na}$. Add one or two drops *directly* to the solution to be tested. Free acid is indicated by a red or reddish-violet color. The reaction becomes more delicate when performed in a similar manner to that suggested for Günzburg's reagent.

3. *Congo-red*.—Use Congo-red paper, prepared by dipping filter-paper into the alkaline indicator solution and drying. Free acid is indicated by the blue color.

4. *Günzburg's Reagent*.—Evaporate 2 drops of the solution to be tested with 1 drop of the indicator, in a porcelain dish, *carefully*, over a water-bath. Upon dryness the presence of free HCl is indicated by the development of a rose-red color.

5. *Boas' Reagent*.—Mix 3 drops of the solution to be tested with the same amount of indicator and evaporate cautiously

in a porcelain dish. Free acid is shown by a rose or vermilion color.

Add 2 or 3 drops of the indicator directly to the solution.

The color will be yellow in acid and acid salt solutions, but red to violet in alkaline. This indicator is especially suited for the determination of the acidity of the urine by titration.

Add 4 drops of the indicator directly to the solution.

The indicator is colorless in neutral and acid reactions, but becomes red in the presence of alkalies. It shows the presence of free, combined, mineral, and organic acids, and acid salts of all kinds. It therefore gives the total acidity. It may indicate an acid reaction when dimethylaminoazobenzene shows alkaline. Explain this.

The Inverting Action of HCl.

To 5 c.c. of a 5 per cent cane-sugar solution add 5 c.c. of 1 per cent HCl and place the mixture in the water-bath for an hour at 40° C. At the end of this time cool, neutralize, and test the reducing power of the mixture. Explain the result.

Although this substance is commonly considered as an abnormal constituent of the stomach contents it may exist

as a normal product formed during the digestion of a meal rich in carbohydrates.

The following experiments are designed to show the ordinary tests for lactic acid and the effect upon the reliability of these tests, of the simultaneous presence of bodies likely to be found in a stomach contents.

(a) To successive portions of 5 c.c. of *Uffelmann's reagent* add a few drops of solutions *a*, *c*, and *d*, under Hydrochloric Acid. Note carefully color changes and make deductions.

Make a very dilute solution of Fe_2Cl_6 in which the yellow color is hardly visible. Such a reagent is much more sensitive than Uffelmann's. Use 5 c.c. of the dilute ferric chloride solution in testing each of the following:

(b) Solutions *a*, *c*, and *d* (under HCl).

(c) A solution of H_2NaPO_4 .

(d) Alcohol, 5 per cent.

(e) A 1 per cent solution of saccharose; glucose.

Make deductions as to the value of the test applied *directly* to gastric contents.

In order that all chances for error may be avoided, lactic acid can be easily separated from disturbing conditions by shaking the stomach contents or gastric juice with ether in which the acid is soluble. Such an ether extract is evaporated carefully on the water-bath, the residue taken up with water and tested with the dilute Fe_2Cl_6 solution.

As this ether extract cannot contain any of the above substances, the presence of which in the stomach contents might interfere with a correct diagnosis, a positive test for lactic acid in this case is decisive evidence of its presence.

PEPSIN AND PEPSINOGEN.

Pepsin is apparently not secreted as such, but appears first in the gastric mucosa in an antecedent form, or zymogen,

pepsinogen. This latter substance shows considerable resistance to reagents (dilute alkalies) which destroy pepsin. Pepsinogen is itself inactive proteolytically, but seems to be converted into active pepsin by the action of the HCl of the gastric juice. Pepsin belongs to the proteolytic enzymes, converting proteins into proteoses, peptones, amino acids, and diamines. The temperature of its optimum activity is from 35–45° C. It is destroyed at 65° C.

Pepsin is only active in acid solutions, and the amount of acidity required for its maximum activity varies according to the character of the acid employed and the form of protein to be digested. Pepsin is easily destroyed by weak alkalies (0.01 per cent), and less readily by strong acids (5–10 per cent).

The following set of experiments should prove these facts:

1. A glycerol extract of a pig's gastric mucosa contains *pepsinogen*.
2. A 0.2% HCl extract of a pig's gastric mucosa contains *pepsin HCl*.
3. A watery extract of a pig's gastric mucosa contains *pepsin*.

Make use of the above extracts numbered 1, 2, and 3 respectively, and in each test-tube add a piece of fibrin, keeping all at 40° C. in the water-bath.

- (a) Fibrin + 5 c.c. of 0.2% HCl.
- (b) " + 5 c.c. of solution 3.
- (c) " + one drop of solution 1 + 5 c.c. of water.
- (d) " + 5 c.c. of solution 2.
- (e) " + one drop of solution 1 + 5 c.c. of 0.2% HCl.
- (f) " + 5 c.c. of solution 2 (the latter having previously been heated to boiling and again cooled).
- (g) Fibrin + one drop of solution 1 (the latter having

previously been heated to boiling with 5 c.c. water and again cooled) + 5 c.c. of 0.2% HCl.

(h) Fibrin + 5 c.c. of solution 3 + 5 c.c. of 0.8% lactic acid.

(k) Fibrin + 5 c.c. of solution 3 + 5 c.c. of 1% oxalic acid.

(m) " + 5 " " " 3 + 5 " " 5% HCl.

(n) " + 5 " " " 3 + 5 " " 0.5% Na_2CO_3 .

(p) " + 5 " " " 3 + 3 " " bile.

Note *carefully*, in each case, the relative rapidity with which the flock of fibrin is disintegrated.

PEPTIC PROTEOLYSIS.

The previous experiments have indicated that the action of pepsin is directed toward the transformation of the proteins by a process of cleavage into soluble and diffusible products. The question concerning the characterization, separation, and identification of these soluble digestive products appears at present to rest in a state of uncertainty, but in a general way they are conventionally divided into the proteoses, peptones, and a mixture of nitrogenous substances ("amino" bodies) only characterized by not giving the biuret reaction. The current method for the separation of these bodies is based, first, upon the precipitation of the proteoses by complete saturation of their solution with $(\text{NH}_4)_2\text{SO}_4$, and, second, upon the precipitation of the peptones in the filtrate (from the proteoses) by means of iodo-potassium iodide. The remaining substances are removed from their solution by the addition of phosphotungstic acid. The proteoses allow of a further separation by means of fractional precipitation with $(\text{NH}_4)_2\text{SO}_4$, and the peptones are divided into two fractions by 95 per cent alcohol. This method isolates

three fractions of proteoses and two of peptones, the procedure for which is the following:

For the purposes of study, a pepsin-hydrochloric acid digestion of meat or fibrin should be prepared, using 0.3% HCl and 0.5 grm. pepsin per liter (Grubler's purissimum or Parke-Davis' scale pepsin). Allow the digestion to proceed at 40° C. for three days. After filtration of the digestion mixture and *exact neutralization* with dilute NaOH the solution is ready for use. Heat a little of it to boiling to show that no coagulable proteins exist in the mixture.

To a given quantity of the digestion mixture add an equal volume of a saturated solution of $(\text{NH}_4)_2\text{SO}_4$. Stir the solutions together and allow the mixture to stand until the resulting precipitate has settled sufficiently to allow of considerable decantation. Filter the remainder. This precipitate obtained by *half saturation of the mixture* represents

Fraction I.—It may be further separated into two parts: one soluble in 95 per cent alcohol—*protoproteose*, and the other remaining insoluble—*heteroproteose*.

In the constitution of *protoproteose* the aromatic groups predominate, while in *heteroproteose* the fatty acid radicals are chiefly found.

To the filtrate from *Fraction I* add one-half its volume of a saturated solution of $(\text{NH}_4)_2\text{SO}_4$. Allow this to stand and proceed as before. This second precipitate obtained by *two-thirds saturation* corresponds to

Fraction II.—It consists of *Deuteroproteose. A* and contains the greater part of the lead-blackening sulphur which was present in the original proteid molecule. On this account it may be termed *Thioproteose*.

Completely saturate the filtrate from *Fraction II* with

$(\text{NH}_4)_2\text{SO}_4$ (in substance), allow the mixture to stand, and treat as previously.

Fraction III.—This precipitate is termed *Deuteroproteose B* or *Synproteose*. It contains the greater part of the carbohydrate nucleus of the original protein molecule and is, therefore, sometimes called *Glycoproteose*.

To the filtrate from *Fraction III* add $\frac{1}{10}$ its volume of a $\frac{\text{N}}{10}$ H_2SO_4 solution, which is saturated with $(\text{NH}_4)_2\text{SO}_4$. Proceed with the precipitation and filtration as before.

Fraction IV.—This fraction, which in the case of some proteins is absent, is designated as *Deuteroproteose C*.

The acid-saturated filtrate from *Fraction IV* is treated with a solution of iodo-potassium iodide (saturated with $(\text{NH}_4)_2\text{SO}_4$) until no further precipitate results. This is allowed to settle and is then filtered off. This precipitate upon treatment with 95 per cent alcohol subdivides into a soluble and insoluble fraction. If the insoluble residue is filtered off it forms

Fraction V, denoted as Peptone A; and

Fraction VI, which consists of the alcoholic filtrate from *Fraction V*, is called Peptone B.

Very little is known regarding the character of these two peptone fractions. Undoubtedly they contain peptides of varying complexity mixed with amino acids. *Fraction V* gives the biuret reaction while *Fraction VI* does not; hence the former fraction must possess the most complex polypeptides.

The “amino” bodies are precipitable with phosphotungstic acid and remain in greater part in the filtrate subsequent to the treatment with iodo-potassium iodide.

More or less of *acid albuminate* may be formed during the initial stage of the digestion (first half-hour), but this quickly disappears and apparently does not participate in the future formation of the proteoses, peptones, and "amino" bodies.

Perform the following tests with the different fractions:

- (a) Try the biuret reaction on each fraction.
- (b) Test *Fractions I* and *II* for lead-blackening sulphur.
- (c) Boil *Fraction III* with 10 per cent H_2SO_4 for an hour. Cool, neutralize, and test the mixture with Fehling's solution.
- (d) Show, first, that the solutions of *Fractions I, II, III, IV* are precipitated by nitric, picric, and trichloroacetic acids, and that the precipitates so produced disappear when heat is applied and reappear upon cooling; and, second, that they are non-coagulable by heat and respond to the acetic acid and potassium ferrocyanide reaction.
- (e) Prove that the peptones (*Fractions V* and *VI*) are not precipitated by nitric or trichloroacetic acids; that Millon's and xanthoproteic reactions give indecisive results; that lead-blackening sulphur is absent; and that both tannic and picric acids combine to form insoluble bodies.

It must be finally emphasized that these various fractions are not to be considered as unit substances. The method of separation just outlined simply attempts to isolate certain mixtures ("fractions") each of which contains in greater part some characteristic component group or complex broken off during the peptic cleavage of the original protein molecule. Thus in Deuteroproteose A the lead-blackening or cystine nucleus predominates; while Deuteroproteose B is characterized by containing the larger part of the carbohydrate complex.

RENNIN OR CHYMOSIN.

This body is classed as a coagulating enzyme acting upon caseinogen with the formation of an albumose-like body and soluble casein. The latter in the presence of calcium salts is precipitated in the form of a coagulum or curd which is termed (insoluble) casein.

Rennin is affected by varying conditions (temperature, reaction, etc.) in a way similar to pepsin.

Make use of the rennin solution given and prepare five test-tube mixtures as follows, using in each case about 5 c.c. of milk and holding them at 40° C. for fifteen minutes; in each experiment add the rennin solution last.

(a) Milk + 15 drops of 0.3% HCl.

(b) " + 1 c.c. of rennin solution.

(c) " + 1 " " " " " + 2 cc. ammonium oxalate.

(d) Milk + 2 c.c. of rennin solution + 5 drops 0.3% HCl.

(e) Milk + 2 c.c. of rennin solution + 10 drops 0.5% Na_2CO_3 .

Note the results carefully. If (c) has not clotted, heat it to boiling to kill the enzyme. Cool and add a few drops of calcium chloride solution. What is this precipitate which settles out? What are the effects of acids and alkalis on the milk alone? Why did not the milk clot in experiment c?

PANCREATIC DIGESTION.

The composition and character of pancreatic juice varies greatly under different conditions. Thus far it has been practically impossible to collect the juice in a manner which will permit of the assumption that it is comparable to that secreted into the intestine.

The juice as obtained from a temporary fistula is clear, viscous, and alkaline in reaction, the alkalinity corresponding to about a 0.5 per cent Na_2CO_3 solution. It differs from the gastric juice in being rich in proteins, having a sp. gr. of about 1.030. The character and quantity of the enzymes which may be present in the fluid varies considerably according to the nature of the diet.

The following have been shown to exist in different extracts of the gland and in the secretion itself at certain times.

1. *Trypsin*—a proteolytic enzyme appearing first in the gland as a zymogen, trypsinogen, entirely comparable to pepsinogen.

2. *Amylopsin* or pancreatic diastase—an amylolytic enzyme almost identical with ptyalin.

3. *Steapsin* or lipase—a lipolytic enzyme probably not characteristic of the pancreatic juice.

4. *Pancreatic rennin*—a coagulating enzyme acting on the caseinogen of milk in a way somewhat similar to the gastric rennin. Various extracts of the pancreatic gland

have been prepared each with a view to obtaining predominating reactions for the specific enzyme. (See Appendix.)

TRYPSIN AND TRYPSINOGEN.

Trypsin is active in an alkaline, faintly acid or neutral solution, but its maximum reaction occurs in a 1 per cent solution of Na_2CO_3 . It is killed at a temperature about 55°C .

Use the proteolytically active pancreatic extract and prepare test-tube digestions in the water-bath at 40°C ., as under gastric digestion.

(a) Fibrin + 5 c.c. of the pancreatic extract.

(b) " + 5 " " " " " " + 2 c.c. 0.5% Na_2CO_3 .

Compare the manner of action of the trypsin upon the fibrin to that of the pepsin.

(c) Fibrin + 5 c.c. of 0.5% Na_2CO_3 .

(d) " + 5 " " " " " " + 5 c.c. pancreatic extract which has been previously boiled and cooled.

TRYPTIC PROTEOLYSIS.

As regards its proteolytic activity, the enzyme trypsin is more energetic and far-reaching than pepsin. Consequently in pancreatic digestion there takes place a more rapid and deep-seated cleavage of the protein molecule than is possible in the stomach. Proteoses and peptones are formed in the middle stages of the digestion, but these are rapidly carried over into substances with a relatively small molecular weight which do not give the biuret reaction and consequently cannot be termed peptones. It is now considered that the end products of a long-continued tryptic digestion of the ordinary proteins comprise the amino-acids,

viz., leucine, tyrosine, aspartic and glutamic acids; the hexone bases, viz., arginine, lysine, and histidine; the so-called tryptophane or proteinochromogen, which is an indole derivative; and probably other bodies whose nature is not well understood.

Prepare a pancreatic digestion as follows:

Place in a large flask (5-10 liters) 500-1000 grms. of fibrin, a liter of an infusion of Kühne's dried pancreas, and 1000-2000 c.c. of 0.25 per cent Na_2CO_3 ; add plenty of chloroform or powdered thymol, and allow the mixture to digest at 40°C . for at least a week.

At the end of this time the solution is exactly neutralized with dilute H_2SO_4 and concentrated to about one-half its volume; it is then filtered. From the filtrate the proteoses are removed by saturation with $(\text{NH}_4)_2\text{SO}_4$ in a nearly boiling solution. These are filtered off and the filtrate still further concentrated. During this procedure relatively large quantities of leucine and tyrosine should crystallize out of the mixture along with the $(\text{NH}_4)_2\text{SO}_4$. These must be filtered off and the $(\text{NH}_4)_2\text{SO}_4$ removed from the filtrate by means of $\text{Ba}(\text{OH})_2$ and BaCO_3 . The BaSO_4 is again removed by filtration and to the new filtrate dilute H_2SO_4 added to just precipitate the excess of Ba in the solution. After filtration the solution will contain the hexone bases.

Compare the quantities of proteoses and peptones obtained in this digestion with those from the peptic proteolysis.

Use the crystals obtained in the pancreatic digestion.

(a) Examine them under the microscope.

(b) Try to dissolve some in cold, then warm water. Then

add about 5 c.c. of Millon's reagent to the test-tube and heat. Why is this result positive?

(c) *Piria's Test*.—Place a few of the tyrosine crystals upon a small watch-glass with two drops of concentrated H_2SO_4 and warm for half an hour on the water-bath. Then transfer it with about 15 c.c. water to a test-tube and neutralize with BaCO_3 in substance. Filter and add a few drops of weak ferric chloride solution (neutral). A positive test is evidenced by the formation of a violet color.

(d) *Mörner's Test*.—To a little of the powder placed in a test-tube add a few c.c. of *Mörner's reagent*, and heat carefully to boiling. A green color develops which is quite lasting.

Make use of the substance obtained from the pancreatic digestion.

(a) Examine some crystals under the microscope.

(b) Place a little in a clean dry test-tube and warm carefully. The leucine sublimes, without melting, on the cold parts of the tube. If the powder is heated too high or suddenly, the substance is decomposed and the odor of amylamine is given off. Write the equation for the formation of this substance.

(c) *Scherer's Test*.—Heat a little leucine with two drops of concentrated HNO_3 upon a platinum foil over a free flame until a colorless residue is obtained. Then add two drops of NaOH and evaporate carefully in the flame. The mass becomes dark red in color and rolls around on the foil like an oil-drop.

AMYLOLYSIS.

Amylopsin is stronger in its action than ptyalin; otherwise the conditions which influence its activity are practically the same as the salivary diastase.

Make use of the glycerol or alcoholic extract of the pancreas.

To 10 c.c. of starch paste add 5 c.c. of pancreatic extract and place in the water-bath at 40° C. Test from time to time with the iodine solution as you did under Salivary Digestion. Do you get an achromic point? Finally test for a reducing sugar. What is the character of this?

LIPOLYSIS.

Steapsin is the least stable of all the pancreatic enzymes, and is particularly sensitive to the action of acids, being destroyed easily by all except the higher fatty acids.

Its action on neutral fats tends toward a breaking down of the fat molecule into fatty acids and glycerol.

For the following experiments use the alkaline glycerol extract of the gland.

(a) To 10 c.c. of "litmus milk" add 5 c.c. of the pancreatic extract. Place the test-tube in the water-bath at 40° C. What is the cause of the change which takes place?

(b) Repeat experiment (a) with the exception that *boiled* pancreatic extract is used. Note any change?

(c) Instead of "litmus milk," try the experiment with neutral ethyl butyrate. What is the equation for the reaction which takes place?

PANCREATIC RENNIN.

The ability of pancreatic juice to cause the clotting of milk has long been known; but only of late has the action been ascribed to a definite enzyme, which, on account of the similarity in its function to the milk-clotting enzyme of the stomach, has been called pancreatic rennin. This substance also possesses the power of bringing about a peculiar changed condition of the caseinogen of the milk by which the protein is rendered liable to coagulation. The coagulable protein is termed *metacasein*, and its appearance is denoted as the *metacasein reaction*. Metacasein is apparently an intermediate stage in the action of the pancreatic rennin upon caseinogen, in which pancreatic casein is the end product. Pancreatic casein differs in some slight degree from true casein.

(a) To 5 c.c. of milk add 1 c.c. of the pancreatic extract and place the mixture at 40° C. Note the formation of the clot.

(b) To 10 c.c. of the milk add 1 c.c. of the pancreatic extract diluted with 2 volumes of water. Place the test-tube at 40° C. and from time to time remove portions (1 c.c.) of the mixture and heat each to boiling. In one of them coagulation in fine flakes will occur. Note the time from the beginning of the experiment.

(c) Make an *exact* duplicate of the preceding experiment, and at the time at which the appearance of the metacasein reaction would be expected, judging from the last experiment, take out the tube from the bath. Examine a drop under the microscope. Stand the tube in a cool place and note the formation of a clot. This will dissolve when replaced in the water-bath.

INTESTINAL PUTREFACTION.

The chief interest in the study of the effects produced by bacteria upon protein matter lies in the direction of the additional insight which can be obtained into the constitution of the protein molecule. This becomes possible since the microorganisms effect a cleavage of the molecule which, while similar in character to that exerted by the digestive enzyme, is more vigorous and deep-seated than even trypsin itself; and the products obtained are so simple in composition and structure that they have yielded easily to investigation.

The most important substances which result from bacterial decomposition of proteins may be divided as follows:

1. Tyrosine and its derivatives, the phenols, aromatic acids, and oxy-acids (e.g., oxyphenylpropionic and oxyphenylacetic acids).
2. Indole and its derivatives, skatole, skatole-carbonic acid, and tryptophane.
3. Fatty acids and derivatives, leucine, etc.
4. Sulphur derivatives and gases, hydrogen sulphide, methyl mercaptan, CH_4 , CO_2 , and NH_3 .
5. Proteoses and "peptones" are present in small amounts as intermediary products.

The following method is employed for the preparation and separation of the various products. It lends itself also for demonstration purposes, and the different distillates can be studied individually.

Prepare a mixture of lean chopped beef and coagulated egg-white in equal amounts, and add thereto about 1 liter of water to each pound of material employed.

If cultures of colon bacillus are available, sterilize the flask and its contents and add some of the culture. Otherwise make the mixture alkaline (add to each liter 100 c.c. of 10% Na_2CO_3), and inoculate with some meat which has been allowed to putrefy. After shaking thoroughly stop the flask with a bored cork which is provided with a glass tube to which is attached a valve (wash-bottle) of mercuric cyanide (3 per cent). This serves to collect the methyl mercaptan and to render the flask less odorous. Allow the flask to remain at 40°C . for two to three weeks. The separation of the products takes place as follows:

Distil the putrefactive mixture with steam until the distillate equals one-half the volume of the original mixture. There is thus provided, 1, a distillate (A); and, 2, a distillation residue (A), which will be treated separately.

Distillate A.

Acidify with weak HCl and redistil (usually one-half volume).

Distillate B.

Make alkaline with KOH and again distil.

Distillation-residue B.
(NH_4Cl .)

Distillate C.

Keep the first few c.c. of this distillation separate from the rest (Indole and Skatole).

Distillation-residue C.
Saturate with CO_2 gas and redistil.

Distillate D.
(Phenols.)

Distillation-residue D.
(Fatty acids.)

Distillation-residue B.

Upon evaporation this can be shown to contain NH_4Cl formed from the NH_3 of the putrefaction product and the HCl added in the manipulation.

Distillate C.

Contains indole and skatole (see Conjugate Sulphates).

The first distillation carries over skatole; this may be tested as follows:

(a) To 2 c.c. add a drop of HNO_3 and a drop or two of KNO_2 solution (2 per cent). Notice the turbidity.

(b) Add some concentrated HCl to some of the distillate.

Try the succeeding tests with the main distillate (*indole*).

(a) Moisten a piece of pine wood with concentrated HCl and dip it into the distillate. A cherry-red color appears on the wood.

(b) *Legal's reaction*.—To a few c.c. of the distillate add 5 drops of a freshly prepared solution of sodium nitroprusside; then make it alkaline with 2 drops of NaOH . A violet color results, which resolves itself into deep blue upon acidification with glacial acetic acid.

(c) Acidify the distillate with HNO_3 and add 2 drops of KNO_2 solution (see Skatole). A red precipitate of nitroso-indole nitrate results.

Distillate D.

Contains phenol and *p.cresol*. (Make *very faintly* alkaline with KOH and concentrate to a small volume.)

For the reactions of phenol see p. 96.

p.cresol gives with ferric chloride solution a dirty-green coloration.

Distillation-residue D.

Acidify this with concentrated HCl and shake it out with a small amount of ether. Upon evaporation of the ether extract, the volatile fatty acids remain.

Distillation-residue A.

Concentrate and filter; then extract with ether.

Filtrate A.

This contains *p*-oxyphenylacetic acid, *p*-oxyphenylpropionic acid, and skatole-carbonic acid.

(a) Try the ferric chloride reaction (see Phenol).

(b) Try Millon's reaction. Which of the three substances are these tests for?

(c) Perform test (c) under Indole and compare the resulting colors and precipitates with those for indole and skatole.

Residue.

See Fatty Acids for tests.

Crystalline Precipitate.

See Leucine and Tyrosine.

Filtrate B.

See Proteoses and "Peptones."

Tryptophane, indoleaminopropionic acid (proteinochrome). This substance is formed as one of the products in the pronounced cleavage of proteins as in tryptic digestion and putrefaction. Its presence in the protein molecule is of considerable importance to the animal economy. It is supposed to stand as the mother substance of the various pigments of the body. Add a few c.c. of chlorine or bromine water to the solution; a reddish-violet precipitate results (proteinochrome).

Finally examine the mercuric cyanide valve which was attached to the flask containing the putrefactive mixture.

Hydrogen sulphide has produced a black precipitate of HgS.

Methyl mercaptan is indicated by the presence of a grayish-green precipitate.

BILE.

The function of the bile is mainly excretory in character, consisting in the removal by way of the intestine of various relatively insoluble substances, such as cholesterol and lecithin. Secondly, it exerts an emulsifying power upon the fats, and fat absorption is markedly decreased in its absence. In this sense it may be classed under the digestive juices.

Bile as it is secreted by the liver forms a clear, limpid solution of a color either yellowish-red, brown, or green according to the species of animal from which it is obtained. It reacts alkaline to litmus, but acid to phenolphthaleïn and possesses a decidedly bitter taste and an odor of musk. By its stay in the gall-bladder the character of the fluid is changed considerably. Absorption of water and admixture of mucus-like substances derived from the walls of the bladder increase the specific gravity from 1.010 to 1.035. When taken from the gall-bladder, bile, therefore, presents a ropy, viscous appearance and contains about 10 per cent of solids. Bile holds the following substances in solution: the salts of the bile-acids, the bile-pigments, mucin or phosphoprotein, cholesterol, lecithin, inorganic salts, and traces of fat, soaps, and urea.

Note the color, consistency, and reaction of the samples presented.

Notice the differences in color between the ox and dog bile.

Use the diluted bile for the following reactions:

(a) Add acetic acid drop by drop to 5 c.c. of the bile. Is this precipitate mucin? How could you prove it? Filter and test the filtrate for protein. What is the most suitable test?

(b) To 1 c.c. of olive-oil add 10 c.c. of water and shake thoroughly. Allow it to stand in the rack.

(c) To 1 c.c. of olive-oil add 10 c.c. of diluted bile. Allow this to stand. Compare the permanency of the emulsion.

(d) To 10 c.c. of a gastric digestion mixture add diluted bile, drop by drop. What is this precipitate and its significance?

CONJUGATE BILE ACIDS.

These are present as the sodium salts of taurocholic and glycocholic acids, and are formed by the combination of cholalic acid with *taurin* and *glycocoll* respectively. Taurocholic acid, by virtue of the taurin, contains sulphur and has a solvent action on the more insoluble glycocholic acid. It is more abundant in the bile of carnivora, while glycocholic acid predominates in that of man and herbivora. Together they tend toward rendering the cholesterol and lecithin more soluble and exert a distinctly inhibitory action on the heart, slowing its rhythm.

Pettenkofer's Test.—Place 5 c.c. of concentrated H_2SO_4 in a clean dry test-tube. In another test-tube place 5 c.c. of diluted bile to which has been added a few drops of a 2 per cent cane-sugar solution or a solution of furfural 1:1000. Pour the diluted bile carefully down the sides of the tube containing the H_2SO_4 so that the two fluids do not mix. (Method of stratification.) Notice the coloration at the line of contact of the two solutions. Shake the tube *slightly*, allowing a little more of the bile to come in contact with the H_2SO_4 .

The temperature must never rise above 70° C; to avoid this, cool the tube under the tap. Upon careful mixing and cooling as described above, the whole solution finally becomes cherry-red or reddish purple. Such a solution shows a definite and characteristic spectrum which distinguishes it from other substances, giving the same reaction, such as phenol, petroleum, fusel-oils, pyrocatechinol, cholesterol, and proteins.

Crystallization of the Bile Salts (Plattner's).

Mix 20 c.c. of the bile with sufficient animal charcoal to form a thick paste and allow the mass to evaporate on the water-bath to dryness. Grind up the dry residue and extract it in a flask with 25 c.c. of absolute alcohol on the water-bath for 15-20 minutes. Filter and to the filtrate add ether until a slight precipitate is visible. Cover the vessel and set it away for a few days. Examine the crystals. Dissolve some in alcohol and try Pettenkofer's Test.

BILE PIGMENTS.

The differences in color which were noticed in the various samples of bile are dependent upon the presence in predominating amounts of certain pigments of which the two most important are *bilirubin* and *biliverdin*. The former is chiefly present in the bile of carnivora, while that of the herbivora contains biliverdin in greater quantities. The bile pigments are closely connected generically and probably chemically with the blood and urinary pigments.

Bilirubin is insoluble in water, somewhat soluble in alcohol and ether, and dissolves readily in chloroform, benzene, and acids and alkalis. It oxidizes in the air to biliverdin.

Biliverdin is soluble in alcohol, partly soluble in ether, and insoluble in chloroform and water.

70 LABORATORY WORK IN PHYSIOLOGICAL CHEMISTRY.

The pigments possess the chemical characteristics of weak acids and are present in biliary calculi as calcium salts.

Use diluted bile for the following tests:

Gmelin's Test.—Stratify 5 c.c. of yellow HNO_3 and 5 c.c. of diluted bile as explained in the previous experiment. Notice the play of colors at the junction of the two liquids.—green, then blue, violet, red, and yellow. To what are these colors due, and what specific substances do they indicate?

Smith's Test.—Stratify 5 c.c. of diluted bile and 3 c.c. of tincture of iodine. Notice the bright green ring.

Huppert's Test.—To 10 c.c. of bile add an equal volume of milk of lime and shake thoroughly. Filter off and after washing once with water remove the precipitate to a small beaker with 25 c.c. of alcohol acidulated with a few drops of HCl . Warm the beaker in a water-bath until the alcohol begins to assume an emerald-green color. What is the chemistry of this reaction?

Hammarsten's Test.—To 5 c.c. of Hammarsten's reagent add five drops of diluted bile. A green color immediately develops. Upon the further addition of the reagent in small quantities to the mixture, the same play of colors may be obtained as in Gmelin's test.

Analysis of a Biliary Calculus.

Place some of the pulverized calculus in a clean dry test-tube with 15 c.c. of ether. Shake thoroughly. Filter off the ether through a dry filter and funnel into a porcelain dish and allow it to evaporate in the air. What is the residue? Test it.

If any of the calculus still remains in the test tube wash it upon the filter paper with some ether; wash the residue of the calculus now on the filter paper with a 10 per cent solu-

tion of HCl; then wash twice with water and dry the paper in the funnel.

When dry pass through the filter again and again about 10 c.c. of chloroform until this assumes a yellowish color, caused by its solvent action on the bilirubin. Finally allow a few c.c. of fresh chloroform to flow through the funnel, and uniting this with the previous extract, let it evaporate in the air.

Treat the residue of the calculus still on the filter-paper with about 10 c.c. of alcohol. What does this extract? Evaporate off the alcohol on the water-bath. Notice the color of the residue and compare it with that of bilirubin.

What was the necessity for the use of the hydrochloric acid in the analysis?

Perform the following tests with the residue of bilirubin:

(a) Gmelin's test.

(b) Dissolve some of the substance in a few c.c. of chloroform and shake it with a dilute solution of Na_2CO_3 . Notice the changes which occur in the aqueous solution.

BLOOD.

Blood may be considered as composed of a fluid plasma in which are suspended the form elements, i.e., the red corpuscles, the leucocytes, and the platelets. When the plasma coagulates, there separates from it the insoluble fibrin or the clot, and the fluid which remains or is pressed out by the contraction of the clot, is designated as the serum. The reaction of the blood is alkaline to litmus, the alkalinity being equivalent to about 0.25 per cent Na_2CO_3 . As was the case with the other body fluids, it is also acid to phenolphthaleïn, since it contains sodium bicarbonate and dihydrogen phosphate.

The specific gravity of the blood varies within rather small limits, 1.055–1.066, and the molecular concentration, as indicated by the depression of the freezing point, is under normal conditions almost constant. Approximately 60 per cent by weight of the blood is composed of corpuscles and the remaining 40 per cent of plasma.

General Reactions.

Test the reaction with litmus paper previously moistened with a concentrated solution of NaCl . To what is this reaction due? Specific gravity—see Hammarsten, p. 223, Hammerschlag's method.

(a) Examine a drop under the microscope.

(b) To 5 c.c. of blood add 10 c.c. of water. Notice changes in the solution and examine a drop under the microscope. What is laky blood?

(c) To 5 c.c. of blood add 10 c.c. of an 0.8 per cent NaCl solution. Examine a drop of this also. What is meant by a solution *isotonic* with blood?

(d) Add a few drops of bile, chloroform, and ether to successive portions of 5 c.c. of blood.

(e) To 5 c.c. of blood add 1 c.c. of hydrogen peroxide. To what is the frothing due?

(f) To about 10 c.c. of water add a few drops of blood and enough freshly prepared tincture of guaiacum to cause a slight turbidity. Then add to the mixture a few c.c. of hydrogen peroxide. Explain the effects produced.

(g) Add 10–12 drops of a saturated glacial acetic acid solution of benzidine to 2–3 c.c. of 3% hydrogen peroxide. Mix and to this add 2 or 3 drops of very dilute blood. Note change in color. Try the same reaction using dilute blood which has previously been boiled and then cooled.

BLOOD SERUM.

Blood serum presents a clear yellow liquid with a specific gravity of 1.027–1.032. It consists of a watery solution of serum albumins, globulins, enzymes, dextrose, fats, fibrin ferment, inorganic salts, and a yellow coloring matter belonging to the class of luteins or lipochromes. In addition it holds in solution traces of nearly every soluble substance which has been found in the body tissues. Of the 8–9 per cent of total solids in the serum, 7 per cent is made up of the proteins. NaCl is present to the extent of about 65 per cent of the inorganic salts.

PROTEINS.

(a) Heat about 25–30 c.c. of serum to boiling with the addition of a drop or two of acetic acid. Filter and test filtrate and precipitate for protein (Millon's and biuret reactions). Retain the filtrate.

(b) Saturate 15 c.c. of the serum with $MgSO_4$. Remove the precipitate by filtration, and to the filtrate add two drops of acetic acid. The filtrate from this second pre-

74 LABORATORY WORK IN PHYSIOLOGICAL CHEMISTRY.

cipitate should be protein-free. Test it. What is the difference between the two precipitates? What is another method for the separation of albumins and globulins?

(c) To 5 c.c. of the serum add 10 c.c. of 95 per cent alcohol.

(d) " 5 " " " " at 30° C add anhydrous Na_2SO_4 to saturation.

(e) To 5 c.c. of the serum add a solution of uranium acetate until no further precipitate is obtained.

After the removal of the precipitates in experiments (c), (d), and (e), test the filtrates for protein.

SUGAR AND SODIUM CHLORIDE.

Use the protein-free filtrate from experiment *a* (under Proteins).

(a) Test a few c.c. of the filtrate with Fehling's solution.

(b) Allow a few c.c. to evaporate on a watch-glass over the water-bath. Examine the glass under the microscope.

(c) Test for chlorides.

BLOOD PLASMA.

(a) To a few c.c. of oxalated plasma add a few drops of a 2 per cent CaCl_2 solution. What is the result? Why?

(b) Dilute 2 c.c. of salted plasma with 10 volumes of water. What happens?

(c) Do the same to some serum. What is the difference, and why?

(d) To 5 c.c. of the salt-plasma add an equal volume of a saturated NaCl solution. What is the precipitate?

FIBRIN.

This substance is formed by the action of the fibrin ferment upon the globulin, fibrinogen, which is present in all the coagulable fluids of the body.

Note the character of the substance presented. It has been obtained by whipping the blood before it has had time to set in a solid mass.

Apply three color protein tests.

Recall the action of 0.2 per cent HCl, pepsin, and trypsin upon fibrin.

FORM ELEMENTS.

These consist of the red blood cells, the leucocytes, and the platelets. Chemically the solid matter of the red corpuscles consists of 90 per cent *hæmoglobin*, 8 per cent proteins and nucleins, and the remainder cholesterol, lecithin, and inorganic salts. The predominating base of these salts is potassium.

The leucocytes, being typical animal cells, contain those substances which are intimately connected with the term protoplasm. Such are proteins (especially nucleoproteins), lecithins, cholesterol, fats, carbohydrates, inorganic salts, and water. Little is known concerning the composition of the blood-platelets. They probably contain a protein of the globulin type and a nucleoprotein.

OXYHÆMOGLOBIN.

Oxyhæmoglobin or hæmoglobin belongs to the class of conjugate proteins often spoken of as the respiratory pigments, and forms the most important constituent of the form elements. When its watery solution is heated to 70° C it decomposes with the formation of an iron-containing pigment, *hæmatin*, and an albuminous body, *globin*.

The function of the oxyhæmoglobin is that of an oxygen carrier. The compound holds the oxygen in a rather loose combination, and in all probability the iron of the hæmatin molecule is directly concerned in the process. This instability

of the oxygen combination renders the compound particularly liable to the action of reducing agents. A reduction of this kind takes place in the capillaries of the tissues, hæmoglobin or reduced hæmoglobin being formed, the presence of which in predominating quantities gives to the venous blood its characteristic dark red color.

Hæmoglobin is very prone to combine to form derivatives with certain compounds such as carbon monoxide, nitric oxide, and hydrogen sulphide. These simple substances, having a greater affinity for the hæmoglobin molecule than the oxygen possesses, replace the latter readily and form stable and more or less toxic compounds which cause death by oxygen starvation. Again, partial decomposition products of hæmoglobin showing characteristic spectra are easily formed under certain conditions; such are methæmoglobin, hæmochromogen, hæmatoporphyrin, etc. Some slight physical and chemical differences seem to exist between the oxy-hæmoglobins obtained from the blood of various animals, a fact which explains why the coloring matter from the blood of different animals does not crystallize in the same form or with the same facility.

Place on a glass slide one drop of defibrinated dog's blood. To this add one drop of water and mix with a platinum wire. Allow the mixture to evaporate at room temperature until the edges of the drop have begun to dry. Then place a cover-glass on the slide and examine under the microscope. Sketch the crystals of oxyhæmoglobin.

The proof for the presence of oxyhæmoglobin is usually adduced by showing under the microscope crystals of hæmin.

Hæmin—Teichmann's Crystals.

Hæmin is the HCl ester of the anhydride of hæmatin.

Place one drop of NaCl solution upon a microscopic slide

and allow it to evaporate to dryness. Then add a very small drop of blood and two drops of glacial acetic acid and cover with a glass. Warm *cautiously* until bubbles of gas begin to form in the mixture under the cover-glass. Examine and sketch under the microscope. The hæmin crystals are rhombic plates, brown in color by transmitted light. In large masses they have a metallic lustre and appear steel-blue by reflected light.

Spectroscopic Examination.

(a) *Oxyhæmoglobin*.—Dilute 1 c.c. of blood with 200 c.c. of water. Examine spectroscopically. At this dilution one broad absorption-band is seen extending from the *D* line (588) to *b* (518). The violet end of the spectrum is also absorbed as far as the *F* line (486). Upon again diluting this solution with an equal volume of water it is noticed that the broad band has resolved itself into two, the one next to *D* being narrower and more intense than the broader one to the right. Between the two bands is a green interspace. Less of the violet end is now absorbed. Upon still further dilution, the bands become narrower and finally disappear simultaneously.

(b) *Hæmoglobin (Reduced Hæmoglobin)*.—Prepare some *Stokes' reagent* as follows: Dissolve 3 grams of ferrous sulphate in a small quantity of water and add to it in watery solution 2 grams of tartaric acid. Make up the mixture to 100 c.c. and just before using add NH_4OH until the precipitate which at first forms is dissolved. This solution of ammonium ferrotartrate is a reducing agent, removing the oxygen which is in weak combination with the oxyhæmoglobin, and thus forming hæmoglobin. To the blood, 200 times diluted, add a few drops of *Stokes' reagent*. Notice the change in color. Examine in the spectroscope. A broad,

less sharply defined absorption-band is seen occupying as much space on the spectrum as the two bands of oxyhæmoglobin, but the hæmoglobin band is moved further to the left. If this solution is shaken in the air the color returns to that of oxyhæmoglobin and the latter's characteristic spectrum also reappears.

(c) *Methæmoglobin*.—Add to blood (diluted 1:15) two drops of a freshly prepared solution (10%) of potassium ferricyanide. The color of the blood becomes brown. The spectrum, in addition to two bands corresponding nearly to those of oxyhæmoglobin, which, however, are only faintly seen, shows a band in the red near *C*. If to such a solution, while still in position before the spectroscope, a drop or two of Stokes' reagent is added, the characteristic absorption-bands of oxyhæmoglobin appear for a second and are then quickly replaced by those of hæmoglobin. Shaking in the air causes the latter to be reoxidized to oxyhæmoglobin with the consequent spectral change.

(d) *Hæmochromogen (Reduced Hæmatin)*. — To blood (diluted 1:15) add two or three drops of strong NaOH and warm gently until the color changes to a brownish green. Then cool and add two drops of Stokes' reagent. Such a solution shows in the spectrum two very dark bands coinciding apparently with those of oxyhæmoglobin; upon careful examination, however, it will be seen that green light appears on the left of the left band and consequently both bands must be moved further to the right than the oxyhæmoglobin ones.

(e) *Hæmatoporphyrin (Iron-free Hæmatin)*.—Place a few c.c. of concentrated H_2SO_4 in a test-tube and add a drop of blood, mixing well. The color changes to wine-red, and spectroscopically the solution shows two bands on the opposite side of the *D* line. The one to the left is narrower and weaker, while that to the right is much more intense,

broad, and more sharply defined. This spectrum is very characteristic.

(f) *Carbon Monoxide Hæmoglobin*.—Carbon monoxide hæmoglobin may be easily prepared by passing ordinary coal-gas through defibrinated blood until the latter assumes a carmine or cherry-red color characteristic of the combination of CO with hæmoglobin. Examined spectroscopically such a solution shows two bands similar to oxyhæmoglobin except that the bands of the CO-hæmoglobin spectrum are nearer to the violet end. Add Stokes' reagent to the solution; the spectrum remains unchanged.

Make the following tests, first with diluted blood (oxyhæmoglobin) and then with diluted CO-hæmoglobin; note the differences carefully:

(a) Add to 5 c.c. of the blood 3 c.c. $(\text{NH}_4)_2\text{S}$.

(b) " " 5 " " " " 10 c.c. of Stokes' reagent.

(c) Shake with air.

What is characteristic and important in the combination of CO with hæmoglobin as shown by the above experiments? Make drawings of all the spectra seen and compare them with those in the text-books.

MILK.

Milk physiologically considered stands as a secretion in the strictest sense of the word, since its organic constituents are specific products of the activity of the cells of the mammary gland. Chemically it presents a perfect emulsion of fat whose menstruum holds in solution three proteins (lactalbumin, lactglobulin, and caseinogen), lactose, inorganic salts (chiefly Ca), gases, and traces of creatinine, lecithin, cholesterol, urea, and citric acid. In the comparison of the composition of human and cow's milk, the chief point to be noted is the relatively low percentage of total solids, protein and fat, and high percentage of lactose in human as against cow's milk. Fresh milk possesses either an amphoteric or alkaline reaction, but with phenolphthaleïn it also has a certain acidity. Upon standing, it develops a definite acid reaction by the change of the lactose into lactic acid. This throws the caseinogen out of solution and the milk is said to be sour. Fresh milk does not coagulate by heat, but forms a scum supposed to consist of some form of caseinogen and calcium salts.

The specific gravity of human and cow's milk is approximately the same (1.028-1.034). It is increased by the removal of the fat (cream), which has a specific gravity lower than that of water.

General Reactions.

(a) Examine under the microscope fresh milk, skimmed milk, and colostrum. What are the differences?

(b) Take the specific gravities and explain the variations in the results.

(c) Test the reaction with litmus paper.

(d) Saturate 10 c.c. of milk with MgSO_4 . What is the precipitate?

(e) Shake 5 c.c. of milk with ether. What takes place? Now add a few drops of NaOH . Explain this change.

(f) Heat some milk in an evaporating-dish. Notice the scum.

(g) Heat 5 c.c. of milk and 5 c.c. of NaOH to boiling. The yellowish-brown coloration is caused by the action of the alkali on the lactose.

(h) Try the guaiac reaction described under blood, using milk instead. Explain the result.

QUALITATIVE SEPARATION OF THE CONSTITUENTS OF MILK.

Dilute 200 c.c. of milk with 800 c.c. of water and add weak acetic acid, drop by drop, until a white flocky precipitate separates out, leaving a fairly clear solution. Allow the precipitate to settle; then filter (Filtrate A) it off and let it drain as dry as possible. Remove the white precipitated *caseinogen* from the paper and place it in a flask with 200 c.c. of a mixture one-half ether and one-half alcohol. Allow the precipitate to stand in contact with the ether for an hour, shaking from time to time. Then filter off the ether mixture and let it evaporate spontaneously. The residue consists of most of the *fat* of the milk, mechanically precipitated adherent to the caseinogen. Keep both the caseinogen and the fatty residue.

Place the filtrate A in an evaporating-dish and heat over a free flame; the solution being acid, any coagulable protein

not precipitated with the acetic acid will be rendered insoluble. When the solution has concentrated to about one-half its original volume, this may be filtered off and the clear filtrate again set evaporating until crystals begin to appear, separating out of the solution (now about 20 c.c.). Keep the coagulable protein precipitate.

After allowing the solution to cool, filter off the crystals of calcium phosphate and again place the filtrate over the water-bath and allow it to evaporate to a thick syrup. Of what is this composed?

From the milk have now been isolated—

1. Caseinogen. 2. Fats. 3. Coagulable protein. 4. Calcium phosphate. 5. Lactose.

These require separate study.

CASEINOGEN.

This substance is a non-coagulable protein of the class of phosphoproteins. It contains about 0.8% phosphorus and 0.7–1.2% sulphur. Caseinogen deports itself chemically as a weak acid combining with bases to form soluble salts or caseinogenates. The acid itself is insoluble. It exists in the milk in the form of Ca-caseinogenate (apparently not in a true solution, however), and when stronger acids are added caseinogen is formed and falls out of solution.

- (a) Try three color-protein tests.
- (b) Test for phosphorus and sulphur by fusion.
- (c) Dissolve some in very *dilute* NH_4OH and reprecipitate with acetic acid.
- (d) Test for lead-blackening sulphur.
- (e) Grind up a little of the substance in a mortar with CaCO_3 and water, then filter and in the filtrate test for caseinogen. What chemical reaction has taken place here?

FATS.

The fats of milk are in general those which occur in adipose tissue. There are present also in small quantities fats formed from the volatile fatty acids, particularly butyric and caproic acids. These give to milk-fat (butter) its characteristic odor when from any cause the fatty acids are liberated (rancidity). The proportion of the various fats is roughly as follows: triolein, $\frac{2}{3}$; tripalmitin, $\frac{1}{3}$; tristearin, $\frac{1}{6}$; tributyrin and tricaproin, $\frac{1}{14}$.

Use the fatty residue in the evaporating-dish (see method of separation). Dissolve the fat in 10 c.c. of ether and add to it 3-5 c.c. of sodium alcoholate. Notice what takes place and compare this saponification with those performed under fats. Evaporate off the ether and replace it with water. The precipitate should dissolve. Now add dilute H_2SO_4 to a distinct acid reaction.

Note the odor. To what is it due? Write the equations for the reactions.

COAGULABLE PROTEIN.

Lactalbumin and lactglobulin together occur to the extent of 1 per cent in cow's milk. They are closely related to, though not identical with, serum albumin and serum globulin.

Try three color-protein tests.

CALCIUM PHOSPHATE.

In the inorganic salts, phosphoric forms the chief acid and calcium the base, except in human milk, where potassium seems to predominate. Strangely enough, iron is only present in traces.

Dissolve some in dilute HNO_3 .

(a) Add an equal volume of ammonium molybdate and warm to 70°C . What is the precipitate? Filter it off, dissolve in NH_4OH and reprecipitate as ammonio-magnesium phosphate.

(b) Make a portion of the HNO_3 solution alkaline with NH_4OH and reacidify with acetic acid. Add a few c.c. of ammonium oxalate. Examine the precipitate microscopically.

LACTOSE.

Lactose occurs only in milk, if the small amounts which are occasionally present in the blood and urine of pregnant women are disregarded. It is a disaccharide and breaks down into a molecule of dextrose and one of galactose. It undergoes yeast fermentation very slowly, but is easily attacked by *bacterium lactis*, in which process lactic acid is formed. With phenylhydrazin, lactose yields osazones, which crystallize in needles and melt at 200°C . Lactose is soluble in water, but does not dissolve in either alcohol or ether. Its solutions are dextrogyrate.

Make use of the syrup derived from the last evaporation in the milk-separation.

(a) Try Fehling's, Moore's, and Nylander's tests.

(b) Apply the fermentation test.

(c) Try the phenylhydrazin test (see p. 5).

(d) To 5 c.c. of the lactose solution add sufficient freshly prepared indigo-carmin solution to render the mixture decidedly blue; then make it alkaline with dilute Na_2CO_3 solution. Upon warming, the mixture becomes red, yellow, and finally colorless. What do these changes indicate?

URINE.

Considered from the physiological point of view, the urine forms the chief exit for those soluble substances which, either on account of their toxicity under certain conditions or from their lack of available potential energy, are unsuited for utilization and further retention in the organism. Chemically, the urine presents a watery solution of organic and inorganic bases and acids, whose degree and manner of combination is dependent upon the conditions (affinity, mass-action, etc.) existent in the solution. The compounds present in the urine represent, on the one hand, the end products of combustion and cellular metabolism, and, on the other, bodies excreted in the same or similar form in which they were ingested. It is this double origin of the constituents of the urine which necessitates a comprehensive knowledge of the daily ingesta, if rational or accurate deductions are to be made from the composition of the urine. Again, as the character and composition of the urine excreted at different periods of the day varies within rather wide limits, it is absolutely essential in every examination of urine that the sample presented for analysis should represent a definite part, if not the whole, of a well-mixed 24-hours excretion. This in part affords an explanation of the now current method of considering the constituents of the urine from the standpoint of weight per diem (grammes) rather than concentration (per cent). Percentage data neglect one

important factor (volume), the consideration of which is indispensable, especially when extreme values are met with.

It is unnecessary to add that the urine should be examined as fresh as possible, or, in the event of this not being feasible, the fluid must be well supplied with antiseptic agents such as powdered thymol, chloroform, NaF, etc.

Physical Properties.

The following comprise the important physical properties of urine to be specially noted in an examination:

Color.—Aromatic alcohols, salol and allied compounds cause the urine to become brown upon standing. Rhubarb and senna impart a reddish color, which, if the urine is alkaline, may be confounded with the appearance of blood. The ingestion of quinine in large quantities gives to the urine a greenish opalescence and methylene blue is followed by the appearance of urine of a decided bluish-green tinge.

It is usual to refer to Vogel's color chart, but unless the color is other than that of some shade of yellow, little can be deduced from the determination of this property.

Odor.—Normal (urinous), ammoniacal, or that peculiar to some substance not normally present, such as acetone, methyl mercaptan, etc.

Transparency.—Clear or cloudy. Usually upon standing, a cloud of mucous-like substance separates, suspending itself in some part of the urine.

Reaction.—Acid, neutral, or alkaline.

As is true of any acid solution, the acidity of the urine is due to the presence of dissociated hydrogen atoms (ions). It is usually assumed that these acid ions are derived from the acid salts, such as H_2NaPO_4 or HNa_2PO_4 (mineral acidity), but in all probability the H ions of the organic acids in the urine also add a not inconsiderable part to the total acidity.

Free mineral acids are never present. By a proper relationship between the dihydrogen and monohydrogen phosphates, a neutral or amphoteric reaction may prevail (litmus). Under normal conditions the 24-hour urine is never alkaline; but portions of urine drawn a few hours after digestion may react alkaline, due to the withdrawal of acidic radicals (acid ions) from the blood in the formation of the acid gastric juice. Alkaline urine may be caused by free or fixed alkalies and when this is the case the urine is always cloudy or turbid. Care should be taken that the observed alkalinity is not a result of bacterial contamination. Volatile alkalies such as ammonia react blue to litmus, but upon warming the paper the red color returns; this will not occur if the reaction is caused by a fixed alkali. Organic acids are burnt in the body to carbonates and the latter decrease the acidity, sometimes, in fact, causing an alkaline reaction to appear.

Volume and Specific Gravity.—Under normal conditions these two factors vary inversely with each other; a large volume of urine is usually accompanied by a low specific gravity and *vice versa*. The volume depends upon the amount of water ingested and that excreted by the bowels, skin, and lungs; it varies between 800 and 1500 c.c. (average 1250 c.c.). The specific gravity may fall between 1.010 and 1.030 (average 1.017–1.020).

The volume should be measured in a graduated cylinder capable of holding two liters. Should it be required to retain the urine for some time, thymol must be added and the fluid placed in a stoppered vessel.

Determination of the specific gravity is made by means of the urinometer (hydrometer). If there is a sediment the urine must be warmed, filtered, and allowed to cool.

Place the cylinder upon the table and fill it to within an inch of the top with urine; then immerse the urinometer.

Read off on the graduation of the spindle the mark where the meniscus of the fluid cuts it.

Sediments.—Notice should be taken as to their color, amount, character, etc. The method for the determination of the identity of such will be taken up later.

Total Solids.—These may be calculated approximately by multiplying the second and third decimals of the specific gravity by Häser's coefficient = 2.33. This gives the number of grammes in 1000 c.c. of the urine, from which must be calculated the total amount in the twenty-four hours.

THE NORMAL CONSTITUENTS OF THE URINE.

INORGANIC COMPOUNDS.

Concerning the exact nature of the combinations formed in the urine by the various bases and acids (ions) but little is known at present. The compounds are present in rather dilute solution and as such are amenable to all the chemical and physical laws of salts in like solutions, complicated, however, by the fact that the condition is not one of a single salt in a simple solution but of an exceedingly complex mixture of salts both inorganic and organic. The dissociation of one salt is influenced by the presence of others, also dissociated, and the particular combinations of the various (ions) bases or acids depend upon their relative masses and avidities. Again, as regards two compounds with like ions each decreases the solubility of the other, while the solubility of unlike ions is increased by their mutual presence.

These factors, dissociation, mass action, and affinity determine the character of the ionic equilibrium which is present in the urine, but as to the exact nature of such equilibrium, our present methods tell next to nothing. The later work

along physical lines has been in this direction, in the hope that the results will be of value for diagnostic purposes.

At present it is customary to determine the quantity of a given base or acid radical and then to express it empirically in terms of a compound of this radical, the quantity of which is considered to predominate in the mixture. Thus chlorine is expressed as NaCl , potassium as K_2O , etc. The ordinary inorganic acidic radicals (anions) of the urine are Cl' , SO_4'' , PO_4''' , and CO_2'' and the basic radicals (kations), NH_4' , K' , Na' , Ca'' , Mg'' , with traces of Fe'' .

ACIDIC RADICALS.

(a) *Chlorides*.—These are present combined principally with Na and K . Acidify some of the urine with HNO_3 and add a drop of AgNO_3 . Prove that this precipitate is AgCl .

(b) *Sulphates*.—These occur in two forms, viz., preformed or sulphate-sulphuric acid and ethereal or combined sulphuric acid. The former precipitates directly from the urine by the addition of BaCl_2 in an acid reaction, the latter only after the previous boiling of the urine with a mineral acid. Acidify some urine with acetic acid and add an excess of BaCl_2 . Prove that this precipitate is BaSO_4 . Filter (filtrate must be clear), and to this add a few c.c. of concentrated HCl and boil a few minutes. A turbidity is indicative of the presence of ethereal sulphates. Explain this.

Sulphur in the organic or lead-blackening form may also be present in the urine as a constituent of taurine, cystine, KSCN , etc. Place some urine in a flask with a few pieces of pure zinc and add enough HCl to cause a gentle evolution of gas. Partially close the mouth with a piece of filter-paper moistened with lead acetate. In the presence of organic sulphur the paper is blackened. Explain this.

(c) *Phosphates*.—These are present combined with NH_4 , Na, K, Ca, and Mg as primary, secondary, or tertiary compounds.

1. Make some urine alkaline with NH_4OH . What is the precipitate? Filter and to the clear filtrate add two drops of BaCl_2 . What is this new precipitate? Filter this off through the same filter as the first and dissolve the whole precipitate in dilute HNO_3 . Test for phosphates.

2. Make the urine acid with acetic acid and add a few drops of uranium nitrate solution. Of what is the precipitate composed?

3. Acidify the urine with a few c.c. of HNO_3 and add some molybdic solution. Warm at 80°C . What is this precipitate?

4. Boil about 10 c.c. of the urine and add one-quarter its volume of Fehling's solution. Notice the color of the precipitate. To what is it due? Then boil 10 c.c. of Fehling's solution and add one-quarter its volume of urine. What occurs? Contrast these two experiments.

Write the equations for the reactions in the above experiments.

BASIC RADICALS.

With the exception of NH_4 these have little or no chemical significance. The proof for the presence of ammoniacal compounds will be taken up under the quantitative determinations.

ORGANIC COMPOUNDS.

Urea or carbamide is present in the urine in about a 2 per cent solution or 30 grms. for 24 hours. It is very soluble in water and alcohol and crystallizes in long rhombic prisms. It possesses weakly basic properties and forms combinations with acids analogous to double salts. When heated gently, urea yields biuret. The *Micrococcus ureæ* splits it into NH_3 and CO_2 .

Prepare some urea from the urine as follows:

Evaporate one-half a liter of urine to syrupy consistency and exhaust the residue with hot alcohol. Filter off the latter and evaporate the filtrate to dryness. This residue is extracted on the water-bath with successive portions of pure acetone, which must be filtered hot. The mixed acetone filtrates are then evaporated slowly to a small volume and allowed to cool. The crystals which separate out may be filtered off and washed with cold acetone.

Make use of these urea crystals for the following reactions:

(a) Dissolve some urea in a few c.c. of water. Place in each of two watch-glasses one drop of the solution. To one add one drop of dilute HNO_3 and to the other a drop of dilute oxalic acid solution. Allow them to stand and then examine the crystals under the microscope. What are these compounds?

(b) Warm carefully a few crystals of urea in a dry test-tube. The substance melts, giving off NH_3 . Continue heat-

ing until the fused mass solidifies. Cool the test-tube and add a few c.c. of water and NaOH drop by drop until the residue is all in solution. Add now a drop or two of very dilute CuSO_4 solution. To what reaction does this correspond? What substance is formed from the urea? Write the equation.

(c) To 5 c.c. of NaOH add some bromine water and drop in a crystal of urea. What is the reaction which takes place?

Uric acid is a diureide and structurally is composed of two urea groups attached to a 3-carbon chain. It is closely related to the purine bases being built up from the "purine" type.

Uric acid acts as a weak dibasic acid and forms three types of salts—neutral, biurate, and quadriurate.

The neutral salts which are formed by the replacement of two hydrogen atoms of the acid by two atoms of the base are very unstable.

The biurates have only one of the hydrogens replaced and form very stable compounds. They present the chief type in which uric acid exists in the urine.

The quadriurates stand between the other urates as regards stability. They are formed by a weak combination of uric acid and biurate molecule for molecule. They therefore contain in the molecule one-fourth the quantity of base that exists in the neutral urate. When uric acid separates out spontaneously from a urine, it is caused by an

interreaction between the acid phosphates and the biurates in which quadriurates are formed; the latter immediately break up into uric acid and biurate, and the former is thrown out of solution.

Pure uric acid forms a white powder. As it separates out from urine in the presence of impurities, it assumes a great diversity of crystalline forms, all of which are characteristic, however; the crystals are always tinged with pigment derived from the urine.

Uric acid is practically insoluble in hot and cold water, alcohol or ether. The sodium, potassium, and lithium salts are soluble, especially the latter. The ammonium, calcium, and magnesium salts are insoluble. The quantity excreted in 24 hours varies according to the individual and the diet, but usually amounts to 0.5—1.0 grms. (average 0.7 gm.). If urine is made sufficiently acid with HCl to react strongly to litmus (about 30 c.c. conc. HCl to the liter) and allowed to stand, uric acid will separate out in crystals of a dark red color.

For the following tests make use of the uric acid prepared in this way:

(a) Examine the crystals under the microscope. Sketch as large a variety as can be found.

(b) Test the solubility in water, NaOH and NH_4OH .

(c) Dissolve some of the crystals in a few c.c. of dilute NaOH and then add NH_4Cl to saturation. What is the precipitate?

(d) Heat a crystal on platinum-foil.

(e) Dissolve some uric acid in a small quantity of dilute NaOH. Add concentrated H_2SO_4 carefully, drop by drop, until the solution is too warm to touch; then add a few c.c. of potassium permanganate solution. What is the reaction which takes place?

(f) Make a concentrated solution of uric acid and pour it into 10 c.c. of Fehling's solution which has previously been brought to boiling. Observe and note result.

(g) Dissolve some uric acid in dilute Na_2CO_3 and place a couple of drops on some filter-paper previously moistened with AgNO_3 . To what is the blackening due?

(h) *Murexide Test*.—Place a few crystals in a clean and dry evaporating-dish and pour upon them two drops of concentrated HNO_3 . Evaporate to dryness *very carefully* over a free flame. A yellowish residue results which upon cooling and the addition of a drop of NH_4OH becomes purple-red. If NaOH instead be used, the color will be purple-violet. What is the chemistry of the reaction?

PURINE BASES.

These substances as they appear in the urine have a double origin. They may be the result of ingested nucleins (exogenic origin) and also the end products of the nuclear metabolism of the tissues (endogenic origin). Although ten different bases have been isolated, it is questionable if many of them are not laboratory products formed during the complicated processes of their isolation. Xanthine, hypoxanthine, guanine, adenine, epiguanine, paraxanthine, and carnine are said to be present in the urine. Taken together the quantity daily excreted is only about 20–100 mg. or about 10 per cent of the uric acid. They are capable of forming insoluble compounds with Ag, Cu, phosphotungstic acid, etc.

To 20 c.c. of urine add an excess of the magnesium mixture. Filter, and to the filtrate add ammoniacal silver nitrate solution. The precipitate is composed of the Ag compounds of all the bases. This is filtered off, suspended in water, decomposed with H_2S , and the Ag_2S removed by filtration. The clear filtrate is evaporated to dryness. This

residue is treated with 3% H_2SO_4 which dissolves the purine bases and leaves the uric acid undissolved. The dissolved purine bases may be reprecipitated with silver nitrate solution.

Creatinine appears in the urine as the result of the ingestion of meat which contains creatine. The amount is also somewhat dependent upon the nitrogenous metabolism, being decreased in starvation. For the relationship between creatine and creatinine and the reactions and tests, see under Muscle. On a mixed diet about 0.1 gm. is excreted in 24 hours.

Creatinine allows of a separation from the urine as follows:

To 50 c.c. of urine add 3 c.c. of a saturated solution of sodium acetate and then 10 c.c. of a saturated solution of HgCl_2 . Filter off the precipitated urates, sulphates, and phosphates, and set the filtrate aside for 24 hours. The mercury compound of creatinine separates out in the form of spherical globules. Examine some under the microscope. This compound of creatinine is readily decomposed by acids or by H_2S . Try Weyl's or Jaffe's test directly on the urine and then on the creatinine isolated by the above method. Test its reducing power.

CONJUGATE SULPHATES.

Of the toxic substances which are formed as the result of intestinal putrefaction of the proteins, phenol, *p*-cresol, indole, and skatole appear in the urine in the form of alkali salts of non-toxic ethereal combinations with sulphuric acid; pyrocatechinol and hydrochinol are also excreted in the same way. Taken together these compounds are denoted

as the ethereal or conjugated sulphates. The strength of combination and manner of detection of the sulphate radical has been studied under the inorganic salts. The amount of the conjugate sulphates varies from 0.09 to 0.6 gm., being dependent, as it would be expected from their origin, upon the extent of putrefaction in the intestine.

The *Phenol* and *p-Cresol* sulphates exist in normal urine in relatively small amounts, about 30 mg. for twenty-four hours. They may be isolated and detected as follows:

Treat the urine with $\frac{1}{4}$ its volume of 25 per cent H_2SO_4 and distil off $\frac{1}{10}$ of the volume of the solution. Use this distillate for the succeeding reactions:

(a) Add some Millon's reagent to a little of the solution. Warm. Compare this with the test under protein and tyrosine.

(b) To a few c.c. of the solution add a trace of *neutral* ferric chloride solution. When did you use this reaction before? Add a drop of HCl.

(c) To 10 c.c. of the distillate add some bromine-water. Note the whitish crystalline precipitate of tribromophenol.

The combinations with *Pyrocatechinol* and *Hydrochinol* originate after the ingestion of these bodies or of phenol. The urine containing them becomes brown on standing ("carbolic urines").

Indole and *Skatole* (β -methyl indole) do not unite with the sulphuric acid directly as such, but first suffer an oxidation by which indoxyl and skatoxyl are formed; they then combine and appear in the urine as potassium indoxyl or skatoxyl sulphate. The former is called animal indican and is not to be confounded with the indican of plants. The skatoxyl compound is only present in minimal quantities and not infrequently is absent entirely.

INDICAN=POTASSIUM INDOXYL SULPHATE,

The following tests are based upon the oxidation of the indican to indigo, and the solvent action of chloroform upon the latter.

(a) *Jaffe's Test*.—To about 10 c.c. of urine add 2 or 3 c.c. of chloroform and mix well with an equal volume, 10 c.c., of concentrated HCl. Then add drop by drop, shaking well between each drop, a concentrated solution of chloride of lime. The indigo is dissolved by the chloroform. Note the color changes.

(b) *Obermayer's Test*.—Perform the test in the same manner except instead of concentrated HCl and lime add an equal volume of Obermayer's reagent.

(c) *Hammarsten's Test*.—The same test, using Hammarsten's reagent. Compare results.

Oxalic acid is present in the normal urine in the form of calcium oxalate, which is held in solution by the presence of the acid phosphates. Frequently the salt separates out in crystalline form (see sediments) and then lays the foundation for renal and vesical calculi. The quantity averages about 30 mg. for 24 hours. In certain conditions which are not well understood the amount is largely increased (oxaluria). The oxalic acid originates in part from the oxalates ingested

in vegetable food, but as it does not disappear in starvation, some must arise from a particular phase of protein metabolism, possibly the nucleins.

The following is the procedure employed for its isolation:

The urine is acidified (20 c.c. of HCl sp. gr. 1.12 for every 1000 c.c.) and extracted with a 10 per cent solution of alcohol in ether. Three extractions are united, evaporated to 10–20 c.c., filtered and the filtrate made slightly alkaline with NH_4OH ; to this is added a few c.c. of 1 per cent CaCl_2 solution and the mixture is rendered faintly acid with acetic acid. Collect the precipitate on a small filter and examine it under the microscope. Heat some on a platinum foil.

This substance is formed in the renal cells by a synthesis of benzoic and aminoacetic acid or glycocoll. Normally it is present as a hippurate to the amount of 0.7 gm. in 24 hours. The quantity is markedly augmented when benzene derivatives such as exist in plants and fruits are ingested. An increase in amount may also result from excessive putrefaction of vegetable material in the intestine. Thus it is that larger quantities of hippuric acid are always found in the urine of herbivora than in that of carnivora. It readily crystallizes in long rhombic prisms or needles formed in rosettes, and is soluble in water and alcohol. If urine containing hippuric acid is acidified (20–30 c.c. of conc. HCl to the liter) and evaporated on the water-bath to a small volume, crystals of this substance will form upon cooling. They may be collected on a filter and washed with a little alcohol saturated with ether.

(a) Heat a few crystals in a dry test-tube. They will melt at 187°C. , and at still higher temperatures will decom-

pose with the formation of a red coloration (decomposition of the glycocoll) and an odor resembling that of the oil of bitter almonds (benzonitrile).

PIGMENTS.

Variation in the yellow color of the urine noticed under certain conditions, is attributable to the presence of different pigments, the most important of which are urochrome, urobilin, and uroerythrin.

UROCHROME.

This is the name of the substance which gives to normal urine its characteristic yellow color. Of its properties and constitution next to nothing is known. It is isolated from urine when the latter is saturated with $(\text{NH}_4)_2\text{SO}_4$ and the filtrate extracted with alcohol. The pigment is soluble in the alcohol and the entire color of the urine passes into the solvent. Urochrome, when acted upon by mild reducing agents, yields a second pigment which is apparently identical with urobilin. The name uroerythrin is applied to the pigment which imparts to the brick-dust sediment of urates (sedimentum lateritium) its pinkish coloration. Solutions of this pigment are rapidly decolorized by light.

UROBILIN.

Although not ordinarily present to such an extent as urochrome, urobilin is frequently increased in diseased conditions, and its importance lies in the knowledge which has been gained concerning its origin from and relation to the biliary pigments. The substance is extremely soluble, dissolving in all ordinary solvents. It exhibits in alcoholic

solutions a green fluorescence which is greatly augmented if ammonia and alcoholic ZnCl_2 solution are added. Acid alcoholic solutions of urobilin also show a distinct absorption-band between b and F .

In constitution urobilin possesses the same acidic properties which characterize all the animal pigments. It forms insoluble compounds with bases, and is completely precipitated from its solution by saturation with $(\text{NH}_4)_2\text{SO}_4$ (differing from urochrome).

(a) To 50 c.c. of urine faintly acidified with H_2SO_4 add ammonium sulphate (in substance) to saturation. Filter off the precipitate and dissolve it in alcohol containing a few drops of concentrated HCl . What sort of a body is urobilin?

(b) To 50 c.c. of urine add an equal mixture of neutral and basic lead acetate until the precipitation is complete. Filter and allow the precipitate to drain as dry as possible. Then place it and the filter-paper in an evaporating-dish half full of 95 per cent alcohol acidulated with HCl . Warm over the water-bath until the alcohol is well colored. Filter and examine the solution in the spectroscope. Now add a few drops of NH_4OH and a few c.c. of an alcoholic zinc chloride solution. Note the fluorescence. In what way is the coloring matter of the urine precipitated?

(c) Shake 25 c.c. of urine with 2 to 3 drops of pure HCl and 5 c.c. of chloroform. Remove the chloroform and stratify upon it a few c.c. of a solution of zinc acetate in alcohol. Notice the green ring; by shaking, the solution becomes fluorescent.

CARBOHYDRATES AND RELATED BODIES.

Under apparently normal conditions, reducing substances of a carbohydrate nature appear in the urine in small

and variable quantities. The most important of these are the pentoses, glycuronates, and possibly dextrose. It is still debatable whether dextrose is present in the urine under absolutely normal conditions, or, better, under conditions which do not permit of the slightest suspicion that the renal function is impaired. If it does exist in the urine in such cases, it is present in amounts which escape detection by the usual methods.

PENTOSSES.

As the methods for isolation and identification of the pentoses improve, the presence of these substances in the urine is being more frequently noted. Xylose, Arabinose, and Rhamnose (methyl-pentose) have been described. They originate in the organism after the ingestion of cherries, plums, grapes, etc., which contain the mother substances of the pentoses, the pentosanes. The pentoses are not readily assimilable, and when ingested appear in part unchanged in the urine. It must be remembered that the tests which were given for pentoses (p. 3) are not absolutely characteristic for these substances, since glycuronic acid and related bodies also react positively with them. The reactions may be used, nevertheless, as confirmatory tests if the polarization and fermentative power of the urine are also determined. The pentoses found in the urine are non-fermentable and inactive toward polarized light, while the glycuronates are lævogyrate.

(a) Test the urine in the polariscope.

(b) Try the orcinol and phloroglucinol tests with 10 c.c. of urine.

(c) Heat to boiling 4-5 c.c. of Bial's reagent and then add a few drops of the urine.

GLYCURONIC ACID.

Glycuronic acid originates as a product of the intermediary metabolism of the carbohydrate nucleus in the protein molecule. Ordinarily it is present in the urine in small amounts combined with phenol, indoxyl, and skatoxyl as conjugate glycuronates; but under certain conditions, either as the result of an abnormal increase in the quantity of these bodies just mentioned, or the ingestion of aromatic substances, such as the camphors, chloral, and naphthol, etc., the conjugate glycuronates appear in such quantities as to give to the urine a left-handed polarization and a decided reducing power on Fehling's solution. (Some glycuronates do not reduce.) Glycuronic acid is dextrogyrate, does not ferment, and gives positive results with the orcinol and phloroglucinol tests (see the pentoses). As glycuronic acid itself is never present in the urine, and since the glycuronates are said to respond negatively to the orcinol test, this test has been used as a means of differentiating between the pentoses and the glycuronates. This is only true, however, of the glycuronates which do not split off the glycuronic acid component during the performance of the orcinol test (boiling on the water-bath with HCl). Glycuronic acid forms with parabromphenylhydrazin an osazone which melts at from 200–216° C. and which, if dissolved in pyridin and alcohol, shows a left-handed rotation of $7^{\circ} 25'$ or $369^{\circ} (a)_D$.

A lævogyrate urine which, upon treatment with Fehling's solution, gives a yellowish precipitate and partial reduction of the copper may be more than suspected of containing conjugate glycuronates.

Examine a sample of urine in the same way as under pentoses.

QUANTITATIVE DETERMINATIONS.

PHYSICAL PROPERTIES.

Determinations of certain physical properties of the urine have come into prominence of late as a means for furnishing additional data concerning the general state of equilibrium which obtains in the fluid. The urine, being in fact merely a somewhat dilute solution of organic and inorganic salts, must possess the same properties and respond to the same physical laws which govern such solutions. The most important property whose determination is attempted is that of the *depression of the freezing-point* below that of the solvent, water.

The law states that the presence of substances dissolved in a solvent causes the freezing-point of the solution to be lower than that of the solvent, and that the amount of the depression is in direct proportion to the number of molecules or (when dissociation has taken place) ions in the solution.

The depression of the freezing-point, which is represented by the Greek letter Δ , is therefore dependent upon the molecular concentration.

The only substances which are present in the urine in a quantity sufficient to exert any influence on Δ are the urea and sodium chloride and possibly the phosphates and sulphates, although the effect of the latter two, even if it did lie outside of the limit of experimental error, could not suffice to cause differences which would influence deductions. The other factors which affect the Δ are those which cause variations in the specific gravity of the urine, and it is stated that a relationship exists between the two which only varies within very small limits. *A priori*, this seems impossible

when it is remembered that solutions of urea and sodium chloride with the same specific gravity would have widely differing Δ , since urea is non-dissociable, while sodium chloride in the strength in which it exists in the urine dissociates to a considerable degree.

The freezing-point determination is made by the use of the Beckmann apparatus (see demonstration). The manipulation is simple, rapid, and requires little experience.

Another property of the urine which is dependent upon the presence of electrolytes in the solution is termed the *electrical conductivity*. Electrolytes are substances which become capable of conveying electricity in virtue of the fact that they become dissociated in solution into (electrically) active, positive or negative parts or ions. Therefore the electrical conductivity is in direct proportion to the degree of dissociation or ionization of the urine. This property is unaffected by variations in the content of urea in the urine. Such determinations are accomplished by the use of a rather complicated apparatus based upon the theory of the Wheatstone bridge. (See demonstration.) The imperfect knowledge which exists in regard to the conditions obtaining in the various fluids of the body does not permit of exact interpretations of the results acquired by these methods.

It must suffice, therefore, to merely mention that various factors are derived from a combination of the estimation of the Δ and that of urinary substances, such as NaCl, total nitrogen, etc., and that such factors are constantly increasing in value as data serving to present a clearer picture of the conditions under which a given sample of urine may have been excreted.

ACIDITY OF THE URINE.

It is quite generally understood that the degree of acidity of the urine is due to the excess of the dihydrogen (acid) over the monohydrogen phosphates, the relation to the total phosphates usually being about 60 per cent of the former to 40 per cent of the latter. Based upon this view, the methods for the quantitative determination of the acidity have consisted in the estimation of the total P_2O_5 and the P_2O_5 present in the form of the acid phosphates, and from which the degree of relative acidity of the urine was derived. Many difficulties arise in the procedure for the quantitative separation and determination of the two types of phosphates, and the present methods have been shown to be full of errors. Theoretical objections have also been raised, and attempts made to show that the "organic" acidity, as opposed to the "mineral" (phosphate) acidity, plays a not inconsiderable rôle in determining the total acidity. How unimportant the estimation of the organic acidity really is can be understood when it is noted that organic acids appearing in a solution of mixed phosphates are able to remove the base from the monohydrogen phosphates, and thus produce an almost equivalent increase in the quantity of the acid phosphates. The presence of the organic acids, therefore, only serves to increase the acid phosphates, and the latter still remain theoretically the most accurate indication of the total acidity. The technical difficulties have led to the reliance upon the method of *direct titration* of the urine with $\frac{N}{10}$ alkali. This procedure has the advantage of rapidity and possesses some value, perhaps, where comparisons only are required. Absolute values in the present state of the question are unattainable.

Quantitative Estimation.

To 25 c.c. of urine in a flask add 15–20 grms. of powdered potassium oxalate and two drops of phenolphthaleïn. Allow the oxalate to partly dissolve by shaking and titrate the mixture immediately with $\frac{N}{10}$ NaOH until the first faint pink coloration is discernible. The acidity in this case is expressed in terms of $\frac{N}{10}$ acid for 100 c.c. of urine.

CHLORIDES.

The quantity of chlorine excreted in the urine during the 24 hours is subject to great variations. On a mixed diet it usually amounts to from 10 to 15 grms., calculated as NaCl. Under normal conditions the quantity eliminated is dependent upon the quantity of chlorine and water ingested; but in pathological states, such as the formation and subsequent absorption of exudates, it is respectively markedly decreased and then increased. In starvation the amount becomes minimal.

Quantitative Estimation.

The principle of the method is the following:

To the urine is added an excess of AgNO_3 over and above what is necessary to precipitate all the chlorides present. The excess of Ag is then determined by means of a sulphocyanide solution, using iron alum as an indicator.

Reagents necessary:

1. A AgNO_3 solution, each c.c. of which precipitates 0.01 grm. NaCl (29.075 grms. AgNO_3 in a liter).
2. A saturated solution of iron alum.
3. Chlorine-free HNO_3 of a specific gravity 1.2.

4. A potassium sulphocyanide solution of which 2 c.c. corresponds to 1 c.c. of the known AgNO_3 solution.

Method: Prepare a *clean* and *dry* graduate. By means of a pipette measure off *accurately* 5 c.c. of urine and run it into the graduate. Add 3 c.c. of the HNO_3 and dilute with water to about 25 c.c. Then allow exactly 10 c.c. of the known AgNO_3 solution to flow in. Add water until the volume equals 50 c.c.; then mix thoroughly. Transfer this now to a clean and dry beaker and clean and dry the graduate again. Prepare also a clean and dry funnel with paper and filter the mixture in the beaker into the graduate. When 25 c.c. of a *water-clear* filtrate has passed through remove the funnel to a test-tube. Add 10 drops of the iron alum to the graduate and titrate with the known potassium sulphocyanide until the first *tinge* of pink appears in the solution.

Calculate the amount of chlorine or NaCl in the 5 c.c. of the urine used, and then in the 24-hour sample.

SULPHATES = SO_3 .

A variable fraction (80 to 90 per cent) of the total sulphur of the urine exists in a completely oxidized form as the salts of sulphuric acid, usually denoted and calculated as SO_3 . Of the total SO_3 , about nine-tenths is combined with bases (preformed) and one-tenth with aromatic radicles (conjugate sulphates). The "organic" sulphur appears as taurine, cystine, KSCN , etc.

Since the food contains merely minimum quantities of SO_3 , that found in the urine must originate as a product of protein metabolism, in which the sulphur of the molecule becomes oxidized. Thus it is that ordinarily the SO_3 output may be considered as indicative of the amount of protein burned in the body. About 1.5–3.0 grms. (average 2.5 grms.) SO_3 are excreted during 24 hours.

Quantitative Estimation.

The procedures for the determination of these various units rest upon the following facts:

Boiling the urine with dilute HCl liberates all the sulphate radicles present in such a form that they may be precipitated with BaCl_2 as BaSO_4 . This gives the amount of total sulphate. If acetic acid be used instead of HCl, the resulting precipitate with BaCl_2 will be made up of the pre-formed or sulphate-sulphate. This may be filtered off and the filtrate treated as for the total sulphates, the result being the amount of ethereal sulphates present. The difference between this and the total will be the amount of the pre-formed sulphate.

TOTAL SO_3 .

In a beaker place 50 c.c. of filtered urine and dilute with 100 c.c. of water, adding 5 c.c. of HCl. Heat to boiling, add *very slowly* 20 c.c. of the BaCl_2 solution and allow it to cool and stand *covered* in a cool place for 24 hours. Filter through a small ash-free filter; the precipitate must be removed *quantitatively* from the beaker to the paper by means of warm water. Now wash the white precipitate with water until the washings give no test for chlorine; then dry at 100°C . When dry, slip out the paper from the funnel, fold it up so that the contained sulphate cannot fall out and place it in a porcelain crucible which has previously been ignited and weighed. Ignite the paper in the crucible carefully and burn until the residue becomes white. Cool the crucible and weigh. The difference in the two weights will give that of the BaSO_4 from 50 c.c. of urine, from which may be calculated the SO_3 in the total 24-hour sample.

CONJUGATE SO_3 .

To 75 c.c. of urine in a beaker add an equal volume of water; acidulate with about 10 drops of acetic acid, heat to boiling and add 10 c.c. of the BaCl_2 solution. Place the covered beaker in a cool place for about 24 hours. The precipitate is then collected on a filter and in the filtrate and combined washings the SO_3 is determined as outlined under total SO_3 . In this case the result corresponds to the SO_3 of the conjugate sulphates.

ORGANIC SULPHUR.

Evaporate 50 c.c. of urine down to dryness, add the fusion mixture and fuse with an alcoholic flame until a white residue remains. Take this up with hot water strongly acidulated with HCl . Determine the amount of SO_3 present in this solution and calculate the amount for the 24 hours, from which may be subtracted the total SO_3 which has been previously estimated in another sample of the same urine. The difference will give the organic sulphur expressed as SO_3 in the 24-hour sample.

PHOSPHATES = P_2O_5 .

Phosphorus is present in the urine to the greatest extent as phosphoric acid combined with bases, but a small quantity (2-2.5 per cent) exists as organic P.; e.g., glycerophosphoric acid, lecithin, etc. It has been stated that the amount of this latter form of phosphorus is a true indication of the nitrogenous metabolism taking place in the body. The phosphoric acid phosphorus is expressed as P_2O_5 , and during 24 hours about 1.5-3.5 grms. (average 2.5 grms.) P_2O_5

are excreted. This amount is made up of alkaline and earthy P_2O_5 in decidedly varying proportions; of the alkaline P_2O_5 about 60 per cent consists of the dihydrogen (acid) phosphate and the remaining 40 per cent as the monohydrogen phosphate.

TOTAL P_2O_5 .

The procedure is based upon the quantitative precipitation of *all* the phosphates in the urine with uranium nitrate, as uranium phosphate, using potassium ferrocyanide as indicator. The phosphates must be present as acid salts.

Quantitative Estimation.

Reagents necessary:

1. A uranium nitrate solution 1 c.c. of which equals 0.005 gm. P_2O_5 (35.461 grms. in a liter). How is this calculated?
2. An accessory solution (100 grms. of sodium acetate and 300 grms. of acetic acid in a liter).
3. A solution of potassium ferrocyanide. What is the reaction which indicates the absolute precipitation of all the P_2O_5 ?

Method: Place 25 c.c. of urine in a large evaporating-dish, add 5 c.c. of the accessory solution (why?), and warm gently over an asbestos board. Keeping the solution warm, add the known uranium nitrate solution from a burette, from time to time removing a drop of the urine on the end of a glass rod and adding it to a drop of the potassium ferrocyanide which has been placed upon a white porcelain dish. When all the P_2O_5 has been precipitated by the uranium and the first excess of the latter appears in the urine solution, the drop of indicator on the plate when tested as above will take on a faint reddish-brown color. The test should be repeated after a minute; if it again causes a color, the titration is complete,

otherwise more of the uranium solution must be added and retested.

From the number of c.c. of the uranium nitrate solution required calculate the P_2O_5 in the 25 c.c. of urine employed and from that determine the total P_2O_5 in the 24-hour urine.

EARTHY P_2O_5 .

In the precipitation of the earthy phosphates by means of NH_4OH various errors creep in which render the determination of doubtful value. Thus, in whatever form the earthy bases (Ca or Mg) were present in the urine, they would form insoluble *phosphates* when the fluid was made alkaline, and the precipitate would therefore contain P_2O_5 not originally combined with the alkaline earths.

The method is given because it is still employed.

Quantitative Estimation.

Place 50 c.c. of urine in a beaker and make it alkaline with NH_4OH . A precipitation of the earthy phosphates occurs. Allow this to stand for a couple of hours and then collect the precipitate upon a small filter. After having washed the precipitate with very dilute NH_4OH transfer it quantitatively to an evaporating-dish by means of dilute acetic acid, which dissolves the P_2O_5 . Dilute the solution to about 25 c.c. and titrate as outlined under total phosphates. The difference between this amount and that of the total will be the amount of alkaline P_2O_5 .

ORGANIC PHOSPHORUS.

The method employed for the determination of the organic phosphorus in the urine is based upon the same principle as that in use for organic sulphur. The total phosphorus is

estimated after fusion as P_2O_5 . From this amount is deducted the total P_2O_5 determined on another sample of the same urine, and the difference corresponds to the organic phosphorus calculated in terms of P_2O_5 .

TOTAL NITROGEN (KJELDAHL).

A well-nourished man eliminates under ordinary conditions with a mixed diet 10–16 grms. nitrogen. The amount is dependent upon the body weight, the diet, and various factors which may influence the metabolism of protein in the organism. Muscular work has no effect upon the nitrogenous excretion. In round numbers 85 per cent of the nitrogen is in the form of urea; 4 to 5 per cent, NH_3 ; 1 to 2 per cent, uric acid; and the remaining extractives, etc., 8 to 10 per cent.

Quantitative Estimation.

The Kjeldahl method for the determination of the total nitrogen of the urine has come into almost universal use.

The principle consists in the decomposition of all the organic matter by heating with sulphuric acid, whereby all of the carbon and hydrogen become oxidized to CO_2 and H_2O , and the nitrogen of such compounds which contain it in combination with hydrogen (such as $=NH$, NH_2 , NH_3), but not with oxygen, appears as ammonia. This is liberated from the acid solution by saturation with $NaOH$; the gas is then distilled over into a known quantity of acid, the amount of which thus neutralized being determined by the titration of the acid still remaining.

Reagents necessary (all nitrogen-free):

1. Concentrated H_2SO_4 (2 parts fuming: 3 parts pure concentrated).
2. Potassium sulphate (powdered).

3. Solution of sodium hydrate, sp. gr. 1.23 (two-thirds saturated).

4. Solution of $\frac{N}{10}$ NaOH.

5. Solution of $\frac{N}{10}$ H₂SO₄.

Detailed Method.—5 or 10 c.c. of urine (according to concentration) are measured out by a pipette and placed in a long-neck digestion (Kjeldahl) flask. To this is added 10 c.c. of the conc. H₂SO₄ and one-half of a teaspoonful of potassium sulphate. The mixture is allowed to boil over a sand-bath or wire gauze until the solution becomes water-clear (3 to 6 hrs.). After the flask has cooled, the contents are removed quantitatively to an Erlenmayer flask (content, 1 liter), using about 400 c.c. of water, and to this solution is added without mixing 40–50 c.c. of the strong NaOH solution. The flask is now quickly connected by means of a rubber cork with a condenser-tube, the other end of which is immersed in a known quantity of $\frac{N}{10}$ H₂SO₄ (50–200 c.c. according to the estimated amount of nitrogen in the urine employed). The vessel used to hold the $\frac{N}{10}$ H₂SO₄ is usually a small Erlenmayer flask. The contents of the large flask are well mixed and a flame placed beneath a wire gauze upon which the flask must rest. After the beginning of ebullition, the boiling should be continued for 45 minutes. This must be regulated so that the NH₃ comes over gradually. At the end of the time the small flask is removed so that the end of the condenser still remains in the flask but is not in contact with the fluid. It must be held in this position for some minutes to allow the condenser-tube to be washed inside with the water still distilling over, and outside with a stream from

the wash-bottle. *Then turn off the flame.* The amount of $\frac{N}{10}$ acid still unneutralized is titrated with $\frac{N}{10}$ NaOH. The difference between this and the amount originally placed in the flask will represent the amount of the $\frac{N}{10}$ acid neutralized by the NH_3 which was distilled over.

1 c.c. $\frac{N}{10}$ $H_2SO_4 = 0.0014$ grm. N., from which may be calculated the quantity of N. in the 10 c.c. used and then in the 24-hour sample.

UREA (HÜFFNER'S METHOD).

For the determination of urea, the following method, whose use has become almost universal, seems sufficiently accurate to warrant its continuance in favor. The rapidity with which it can be performed offsets the disadvantage of the mere approximation of results and renders it pre-eminently suited for clinical purposes.

Only about 92 per cent of the nitrogen of the urea actually present in the urine is obtained as a gas, although the theoretical reaction is quantitative,

(see experiment (e) under Urea), but the deficit is partially diminished by the fact that other substances (e.g., uric acid) also yield up some of their nitrogen as a gas by the decomposition with hypobromite.

The more accurate methods (Mörner-Sjöqvist or Folin's) will be demonstrated.

Quantitative Estimation.

Reagents necessary:

Hypobromite solution (see Appendix).

In making use of the Doremus or similar ureometers, it is advisable to so dilute the urine that the content of urea will not exceed approximately 0.5 per cent.

Rinse the ureometer first with water and then fill it with the hypobromite solution, so that when the apparatus is perpendicular and no air is at the top, the amount of fluid in the bulb covers the opening from it to the upright tube. Then draw up into the pipette the exact amount of urine, and placing it under the surface of the solution with the pipette tip well into the space below the upright tube, force the urine out of the pipette slowly, noting that the bubbles generated all pass upward into the closed tube. Care must be taken that the last drop of urine is expelled from the pipette without allowing any air to escape from it also. Allow the reaction and collection of gas to go on for half an hour, then make a reading on the scale on the areometer of the apparatus. The readings become more accurate when the whole apparatus is immersed in a beaker of water in such a manner that the levels of the fluids in the two vessels correspond. The figures on the scale of the instrument indicate grammes of urea in 1 c.c. of the urine.

URIC ACID (HOPKINS-FOLIN METHOD).

The following method seems to combine considerable accuracy with simplicity and rapidity of performance. It is based upon the fact of the precipitation of the soluble urates by saturation with ammonium salts (Hopkins); and additional accuracy has been obtained by the previous removal of certain substances, e.g., mucoids, phosphates

(Folin). The urates are finally titrated with $\frac{N}{20}$ potassium permanganate.

Quantitative Estimation.

Reagents necessary:

1. 500 grms. $(\text{NH}_4)_2\text{SO}_4$, 5 grms. uranium acetate, and 60 c.c. of 10 per cent acetic acid dissolved in water and the mixture made up to a liter.

2. $\frac{N}{20}$ $\text{K}_2\text{Mn}_2\text{O}_8$ titrated against a $\frac{N}{20}$ solution of oxalic acid.

Place 300 c.c. of urine in a beaker, add 75 c.c. of the ammonium sulphate reagent and mix thoroughly. After the resulting precipitate has settled sufficiently (5 minutes), the mixture is filtered through a double-folded filter. When 250 c.c. of the filtrate have passed through, this volume is divided into two portions of 125 c.c. each, to serve as duplicates. To each portion add 5 c.c. of concentrated NH_4OH , mix thoroughly, and allow them to stand for 24 hours. The precipitated ammonium urate is then transferred quantitatively to a filter, using a 10 per cent $(\text{NH}_4)_2\text{SO}_4$ solution to remove the last portions of the precipitate from the beaker. After removing the filter-paper from the funnel and opening it up, the precipitate is washed with about 100 c.c. of water into the same beaker in which the ammonium urate was precipitated; to this 15 c.c. of conc. H_2SO_4 is added and the mixture immediately titrated with the $\frac{N}{20}$ $\text{K}_2\text{Mn}_2\text{O}_8$ solution, which is added from a burette until the first permanent tinge of pink color appears. 1 c.c. of $\frac{N}{20}$ $\text{K}_2\text{Mn}_2\text{O}_8$ solution = 3.75 mg. uric acid. Calculated the quantity of uric acid in the 24-hour sample.

AMMONIA.

Ammonia in the form of ammonium salts is present in the urine of carnivora to the extent of 0.3–1.2 (average 0.7) grms. for 24 hours. Its function in the body consists in combining with and thus rendering non-toxic the mineral and organic acids which appear in the organism, either after their ingestion or their formation in metabolism. The amount found in the urine is, therefore, markedly increased in certain pathological states (e.g. diabetes) in which more or less large quantities of organic acids make their appearance in the blood as a result of disordered metabolism.

Quantitative Estimation.

Prepare two 500-cc. wide-mouthed Erlenmeyer flasks and one tall cylinder (100 c.c. graduate); into the neck of each insert a two-hole rubber stopper, one hole of which is provided with an L-shaped glass tube, each arm being about 3 inches long, the other hole provided with an L tube, the long arm extending to within one-quarter inch of the bottom of the vessels. A Folin absorption tube may be employed instead of the long arm in the absorption flasks.

Place 25 c.c. of urine in the tall cylinder with about one gram of dry sodium carbonate; cover the urine with one-half inch of crude petroleum to prevent foaming.

To each flask add 20 c.c. $\frac{N}{10}$ H_2SO_4 , 200 c.c. of distilled water and a few drops of an indicator (Congo red or alizarin red). Place the three bottles in series with the cylinder in the middle, and connected in such a manner that the short arm of one bottle is attached to the long arm of the succeeding one. The long arm of the first flask is open to the air; the short arm of the last bottle must be connected with a suction pump. The purpose of the first flask is to insure the passage

of ammonia-free air into the cylinder; the acid in the second flask absorbs the ammonia liberated by the urine and carried over by the current of air sucked through the system. Suction should be continued for about two hours.

The collection flask should finally be titrated with $\frac{N}{10}$ NaOH and the amount necessary for neutralization subtracted from the number of cubic centimeters of acid originally taken. The remainder, when multiplied by 0.0017, gives the amount (grams) of ammonia in 25 c.c. of urine.

PROTEIN.

Quantitative Estimation.

Of the numberless methods which have been suggested for the quantitative determination of the total protein in the urine, the following has received perhaps the most marked approbation from the clinicians. It is based upon the quantitative precipitation by picric acid in the presence of an organic acid, of all forms of proteins which appear in the urine under abnormal conditions. The results obtained are sufficiently accurate if the urine does not contain over 0.4 per cent protein. When more than this is present, the urine must be diluted with water in such amounts as to allow the percentage to fall below that figure.

The procedure is as follows:

Fill the albuminometer to the mark "u" with urine acidified with a few drops of dilute acetic acid, and add Esbach's reagent to "R". Stop the end of the tube with a cork. By inverting several times the contents of the tube can be thoroughly mixed without producing any froth on the top of the mixture. Allow the corked tube to stand upright for 24 hours and then read off the height of the precipitate on the scale, which indicates directly the number of grammes of dry protein contained in a liter of the urine.

DEXTROSE.

All of the methods for the quantitative estimation of dextrose in the urine are founded upon the following properties of this carbohydrate:

1. Reducing action on copper and bismuth.
2. Effect on the plane of polarized light.
3. Fermentation by yeast.

1. The methods of this type assume that the total reducing power of the urine may be ascribed to dextrose. This is probably true in most instances, but the fact of the possible presence in appreciable quantities of other copper-reducing bodies must not be neglected in critical examinations, especially when the reduction is small and at the same time atypical.

The Allihn method is by far the most accurate in results and satisfactory in its performance. Its accuracy, however, is entirely dependent upon the strict attention with which all the details of the procedure are followed out. The cuprous oxide produced by the reducing action of an unknown amount of dextrose upon a definite volume of Fehling's solution is filtered off and further reduced to metallic copper by a stream of hydrogen gas. This metallic copper is then weighed. The quantity of sugar which corresponds to this weight of copper is found upon consultation of tables. The details are too numerous to allow of their explanation in a limited space, but the method is to be recommended highly.

The most common clinical method depends upon the complete reduction as indicated by the entire loss of color, of a given quantity of Fehling's solution, by means of titration with diluted urine.

Method: 10 c.c. of quantitative Fehling's solution are placed in an evaporating-dish and diluted with 40 c.c. of water. The solution is brought to boiling and kept so while the urine (usually diluted ten times) is run in slowly from a burette, until the blue color of the copper solution has entirely disappeared. An accurate determination of the end point of the reaction requires considerable experience.

Since 10 c.c. of Fehling's solution are completely reduced by 0.05 gm. of dextrose, the quantity of urine required to produce the end reaction must contain that quantity of dextrose. From this can be easily calculated the total amount of dextrose in the 24-hour urine.

2. The possible simultaneous appearance in the urine of lvogyrate bodies (e.g. glycuronates, β -oxybutyric acid) renders the application of methods of this type of doubtful value. Even when substances of this kind are apparently absent, comparative values obtained by polarization and reduction methods seldom agree within wide limits. Whenever polarization determinations are made, they must be followed by a second estimation after fermentation.

The urine may be decolorized by the addition of a crystal or two of lead acetate and subsequent stirring and filtration (see Polarization, p. 5).

3. For rough approximations this method, using Einhorn's saccharometer, serves the purpose better than any other. The procedure is described under Monosaccharides, p. 5. A method of this type has also been suggested, based upon the difference of the specific gravity of urine observed before and after fermentation.

PATHOLOGICAL URINARY CONSTITUENTS.

PROTEINS.

Representatives of nearly every class of proteins have been detected in urine at various times. Normal urine undoubtedly contains traces of these substances; thus, for example, in the "mucous cloud" which separates out from many urines upon standing a phosphoprotein can be demonstrated. Of the proteins which appear under well-defined morbid conditions, the most important are the albumins, the globulins, and the proteoses. So-called peptone has also been noticed. Since, however, the conception of the properties of the mixture which passes under this name is so ill-defined and changeable, it is probably better in the future to dismiss the term peptonuria. Hæmoglobin and related substances may also escape from the blood into the urine.

ALBUMIN AND GLOBULIN.

Serum albumin and serum globulin usually appear together in the urine under the name of albuminuria; but concerning the relative quantities of the two bodies under the various conditions very little is known. The two proteins allow of separation in the same manner as was employed under Blood Proteins, p. 73.

In testing urines suspected of containing protein, the fluid should always be perfectly clear. This can be accomplished either by repeated filtration through paper or asbestos or by shaking with magnesia.

The following tests are best suited for ordinary conditions:

(a) *Heat Test*.—Heat 5 c.c. of clear urine to boiling and add

1 to 3 drops of dilute acetic acid. If the urine contains more than a small amount of albumin, this will settle out in flocks after the addition of acid. When mere traces are present the solution may only become turbid and should then be compared to the urine before heating, but to which the same amount of acid has been added. This test is only of value as a *positive* one. When a negative result is obtained other tests should be tried with a view to confirmation.

In some cases the addition of acetic acid to boiled urine may give rise to a faint precipitate or turbidity which disappears upon shaking. This is caused by the formation of acid albumin, which may be salted out by one-third saturation with NaCl after the addition of more acetic acid to keep the phosphates in solution. Faintly alkaline or amphoteric urine may sometimes give on heating a precipitate due to phosphates, which is sometimes difficult to distinguish from a precipitate of albumin. Again, such a urine may remain perfectly clear and still contain albumin. The addition of a small amount of acid will, in the first case, dissolve the phosphates, and, in the second, precipitate any albumin remaining soluble. Phosphoproteins nor proteoses do not react similarly, since both bodies are soluble in hot acid solutions; but a precipitate settling out upon cooling may point to the presence of such substances. Resins resulting from the administration of petroleum, turpentine, oil of sandal-wood, tolu-balsam, etc., may be present in the urine, and if so, will be precipitated by the acid. Such precipitates easily dissolve in alcohol.

(b) *Heller's Test*.—Place 5 c.c. of conc. HNO_3 in a test-tube and allow a few c.c. of the urine which is being filtered to flow from the funnel down the sides of the tube. The urine will thus stratify itself on top of the acid, and at the *surface of contact* of the two liquids the albumin will be precipitated

and will appear as a *white ring*. As the HNO_3 upon standing diffuses upwards into the urine the ring may become broader. This test is delicate enough for ordinary clinical purposes, but will not show the presence of traces. Urines containing an excessive amount of urea may form a crystalline precipitate in this test, but such a precipitate cannot be confused with albumin. Colored rings may also form, due to the oxidation of the urinary pigments, and a blue coloration is produced by indican. Substances mentioned under the heat test, such as resins, etc., may form a cloud, but simple tests such as those indicated will exclude them.

(c) *Roberts' Modification of Heller's Test*.—This test is performed similarly to the last by the stratification of a few c.c. of the urine upon 5 c.c. of Roberts' reagent. It has some optical advantages and colored rings never appear in its use.

(d) *Acetic Acid and Potassium Ferrocyanide Test*.—Acidify 5 c.c. of urine with two drops of acetic acid and add, drop by drop, a dilute solution of K_4FeCN_6 . In the presence of albumin a white precipitate occurs which dissolves in a large excess of the reagent. Traces of albumin may be detected with this reaction. Should the precipitate dissolve upon heating, proteoses may be suspected. The presence of a considerable amount of mucin or phosphoprotein in the urine may sometimes give rise to a precipitate with acetic acid alone. This must be removed by filtration before the ferrocyanide test can be completed.

(e) *Trichloroacetic Acid Test*.—Stratify a few c.c. of a concentrated aqueous solution of this reagent with 5 c.c. of urine. A white ring sharply defined indicates the presence of albumin. The precipitate may also be proteoses, but in this case the ring dissolves with cautious warming. This test is more delicate than Heller's, and by its use smaller quanti-

ties of albumin are demonstrable in urines with which the more common tests yield negative results.

(f) *Spiegler's Test*.—Stratify 5 c.c. of urine which has been slightly acidified with acetic acid, upon a few c.c. of Spiegler's reagent. In the presence of albumin a white ring appears at the line of contact of the two liquids. This test is very sensitive, showing albumin in a dilution 1:250,000. In fact most normal urines indicate protein with the reagent. This must be borne in mind in making deductions from its use. Urines containing iodides give a precipitate of HgI_2 .

(g) *Tanret's (Bouchardat) Test*.—This reagent is added, drop by drop, to 5–10 c.c. of the urine until a turbidity or precipitate appears. The reagent precipitates besides albumin, mucin, peptone, and alkaloids. In cases where the presence of alkaloids in the urine is suspected, the peptone and alkaloids may be dissolved in potassio-mercuric iodide and the solution shaken out with ether, whereby the alkaloid is dissolved.

PROTEOSES.

Non-coagulable bodies which are precipitated by saturation with $(\text{NH}_4)_2\text{SO}_4$ and which give the biuret reaction, have been demonstrated and isolated from urines under different conditions. These substances react positively to nearly all of the proteose reactions, and it seems definitely decided that true proteoses actually appear at certain times in the urine. Of especial interest is the presence of an albumose-like substance (Bence-Jones' body) associated with multiple myelomata of the bone.

The identification of these bodies follows from the use of the same tests employed under Digestion, p. 54.

DEXTROSE.

Before testing for dextrose in the urine, protein, if present, *must* be removed by heat and acetic acid. The following tests depend upon the power of dextrose to reduce metallic oxides, as evidenced by the formation of precipitates or color changes. It must be remembered that the urine may also contain other bodies such as creatinine, uric acid, allantoin, hydroquinol, alkaptonic acid, urine and bile pigments, and conjugate glycuronic acids, which also reduce metallic oxides to a slighter degree, however. It is, therefore, better never to base a decision entirely upon reduction tests.

Never allow the urine to boil more than a few seconds in performing the tests. This will tend to eliminate the possibility of a reduction caused by the above-mentioned substances.

(a) *Trommer's, Fehling's, and Fermentation Tests.*—Perform these tests as suggested under Monosaccharides (p. 4), using the urine instead of the dextrose solution.

(b) *Benedict's Modification of Fehling's Test.*—To 5 c.c. of Benedict's solution add five to eight drops of urine. Boil the mixture vigorously for one or two minutes and allow the test tube and contents to cool on the rack. A precipitate will form in the solution, the color of which may be red, yellow, or green, according to the amount of dextrose present. This test is more sensitive than Fehling's and has the advantage that the test solution does not deteriorate upon long standing.

(c) *Nylander's Test.*—To 10 volumes of urine add 1 volume of Nylander's reagent and heat. The presence of sugar is indicated by a dark coloration of the urine followed by a separation of a black precipitate. By this very sensitive test the reducing character of some normal urines may be shown.

Urines containing sulphides cannot be tested by this method. Why?

(d) *Phenylhydrazin Test*.—Perform this test as given under the Monosaccharides, using 15 c.c. of urine. As other substances in the urine may give a precipitate with the reagent, a mere separation of an insoluble body is not sufficient evidence for the presence of a sugar. The precipitate must be yellow and must be examined *carefully* and *critically* under the microscope. If sufficient quantities are obtainable for a melting-point determination, this procedure should be carried out. Phenylglucosazone melts at 204–205° C.

(e) *Polarization Test*.—As it was stated in the discussion of the value of this test for quantitative purposes, the results obtained are apt to be misleading, especially when no polarization of the urine can be detected. When taken as evidence confirmatory of other tests it may serve a useful purpose. The dextro-rotation must completely disappear after fermentation.

BILE.

The presence of bile imparts to the urine a saffron color, which upon standing takes on a greenish tinge. Icteric urine is usually cloudy or turbid and the sediment, if any, is rather strongly colored.

In order to insure a positive identification of icteric urine by means of the bile *acids* it is necessary to separate them from the urine by a long and laborious procedure and then to perform Pettenkofer's test. Though this is the more accurate method, still it is more usual clinically to perform tests for the presence of the biliary *pigments* directly on the urine.

(a) Perform Gmelin's, Smith's, Hammarsten's, and Huppert's tests.

(b) *Rosenbach's Modification of Gmelin's Test*.—Filter

some icteric urine and to the moistened paper add *one* drop of HNO_3 . Colored rings around the drop correspond in color and arrangement to those obtained in Gmelin's test. Caution: Impure filter-paper may give this test, so make a control test in using strange paper.

(c) *Haycraft's Test*.—Fill a test-tube half full of *fresh* urine and sprinkle on the surface powdered sulphur. If the sulphur sinks to the bottom the presence of bile salts is indicated. This can only be used as a confirmatory test.

BLOOD AND BLOOD PIGMENTS.

Blood, as such, may be present in the urine (hæmaturia). In these cases the urine is cloudy, brownish-red in color, and contains serum albumin and serum globulin. Upon microscopical examination blood corpuscles are found in the sediment.

In hæmoglobinuria the form elements are absent and the urine holds the oxyhæmoglobin in solution; methæmoglobin often accompanies it, and hæmatin appears under some conditions. In a number of diseases, but especially after the use of sulphonal and similar therapeutic agents, hæmatoporphyrin has been observed in large quantities. Reduced hæmoglobin is never present.

OXYHÆMOGLOBIN.

(a) Notice the color of the urine. If fresh it has a reddish tinge and is turbid. Try the *benzidine reaction*, (g) p. 73.

(b) Examine it with the spectroscope. If the urine is not fresh be on the lookout for methæmoglobin. Warm a portion of the urine with an excess of NaOH ; filter and add a few drops of $(\text{NH}_4)_2\text{S}$. Oxyhæmoglobin is changed to hæmochromogen with its characteristic spectrum.

(c) *Heller's Test* —Make the urine strongly alkaline with

NaOH and heat it. The oxyhæmoglobin is split into hæmatin and protein, and the earthy phosphates being precipitated, drag down the hæmatin with them. Filter off the precipitate, which should be of a brownish color, and after drying it, try to obtain Teichmann's crystals (p. 76).

(d) *Struve's Test*.—Make a portion of the urine alkaline with NaOH and precipitate with tannic acid. Test this precipitate for hæmin.

METHÆMOGLOBIN.

Examine the urine with the spectroscope, add a few drops of NH_4OH and filter; a two-banded spectrum appears similar to that of oxyhæmoglobin. Do not confound the spectra of methæmoglobin and of hæmatin in acid solution.

HÆMATIN.

1. Examine in the spectroscope. If a single band is present add $(\text{NH}_4)_2\text{S}$ to the urine, filter, and again examine it. The two bands of reduced hæmatin should appear.
2. Make tests *c* and *d* under Oxyhæmoglobin.

HÆMATOPORPHYRIN.

The preparation of this substance from the urine for the purposes of identification and spectroscopic examination is as follows:

Method of Garrod.—To 100 c.c. of urine add 20 c.c. of 10 per cent NH_4OH . The phosphates of the earthy metals are precipitated and with them the hæmatoporphyrin. This precipitate is filtered off, washed, and warmed in a flask with acidulated alcohol. The pigment goes into solution. Use

this for the spectroscope. Upon the addition of a small amount of water the alcoholic extract will exhibit a red fluorescence. For tests see under Blood, p. 78.

This substance has come into prominence of late as a protein decomposition product containing sulphur in the neutral or lead-blackening form. In fact, the cystine which can be obtained in the decomposition of certain proteins contains an amount of *lead-blackening* sulphur which corresponds closely to the *total* sulphur of the original protein molecule. This would seem to imply that the sulphur of some proteins was present in the molecule as a cystine nucleus, and that proteins containing large amounts of neutral sulphur have present large numbers of cystine groups in the molecule. Cystine is also closely related to Taurine,

a substance which contains oxidized sulphur in organic form and which probably represents an intermediate stage of oxidation between the neutral sulphur atom of cystine and the mineral sulphates of the urine. Cystine, upon standing, separates out of the urine as colorless six-sided plates, insoluble in water, acetic acid, alcohol, and ether. It sometimes is found in the form of calculi. Cystineuria has repeatedly been observed as an apparently anomalous metabolism of

the sulphur, peculiar to certain families, all the members of which excrete normally relatively large quantities of the substance (0.5–1.0 grm. for 24 hours).

The appearance of the characteristic crystals and an abnormal amount of lead-blackening sulphur in the urine is sufficient proof of the presence of cystine.

FATTY ACID DERIVATIVES.

Associated with certain peculiar metabolic disturbances, three closely related substances (β -oxybutyric acid, diacetic acid, and acetone) may appear in the urine either separately or together. They have been noticed in severe cases of diabetes, scarlet fever, cachexia, etc., and are the cause of that aromatic fruity (apple-like) odor which is so frequently present in diabetic urines. β -oxybutyric acid only appears in conjunction with acetone or diacetic acid, but the latter two are often found alone in the urine.

It is only necessary to test for this substance in urine which contains diacetic acid. Since this body is lævogyrate and non-fermentable, the urine after fermentation should turn the plane of polarized light to the left. This is not sufficient evidence, however, since the conjugate glucuronates also are lævogyrate after fermentation. The following test had better be added as confirmatory of β -oxybutyric acid.

Evaporate some fermented urine to a syrup and after the addition of an equal volume of concentrated H_2SO_4 distil directly without cooling; α -crotonic acid $\text{CH}_3\text{—CH}=\text{CH} \cdot \text{C} \begin{array}{l} \diagup \text{O} \\ \diagdown \text{OH} \end{array}$ is formed and distils over, and the acid

crystallizes out from the distillate. The crystals are soluble in ether and melt at 72° C.

The urine must be tested soon after voiding, as this body disappears upon standing.

Strongly acidify some urine with H_2SO_4 and shake it out with ether. Separate the ether from the solution and shake out the former with water which is just colored with Fe_2Cl_6 . The watery solution becomes violet and upon the addition of more Fe_2Cl_6 turns bordeaux-red.

The ferric chloride solution may also be added to the urine directly. In this case the phosphates must be completely removed by the Fe_2Cl_6 and filtration. Then add more Fe_2Cl_6 to the filtrate.

Salicylic acid and salicylates give a similar reaction. When these bodies are present they may be removed from the acidified urine by shaking it out with chloroform or benzene in which the diacetic acid is not soluble. The urine is then treated with ether as above.

Distil 100 c.c. of urine to which has been added 2 c.c. of 50 per cent acetic acid. Take the first 50 c.c. of the distillate, add 1 c.c. of concentrated H_2SO_4 diluted 8 times and redistil over 25 c.c. Make the following tests with this solution:

(a) *Lieben's Test*.—Place some of the solution in a test-tube and make it alkaline with sodium carbonate. Add

enough iodo-potassium iodide to give the solution a decided yellow color. Warm at 65° C. for 5 minutes and allow to cool. A yellow precipitate of iodoform settles out, recognizable by its odor and its hexagonal crystals. This test is also given by alcohol.

(b) *Gunning's Modification.*—In this test ammonia is substituted for the sodium carbonate and a tincture of iodine in place of the iodopotassium iodide. A black precipitate of iodide of nitrogen is first formed, but this gradually disappears on standing, leaving the iodoform visible, if present. Alcohol or aldehydes do not give this reaction.

(c) *Legal's Test.*—To a few c.c. of the distillate add a few drops of a freshly prepared solution of sodium nitroprusside and make the solution alkaline with NaOH. A ruby-red color is produced which quickly disappears. Creatinine also gives this reaction. If the alkaline acetone solution is treated with a large excess of acetic acid, the color becomes red, whereas in the case of creatinine it is changed to green and then blue.

(d) *Lange's Modification of Legal's Test.*—To 15 c.c. of urine add one c.c. of glacial acetic acid, and two or three drops of a freshly prepared solution of sodium nitroprusside. Mix this solution well and carefully stratify upon it concentrated ammonium hydroxide. At the line of contact a violet ring will appear. This reaction is not given with creatinine, alcohols or aldehydes.

EHRlich's DIAZO REACTION.

Diazobenzenesulphonic acid comes into prominence as a reagent in various connections.

It is employed in the detection of sugar, protein, bilirubin, and an unknown chromogen of the urine which is so commonly present associated with certain pathological conditions (typhoid, pulmonary tuberculosis, etc.). It is especially as a test for this unknown substance or substances that

diazobenzenesulphonic acid was used by Ehrlich and has since been designated by the clinicians as Ehrlich's Diazo-reaction. The fact must not be forgotten, however, that, as a test for bilirubin this reagent has also been employed by Ehrlich, and by others to determine the presence of sugar and protein in the urine. The color obtained in the reaction in the case of these two latter substances does not differ greatly from that of the so-called Ehrlich's reaction, and on this account the possibility of error in interpretation must always be borne in mind.

Ehrlich's diazo-reaction is usually performed as follows:

An equal volume of a freshly prepared solution of diazobenzenesulphonic acid is added to the urine and the mixture is then rendered alkaline with an excess of NH_4OH . An orange color develops in ordinary cases, but in certain urines there results a *red* color which may vary from a carmine to a deep ruby-red; upon shaking the solution the froth also partakes of the color. Sometimes a green or violet precipitate will settle out upon standing.

The formation of the fresh diazobenzenesulphonic acid is effected by the previous preparation of the two following solutions:

1. 1 gram. sulphanilic acid and 50 c.c. of conc. HCl dissolved in a liter of water.

2. 5.0 gram. sodium nitrite dissolved in a liter of water.

Just previous to use these two solutions are mixed in the proportion 40:1 and the mixture used as above.

The free nitrous acid which is liberated by the action of the HCl upon the NaNO_2 , reacts with the sulphanilic acid with the formation of diazobenzenesulphonic acid, according to the equation

SEDIMENTS.

UNORGANIZED.

Separating from a urine which is **acid in reaction**, the following sediments may be present:

1. CRYSTALLINE TYPE.

(a) *Uric Acid*.—Color, golden brown. To what is this due? Does not dissolve upon warming. Soluble in NaOH and reprecipitated by HCl. Responds to the murexide test. Very characteristic crystalline form under the microscope.

(b) *Calcium Oxalate*.—Usually present mixed with uric acid. Colorless. Dissolves easily in HCl, but is insoluble in acetic acid. (See Triple Phosphate, with which there is the possibility of confusion.) Under the microscope the crystals are transparent, refractive, octahedral (envelope shape).

(c) *Bilirubin and Hæmatoidin*.—The former crystallizes in golden or brown rhombic plates or needles. Dissolves easily in alkalies and chloroform and gives Gmelin's reaction. The latter is similar in crystalline form, but does not dissolve in alkali and gives a blue coloration with HNO_3 .

(d) *Cystine*.—Under the microscope it appears as superimposed six-sided plates, which are insoluble in acetic acid, but soluble in NH_4OH (differing from uric acid).

(e) *Tyrosine, Leucine, and Xanthine*.—Very rare. For tests see under these substances.

(f) *Phosphates*.—1. Magnesium Phosphate. Rhombic plates, soluble in acetic acid, slightly attacked by ammonium carbonate. 2. Calcium Phosphate. Soluble in acetic acid. Crystals wedge-shaped, seldom found. 3. Ammonio-magnesium Phosphate (Triple Phosphate). These separate only when the reaction is *weakly acid* or amphoteric.

(g) *Potassium Sulphate*.—Long colorless needles, insoluble in NH_4OH or acids; seldom found.

2. AMORPHOUS TYPE.

(a) *Uric Acid Salts* (Acid Urates).—Brick-red or brownish-red in color. Dissolves upon warming and gives the murexide test. Upon the addition of a mineral acid, free uric acid separates out in small crystalline form.

(b) *Calcium Oxalate*.—Dumb-bell shape. See above for detection.

(c) *Calcium Sulphate*.—Dumb-bell shape, insoluble in HCl .

(d) *Fat*.—Strongly refracting round drops, soluble in ether.

Sediments separating from an **alkaline** reacting urine:

1. CRYSTALLINE TYPE.

(a) *Triple Phosphate*.—Dissolves easily in acetic acid; unchanged by ammonium carbonate (see Magnesium Phosphate); appears under the microscope as large colorless prisms (coffin-cover shape). Upon warming gives off NH_3 .

(b) *Ammonium Urate*.—Dissolves in HCl or acetic acid, followed by the separation of free uric acid crystals (rhombic form). Forms dark balls with needles radiating from the circumference (chestnut-burs). Gives off NH_3 upon heating on a platinum foil.

(c) *Magnesium Phosphate*.—See under Acid Urine.

2. AMORPHOUS TYPE.

(a) *Earthy Phosphate*.—Dissolves in acetic acid without the development of gas.

(b) *Earthy Carbonate*.—Dissolves in acetic acid with effervescence.

(c) *Calcium Carbonate*.—Dumb-bell shape. Soluble in acetic acid, with an escape of gas. (Compare Calcium Oxalate.)

SCHEME FOR IDENTIFYING SEDIMENTS.

On heating the sediment on a platinum foil it					
Does not char.			Does char.		
Fresh sediment treated with HCl			Fresh sediment gives the murexide test.		
Does not effervesce.			Effervesces.	Sediment treated with NaOH gives	
Fresh sediment gently heated and then treated with HCl				Ammonia.	No ammonia.
Does not effervesce.				Ammonium urate.	Uric acid or urates.
Fresh sediment moistened with NaOH					
NH ₃	No NH ₃	Effervesces.	Calcium carbonate.		
Triple phosphate.	Ca or Mg phosphate.	Calcium oxalate.			

APPENDIX.

The methods for the preparation of the solutions, reagents, and material of which use is made in the laboratory work outlined in the preceding pages, are as follows:

SOLUTIONS.

Hydrochloric acid, 0.2 per cent; contains 6 c.c. of conc. HCl to the liter of water.

Acetic acid, 20 per cent in water.

Silver nitrate, 2 per cent in distilled water.

Barium chloride, 10 per cent in distilled water.

Picric acid, a cold saturated solution.

Tannic acid, 5 per cent in water.

Phosphotungstic acid, 10 per cent in water.

Zinc acetate, 1 per cent in 95 per cent alcohol.

Zinc chloride, saturated water solution, diluted with 95 per cent alcohol to a sp. gr. 1.20; filtered.

Ammonium oxalate, a cold saturated solution.

Alcoholic potash, 10 per cent KOH in 95 per cent alcohol.

Iodine solution, 0.5 per cent solution of potassium iodide saturated with iodine.

Sodium alcoholate, sodium dissolved in absolute alcohol.

Potassium permanganate $\frac{N}{10}$, 3.16 grms. in a liter.

Potassium mercuric iodide, same as Tanret's reagent without the addition of acetic acid.

REAGENTS.

Ammonium Molybdate.

50 grms. molybdic acid dissolved in 200 grms. 10 per cent NH_4OH ; pour this solution slowly into 750 grms. HNO_3 (sp. gr. 1.2); allow the mixture to stand for several days; then filter.

Ammoniacal Silver Nitrate.

2.5 per cent in water; to this is added NH_4OH until the precipitate is completely dissolved.

Barfoed's Reagent.

4 per cent copper acetate in water; the solution made faintly acid with acetic acid and filtered.

Benedict's Solution.

Dissolve with heat 173 grams of sodium citrate and 100 grams of anhydrous sodium carbonate in about 600 c.c. of water. Dissolve 17.3 grams of cupric sulphate in about 150 c.c. of water. Mix the two solutions by slowly pouring the cupric sulphate into the carbonate-citrate solution. Make the volume up to 1000 c.c.

Bial's Reagent.

500 c.c. of 30 per cent HCl in which are dissolved 1 gm. of orcinol and 25 drops of a 63 per cent solution of crystalline ferric chloride.

Esbach's Reagent.

10 grms. picric acid and 20 grms. citric acid dissolved in a liter of water.

Fehling's Solution.

A mixture in equal volumes of the following solutions:

1. Copper sulphate solution, 69.28 grms. *crystalline* CuSO_4 dissolved in water and made up to a liter.

2. Alkaline tartrate solution, 346 grms. crystalline Rochelle salts dissolved in 350 c.c. of water and 250 grms. NaOH dissolved in 300 c.c. of water; these two mixed and the volume made up to a liter.

Hammarsten's Reagent.

1 volume HNO_3 and 19 volumes HCl (both acids about 25 per cent). This acid mixture should be kept at least a year. Then mix with it four times its volume of 95 per cent alcohol.

Hopkins-Cole Reagent.

60 grms. sodium amalgam added to 1 liter of a saturated solution of oxalic acid. After the development of gas has ended, filter and dilute with 2 or 3 volumes of water.

Hypobromite Solution.

Mix 100 c.c. of each of the following solutions and dilute with 300 c.c. of water.

1. 125 grms. bromine and 125 grms. sodium bromide dissolved in water and the volume made up to 1000 c.c. Keep in stoppered bottle.

2. A solution of sodium hydrate with a sp. gr. 1.250.

Magnesium Mixture.

100 grms. MgSO_4 and 200 grms. NH_4Cl dissolved in 800 c.c. of water; to this add 400 grms. concentrated NH_4OH . Mix thoroughly and keep in glass-stoppered bottle.

Millon's Reagent.

To a given amount of metallic mercury add twice its weight of HNO_3 (sp. gr. 1.42); after the evolution of gas has ceased, warm slightly and then dilute the mixture with 2 volumes of water. Allow this to stand at least 24 hours; then filter.

Mörner's Reagent.

1 volume formalin, 45 volumes distilled water and 55 volumes concentrated H_2SO_4 ; thoroughly mixed.

Nylander's Reagent.

2 grms. bismuth subnitrate and 4 grms. Rochelle salts digested on the water in 100 c.c. of 10 per cent NaOH ; cool and filter.

Obermayer's Reagent.

1 liter of fuming concentrated HCl to which has been added 2-4 grms. ferric chloride.

Roberts' Reagent.

1 volume concentrated HNO_3 mixed with 5 volumes of a saturated solution of MgSO_4 .

Spiegler's Reagent.

8 grms. mercuric chloride, 4 grms. tartaric acid, and 20 grms. glycerol dissolved in 200 c.c. of water.

Tanret's Reagent (Potassium-Mercuric-Iodide).

3.32 grms. potassium iodide dissolved in 20 c.c. of water; to this add 1.35 grms. mercuric chloride also dissolved in 20 c.c. of water; dilute the mixture to 60 c.c. and mix with it 20 c.c. glacial acetic acid.

Uffelmann's Reagent.

1 per cent solution of carbolic acid in water colored faintly amethyst with a solution of ferric chloride.

INDICATORS.

Alizarin.

1 grm. dissolved in 100 c.c. of water and filtered.

Boas' Reagent.

5 grms. resorcinol and 3 grms. saccharose dissolved in 100 c.c. 95 per cent alcohol.

Congo Red.

1 grm. dissolved in 90 c.c. of water to which is added 10 c.c. of 95 per cent alcohol.

Dimethylaminoazobenzene.

0.05 grm. dissolved in 100 c.c. 95 per cent alcohol.

Günzburg's Reagent.

2 grms. phloroglucinol and 1 grm. vanillin dissolved in 100 c.c. 95 per cent alcohol. (Does not keep well.)

Phenolphthaleïn.

1 grm. dissolved in 100 c.c. 95 per cent alcohol.

Tropæolin OO.

0.05 grm. dissolved in 100 c.c. 50 per cent alcohol.

MATERIAL.

Fusion Mixture.

5 parts Na_2CO_3 in substance and 1 part KNO_3 in substance, intimately mixed.

Litmus Milk.

Add a strong filtered solution of litmus to fresh milk until the latter is decided bluish in color.

Neutral Olive-oil.

Ordinary olive-oil thoroughly shaken with a 10 per cent solution of Na_2CO_3 ; then extract the mixture with ether. The residue after the evaporation of the ether is neutral fat.

Oxalated Blood Plasma.

As the blood flows from an artery, allow it to mix thoroughly in a beaker with an equal volume of 0.2 per cent ammonium oxalate solution.

Salted Blood Plasma.

Allow the blood flowing from an artery to mix thoroughly with an equal volume of a saturated solution of Na_2SO_4 or 10 per cent NaCl . Place the mixture away in a cold place and let it remain for 24 hours.

Pancreatic Extracts.

Proteolytically active: finely divided gland digested for three days with water containing 5-10 c.c. of chloroform to the liter, or with a saturated solution of NaCl .

Amylolytically active: water or glycerol extract of the gland previously allowed to stand exposed to the air for

24 hours; or finely divided gland digested with saturated solution of NaCl; or digestion of the finely comminuted gland at 40° C. in 0.7 per cent NaCl.

Lipolytically active: slightly alkaline watery infusion of the gland; or an alkaline glycerol extract of the *fresh* gland (9 parts glycerol and 1 part 1 per cent Na_2CO_3 solution).

Rennetically active: finely minced fresh gland digested with 4 times its weight of 25 per cent alcohol for 4 to 5 days. Then filter after the addition of a *trace* of acetic acid.

INDEX.

	PAGE		PAGE
Acetone	131	Basic radicals in urine	90
Acetic acid and potassium ferrocyanide test	19, 123	Bayberry wax, saponification	10
Achroödextrin	44	Benedict's reagent	138
Acid albuminate	54	test for dextrose	125
Acidic radicals in urine	89	Benzidine reaction	73, 127
Acidity of urine, quantitative determination of	105	Bial's reagent	101, 138
Adamkiewicz' test	18	Bile	67
Albumin	21	acids, conjugate	68
, coagulation of	21	pigments, tests for	69, 70
in urine	121-124	salts, crystallized (Platt- ner's)	69
Albuminates, acid and alkali	24	Biliary calculus, analysis of	70
Albuminoids	27, 28	Bilirubin	69
Alcohol as protein precipitant	20	in sediments	134
Alizarin as indicator	48	Biliverdin	69
, preparation of	141	Biurates	92
Amino bodies	54	Biuret test	18
Ammonia in urine	117	Blood, general	72
, quantitative deter- mination of	117	pigments	75
Ammoniacal silver nitrate, preparation of	138	in urine	127
Ammonium molybdate, prep- aration of	138	, spectroscopic examination of	77
sulphate as pro- tein precipitant	20	plasma	74
urate	135	, oxalated	74
Amygdalin	44	, prepara- tion of	142
Amylolysis	44, 60	, salted	74
Amylopsin	56, 60	, prepara- tion of	142
Amyloses	1, 7, 8	platelets	75
Arabinose	2	Blood proteins	73
Appendix	137	in urine	127
<i>Bacterium Micrococcus ureæ</i>	91	Boas' reagent, test for HCl	47
Barfoed's test	4	, preparation of	141
reagent, prepara- tion of	138	Boettger's test	125
		Bone	36
		, mineral constituents	36
		, ossein of	36

	PAGE		PAGE
Calcium carbonate in sedi-		Depression of freezing-point	
ment	136	of urine	103
oxalate in sediment. . . .	134	Deuteroproteoses	52
phosphate in bone		Dextrin	7
ash	36	Dextrose, tests for	4
phosphate in milk ..	83	in blood	74
in sediment	135	in urine	125
sulphate in sediment	135	Diacetic acid in urine	131
Cane-sugar	6	Dialysis experiment	23
Carbohydrates	1	Digestion, gastric	46
in urine	100	, peptic	51
Carbon, test for	2	, pancreatic	56
Carbonates in bone ash	37	, tryptic	57
, earthy, in sediment	136	Dimethylaminoazobenzene as	
Casein	55	indicator	47
, pancreatic	61	, preparation of	141
Caseinogen, test for	82	Disaccharides	1, 5, 7
Celluloses	7	Edestin	23
Cerebrin	40	Ehrlich's diazo reaction. . . .	132, 134
Cerebrosides	38, 40	Electrical conductivity of	
Chlorides in bone ash	36	urine	104
in urine	89	Emulsification of fats	13
, quantitative de-		Enzymes, preparation of. . . .	142
termination of. . . .	106	Erythrodextrin	44
Cholesterol in bile	70	Esbach's reagent, preparation	
in biliary calculus	70	of	138
in nervous tissue. . . .	41	Fat	9
Chymosin (<i>see</i> Rennin).		, emulsification of	13
Coagulation of proteins. . . .	21	in milk	83
, method of de-		in sediment	135
termination	22	, saponification of. . . .	10, 11
CO-hæmoglobin	79	Fatty acids, derivatives in	
Collagen	27	urine	130
Color reactions for proteins. .	17	in nervous tissue	38
of urine	86	, preparation of. . . .	11
Conductivity, electrical, of		, reactions for. . . .	12
urine	104	Fehling's test	4
Congo-red as indicator	47	solution, prepara-	
, preparation of. . . .	141	tion of	139
Conjugate sulphates. . . .	95, 109	Fermentation of dextrose. . .	5
proteins. . . .	15, 25-27	of saccharose. . . .	7
Copper sulphate as protein		Fibrin	74
precipitant	20	Form elements in blood	75
Creatine in muscle	31	Freezing-point, depression of,	
, transformation into		in urine	103
creatinine	31	Fusion mixture, preparation of	142
Creatinine, preparation from		Galactose	3
creatine	31	Garrod's method for hæmato-	
in urine	95	porphyrin	128
Cresol, p-	64, 96		
Cystine in urine	129		
in sediment	134		

	PAGE		PAGE
Gastric digestion	46	Hopkin's-Folin method for	
juice	46	uric acid	115
Gelatin, tests for	28	Hüfner's method for urea . . .	114
Globulin in urine	121	Huppert's test for bile pig-	
, tests for	23	ments.	70
Glucose (<i>see</i> Dextrose).		Hydrochinol, conjugate in	
Glycerol	14	urine	96
Glycocholic acid	68	Hydrochloric acid in gastric	
Glycogen in muscle	34	juice	46
, tests for	35	Hydrogen, test for	2
Glycoprotein	25	Hypobromite solution, prepa-	
Glycoproteose	53	ration of	139
Glycuronic acid	102	Hypoxanthine	32
Gmelin's test for bile pigments	70		
Guaiac reaction for blood . . .	73	Indican	97
for milk	81	Indicators	47
Gum arabic	2	Indigo-carmin test for lactose	84
cherry	2	Indole in intestinal putrefac-	
Gunnings' modification of		tion	62-64
Legal's test for acetone . . .	132	conjugate in urine . . .	96
Günzburg's reagent, test for		Inorganic compounds in urine	88
HCl	47	Intestinal putrefaction	62
, prepara-		Inversion	6
tion of	141	Invertin	6
		Iron in bone ash	37
Hæmatin	75	in protein	17
in urine	125	, qualitative test for	17
, reduced	78	Jaffe's test for creatine	37
Hæmatoidin in sediment . . .	134	for indican	97
Hæmatoporphyrin	78		
in urine	128	Keratin, test for	28
Hæmaturia	128		
Hæmin crystals, test for blood	76	Lactic acid, test for, in stom-	
Hæmochromogen	78	ach contents	47, 48
Hæmoglobin	75, 77	Lactose	5
carbon monoxide		in milk	84
(<i>see</i> CO-hæmoglobin).		Lard, saponification of	11
Hæmoglobinuria	127	Lead oleate	12
reagent, prepa-		Lecithins	39
ration of	139	Lecithoproteins	15
Hammarsten's test for bile		Legal's reaction for acetone .	132
pigments	70	for indole	64
for indican	97	Leucine, tests for	59
Haycraft's test	127	in sediment	134
Heller's test for protein	19	Levulose	3
for oxyhæmoglob-		Lieben's test for acetone . . .	131
in	127	Liebermann's test for cholest-	
Heteroproteose	52	erol	42
Hexoses	1	Lipoids of nervous tissue . . .	38
Hippuric acid	98	Lipolysis	60
Hopkins-Cole reagent, prepa-		Litmus milk	60
ration of	139	, preparation of	142

	PAGE		PAGE
Magnesium in bone ash	37	Nitrogenous extractives	
mixture, preparation		of muscular tissue	30
tion of	139	of nervous tissue	38
Magnesium sulphate as pro-		Nucleins,	26
tein precipitant	20	, tests for	27
Maltodextrin	44	Nucleoproteins	26
Maltose	5, 44	Nylander's reagent, prepara-	
Metacasein reaction	61	tion of	140
Methæmoglobin	78	test	4, 125
Milk, general	80		
, qualitative separation		Obermeyer's reagent, prepar-	
of the constituents of . .	81	tion of	140
Millon's reagent, preparation		test for indican	97
of	140	Odor of the urine	86
test for proteins	18	Oleate, lead	12
Mineral constituents of bone .	36	Oleic acid	12
Monosaccharides	1, 3, 5	Olive-oil, neutral	13
Moore's test for hexoses . . .	4	, preparation of	141
Mörner's reagent, preparation		Orcinol as test for pentoses .	3
of	140	Ossein of bone	27, 36
test	59	Oxalic acid	97
Mucins in bile	67	preparation from urine	98
in saliva	43	β -oxybutyric acid in urine . .	130
, tests for	25	Oxyhæmoglobin	75, 127
Murexide test	94		
Muscular tissue	29	Palmitic acid	11
, nitrogenous		Pancreatic digestion	56
extractives of	30	extracts, prepara-	
, non-nitroge-		tion of	138
nous ex-		rennin	56, 61
tractives of	34	Pentosanases	2
, proteins of	29	Pentoses, tests for	2
Myogen	29	in urine	101
Myosin	30	Pepsin	49-51
		Pepsinogen	50
Nervous tissue	38	Peptic proteolysis	51
, cerebrin of	40	Peptones	51, 53, 54
, cholesterol of	38, 41	in intestinal putre-	
, fatty acids of	38	faction	62
, lecithins of	39	Pettenkofer's test for bile	
, lipoids of	38	acids	68
, neurokeratin		Phenol, conjugated in urine .	96
of	38	Phenolphthalein as indicator .	48
, proteins of	38	, preparation	
Neurokeratin	38	of	140
Neutral fats (<i>see</i> Fats).		Phenylhydrazin reaction . .	5, 126
Neutral olive-oil	13	Phloroglucinol, as test for pen-	
, preparation of	142	tose	3
Nitrogen, qualitative tests for	15	Phosphates, earthy, in urine .	111
, total, quantitative		in sediment	135
determination of		in urine	109
(Kjeldahl)	112-114		

	PAGE		PAGE
Phosphates, quantitative de- termination of	109, 110	Reaction of the urine	86
Phosphoric acid in bone ash . .	36	Reagents, preparation of	138
Phosphorus in protein	17	Reduced hæmatin	78
, organic, in urine.	110	hæmoglobin	77
, qualitative test for, in urine	111	Rennin	55
Phosphoprotein	26, 122	Rhamnose	101
Phosphotungstic acid, as pro- tein precipitant	20	Robert's modification of Hel- ler's test	123
Picric acid as protein precipi- tant	20	reagent, preparation of	140
Pigments, biliary	69	Rosenbach's modification of Gmelin's test	126
, blood, in urine	127	Saccharose	1, 5-7
, urinary	99	Salivary digestion	43
Piria's test for tyrosine	59	Salkowski's test for choleste- rol	42
Plattner's crystallized bile . .	69	Saponification of bayberry wax	10
Polarization	5	of lard	11
of urine	126	Scherer's test for leucine . . .	59
Polysaccharides	1, 7, 8	Schlösing's method for ammo- nia	117
Potassium mercuric iodide . .	20	Sediments in acid reaction . .	134
sulphate in sedi- ment	135	in alkaline reaction	135
sulphocyanide in saliva	43	, scheme for identi- fying	136
Precipitation reactions	19	unorganized	134
Protagon	38	Serum albumin	73
Proteins	15	globulin	73
, coagulation of	21, 22	Skatole-carbonic acid	62, 65
, compound	15, 25, 27	conjugated in urine	96
in blood serum	73	in intestinal putre- faction	62-64
in milk	83	Soaps	12
in nervous tissue	29	Sodium alcoholate	83
in urine	121	chloride in blood- cerum	74
, quantitative deter- mination of	118	Solutions, preparation of	137
Proteoses	51, 124	Smith's test for bile pigments .	70
in intestinal putre- faction	62	Specific gravity of the urine . .	87
in urine	124	Spectroscopic examination of the blood	77
Protoproteose	52	Spiegler's reagent, prepara- tion of	140
Ptyalin	44	test	124
Purine bases in muscle	32	Starches	7
in urine	94	Starch paste	8
, method of sep- aration	33	Steapsin	56, 60
Putrefaction experiment and separation	63	Stokes' reagent	77
Pyrocatechinol, conjugated in urine	96	Struve's test for oxyhæmoglo- bin	128
Quadiurates	92		

	PAGE		PAGE
Sugar in blood	74	Urea	91
Sulphates, conjugate, in urine	109	, preparation of	91
, quantitative determination of	107	, quantitative determination of	114
, total	108	Uric acid	92
Sulphur in urine neutral	89	, preparation of	93
, lead-blackening	15	, quantitative determination	115
, oxidized	16	Uric acid in sediment	134
, qualitative test for	15	, tests for	93
Synproteose	53	Urine	85
Tannic acid, as protein precipitant	19	, acidity of	105
Tanret's reagent, preparation of	140	, quantitative determination of	86
test	124	, color of	86
Taurocholic acid	68	, odor of	86
Teichmann's crystals, test for blood	75	, physical properties of	86
Tetroses	1	, quantitative determination of	103, 104
Thioproteose	53	, reaction of	86
Total solids in urine	88	, sediments in	88, 134
Trichloroacetic acid, test for proteins	20, 123	, specific gravity of	87
Trioses	1	, tests for normal constituents of	88
Trommer's test for hexoses	4	, total solids of	88
Tropæolin OO as indicator	47	, transparency of	86
, preparation of	141	, volume of	87
Trypsin	56, 57	Urinary pigments	99
Trypsinogen	57	Urobilin	99
Tryptic proteolysis	57	, test for	100
Tryptophane	62, 66	Urochrome	99
Tyrosine, test for	58	Volume of the urine	87
in sediment	134	Water, test for hardness	12
Uffelmann's reagent, test for lactic acid	49	Weyl's test for creatinine	32
, preparation of	141	Xanthine	32
Urates	92	in sediments	134
, ammonium, in sediments	135	Xanthoproteic reaction	12
		Xylose	8

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