Practical physiological chemistry / Sydney W. Cole.

PRACTICAL PHYSIOLOGICAL CHEMISTRY

S. W. COLE

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PRACTICAL PHYSIOLOGICAL CHEMISTRY.

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Practical Physiological Chemistry

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FOURTH EDITION

CAMBRIDGE:
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1914

PREFACE TO THE FIRST EDITION.

My aim in writing this book has been to present to the student a series of exercises which can be successfully carried through in ordinary class work.

Too often a student is discouraged in his work and displeased with his Text-Book by finding that a minute care in following the instruction given fails to produce the specified result. I trust that no such difficulty will be met with in working through this Book. Each and every exercise given here I have first worked through and obtained the result stated. All I ask of the student is a zealous and interested care and he will then have no difficulty in performing the experiments and learning the lessons they teach.

The ground covered is more than is at present necessary for most examinations in medicine, but I feel that this is justified by the growing importance of the subject and the increasing standard of the knowledge of it required of candidates at these examinations.

A special feature of the book is the notes that follow certain of the exercises. These notes summarise a series of exercises, indicate the special precautions that are necessary for success or give the probable reasons for an apparent failure in the performance of a given exercise. They should be carefully studied both before and after the exercise to which they refer. At the end of the book spaces are provided for the student to draw various crystalline forms from preparations made by himself. I consider this a more instructive plan than giving illustrations of typical crystals, which often differ considerably from those prepared in class work. A blank chart for recording the absorption spectra of various pigment solutions and colour reactions is also added. The drawings should be shown to the demonstrator of the class for comments or corrections.

SYDNEY W. COLE.

Trinity College,
Cambridge.
November, 1904.

PREFACE TO THE THIRD EDITION.

THE present volume is an outcome of the two editions of the Author's "Practical Exercises in Physiological Chemistry."

The increasing importance of the science to medical men has created a demand for a book that embodies precise instruction for class-work with an account of the properties and significance of the more important physiological substances. The present work is an attempt to realise these desiderata.

The Author wishes to draw particular attention to the analytical methods. It is lamentable that for the investigation of the nitrogenous excretion of a patient, the average medical man has at present only one method at his command. That method, the hypobromite, is notoriously unreliable, and the conclusions drawn from it may be extremely misleading. It is sincerely hoped that all medical students will be taught the microchemical methods of urinary analysis introduced by Folin. The Author is convinced that they are reliable, and that the average medical man could conduct them rapidly with a very small amount of special apparatus. If such training were universally adopted in England, an enormous amount of clinical material that is now wasted would become available for research, and a rapid increase in our knowledge of physiology and pathology would inevitably follow.

The qualitative methods for urinary analysis also have been considerably modified in recent years, especially in regard to sugar. Fehling's method, that has for so long been the crucial test, is unreliable. It should be supplanted as soon as possible by more conclusive methods, such as those described in the section on glucose in urine.

By a judicious selection of exercises the book can be adapted for elementary or advanced classes.

The Author gratefully acknowledges his indebtedness to Mr. H. M. Spiers, of Caius College, for invaluable help in reading the proofs, and to Messrs. J. Griffin & Sons and Messrs. Baird & Tatlock for the loan of certain of the diagrams.

SYDNEY W. COLE.

TRINITY COLLEGE,
CAMBRIDGE,
April, 1913

PREFACE TO THE FOURTH EDITION.

LITTLE more than a year has elapsed since the third edition of this book was issued, but my publishers inform me that the edition is exhausted. I venture to hope that this rapid sale indicates that both medical students and practitioners are realising the immense importance of accurate analytical methods, and that the old so-called clinical methods are essentially of the past.

In order that the third edition may not be rendered obsolete and unusable by the issue of this edition I have not altered the arrangement of the book but I have enclosed in an appendix a number of new or improved methods. In particular I would draw attention to the recent methods for the microchemical analysis of sugar in blood. Bang's method can be performed on about three drops of blood drawn from a finger prick, and I have completely satisfied myself of its accuracy. Its extended use may do something towards the elucidation of the mystery of diabetes. Improved methods are given for the analysis of glucose and lactose in pure solutions, for the microchemical analysis of uric acid in urine, for chlorides in blood and urine, and for acetone and aceto-acetic acid. I have also added two recent tests of my own for minute amounts of glucose in urine and for bile pigments. Finally I have

included Wohlgemuth's method for the estimation of urinary diastase which has proved of such service in the diagnosis of pancreatitis.

I have to thank Professor Langley for the loan of Fig. 10, and Messrs. J. J. Griffin and Sons for Figs. 4, 5, 11, 12 and 13.

In conclusion, I desire to thank the many teachers who have sent me letters of advice, friendly criticism and encouragement. If this book has helped to further the study of analytical methods in a number of physiological schools both in England and abroad I shall feel that one piece of good work has been accomplished.

SYDNEY W. COLE.

CAMBRIDGE,
May, 1914.

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CHAPTER I.

THE PROTEINS.

These bodies are composed of certain amino-acids and bases condensed in varying proportions.

A. Classification.

1. **Protamines.** Basic substances, containing a high percentage of nitrogen and formed almost entirely of bases. They are found in ripe spermatozoa and ova. They form salts with acids.

2. **Histones.** Similar to the protamines, but less rich in nitrogen and bases. Found in unripe spermatozoa, the stroma of red corpuscles, and in the thymus. They are precipitated by ammonia.

3. **Globulins** insoluble in water
4. **Albumins** soluble in water

} coagulated by boiling.

5. **Glutelins.** Insoluble in water and alcohol: soluble in dilute acid or alkali
6. **Gliadins.** Insoluble in water: soluble in 75 % alcohol

} Found in cereals.

7. **Sclero-proteins.** Forming the skeletal structure of animals; *e.g.* keratin, elastin, collagen (the anhydride of gelatin).

8. **Phospho-proteins.** Proteins rich in phosphorus, *e.g.* caseinogen of milk and vitellin of egg-yolk.

9. **Conjugated-proteins.** Proteins joined to a non-protein ("prosthetic") group.

(i) *Chromoproteins.* Protein + pigment molecule, *e.g.* haemoglobin.

(ii) *Nucleoproteins.* Protein + nuclein or nucleic acid.

(iii) *Glucoproteins.* Protein + carbohydrate, *e.g.* mucin.

10. **Hydrolysed Proteins.** Proteins formed by the action of acids, alkalies and certain enzymes, on native proteins.

(i) Metaproteins.

(ii) Albumoses or Proteoses.

(iii) Peptones.

(iv) Polypeptides—formed of amino-acids only.

B. General Properties.

1. Certain colour reactions are given dependent on the existence of certain constituent radicles. (See Exs. 1-6.)

2. They are precipitated by the so-called alkaloidal reagents (Ex. 14-18).

3. They are precipitated by salts of the heavy metals (Ex. 19-22).

4. They are colloidal. That is, they do not diffuse through animal membranes, and the large molecules tend to aggregate together under the influence of heat, neutral salts, etc., to form a precipitate or coagulum.

Solubilities of the chief proteins.

S = Soluble. I = Insoluble.

	Water.	Dilute Salts.	Dilute Acetic Acid.	Dilute Alkalies.	½-sat'd. Am_2SO_4.	Sat'd. Am_2SO_4.
Globulin	I	S	S	S	I	I
Albumin	S	S	S	S	?	I
Metaprotein	I	I	S	S	I	I
Primary Albumose	S	S	S	S	I	I
Secondary Albumose	S	S	S	S	S	I
Peptone	S	S	S	S	S	S
Caseinogen	I	I	I	S	I	I
Nucleoprotein	I	I	I	S	I	I
Mucin	I	I	I	S	I	I
Gelatin	S*	S*	S*	S*	I	I
Keratin	I	I	I	I	I	I

* If warmed.

C. The Colour Reactions of Proteins.

For the following reactions use egg-white that has been well beaten with six times its volume of water, or serum that has been diluted ten times with water.

1. **The Xanthoproteic reaction.** To 5 c.c. of the protein solution in a test-tube add about one-third of its volume of strong nitric acid. A white precipitate is formed. Boil for a minute. The precipitate turns yellow and partly dissolves to give a yellow solution. Cool under the tap and add strong ammonia till the reaction is alkaline. The yellow colour is turned to orange.

NOTES.—1. The essential features of the reaction are that a yellow colour is obtained when the solution is boiled with strong nitric acid, and that this yellow colour is intensified on the subsequent addition of ammonia.

2. The precipitate is due to the formation of metaprotein by the action of nitric acid on albumins or globulins, this metaprotein being insoluble in strong mineral acids. (See Ex. 13.) It follows that albumoses and peptones, etc., do not give the precipitate with nitric acid.

3. The yellow colour is due to the formation of a nitro-compound of some aromatic substance, *i.e.* a substance containing the benzene ring.

4. The aromatic substances in the protein molecule that are responsible for the reaction are tyrosine, tryptophane and phenyl alanine.

5. Oleic acid, olive oil and most vegetable oils give a well-marked xanthoproteic reaction.

2. **Millon's reaction.** Treat 5 c.c. of the protein solution with half its volume of Millon's reagent. A white precipitate is formed. Boil the mixture. The precipitate turns brick-red in colour, or disappears and leaves a red solution.

NOTES.—1. The essential feature of the reaction is the red colour on boiling. The white precipitate in the cold is due to the action of the mercuric nitrate on the proteins. (See Ex. 19.)

2. A white precipitate is also obtained with solutions of urea. (See Ex. 251.)

3. Sulphates give a white precipitate of mercurous sulphate.

4. The reagent is made by dissolving 30 c.c. of mercury in 570 c.c. of concentrated nitric acid and diluting with twice its bulk of water. It contains mercurous and mercuric nitrates, excess of nitric acid, and a small amount of nitrous acid.

5. The reaction should never be attempted with a strongly alkaline fluid, since the alkali will precipitate the yellow or black oxides of mercury.

6. The reaction is given with all aromatic substances that contain a hydroxyl group attached to the benzene ring. Thus it is given by phenol, salicylic acid, and naphthol, but is not given by benzoic acid.

7. The aromatic substance derived from protein that is responsible for the reaction is tyrosine. $C_6H_4\begin{cases}OH & \ldots\ldots\ldots\ldots\ldots\ldots\ (1)\\ CH_2.CH.NH_2.COOH & (4)\end{cases}$

3. **The glyoxylic reaction** (Hopkins and Cole). Treat 2 or 3 c.c. of the fluid with the same bulk of "reduced oxalic acid." Mix and add an equal volume of concentrated sulphuric acid, pouring it down the side of the tube. A purple ring forms at the junction of the fluids. Mix the fluids by shaking the tubes gently from side to side. The purple colour spreads through the whole fluid.

NOTES.—1. Reduced oxalic acid is prepared by one of the following methods:—

A. Treat half a litre of saturated solution of oxalic acid with 40 grammes of 2 per cent. sodium amalgam in a tall cylinder. When all the hydrogen has been evolved the solution is filtered and diluted with twice its volume of distilled water. The solution now contains oxalic acid, sodium binoxalate, and glyoxylic acid (COOH.CHO). It should be kept in a closed bottle containing a little chloroform.

B. In a flask place 10 grammes of powdered magnesium and just cover with distilled water. Slowly add 250 c.c. of saturated oxalic acid, cooling under the tap at intervals. Filter off the insoluble magnesium oxalate, acidify with acetic acid, dilute to one litre with distilled water, and bottle as above.

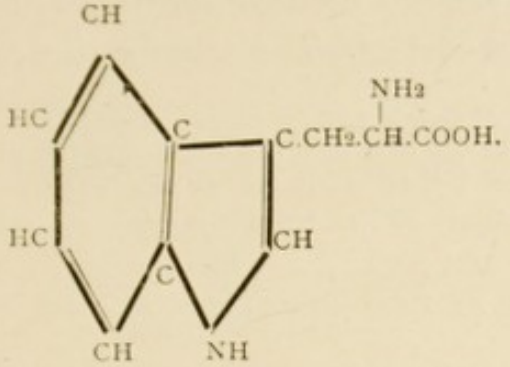
2. The reaction does not succeed in the presence of nitrates, chlorates, nitrites, or excess of chlorides.

3. The colour is not well seen if the protein is mixed with certain carbohydrates (*e.g.* cane-sugar), owing to the char produced by the strong sulphuric acid.

4. It is most important to use pure sulphuric acid for this test. It some times fails owing to the presence of impurities in the acid employed.

5. In performing the test on a solid substance, like fibrin or keratin, a small amount of the material should be heated with a few c.c. of the reduced oxalic acid and an equal volume of strong sulphuric acid. The mixture is shaken, and as the protein dissolves in the strong acid both the fluid and the solid particles assume a purple colour.

6. The substance in the protein molecule that is responsible for the reaction is tryptophane (indol-amino-propionic-acid) $C_{11}H_{12}N_2O_2$.



4. **Piotrowski's reaction (the biuret reaction).** Treat 5 c.c. of the solution with an excess of sodium hydrate and a drop of a 1 per cent. solution of copper sulphate. A violet or pink colour is produced.

NOTES.—1. The reaction is of especial importance in testing for albumoses and peptones, which give a rose colour. It is generally stated that other proteins give a voilet, but usually egg-albumin gives a distinct rose tint.

2. The nomenclature of the reaction is somewhat varied. Some writers use the term "biuret reaction" even when performed on serum-proteins, but others restrict this term to the reaction given by albumoses and peptones, employing the term "Piotrowski's reaction" to that given by albumins and globulins.

3. The test cannot be applied in the presence of a large amount of magnesium sulphate, owing to the precipitation of magnesium hydrate by the alkali.

4. If the solution contains much ammonium sulphate it must be treated with a large excess of strong sodium hydrate as directed in Ex. 54.

5. The reaction is given by nearly all substances containing two CONH groups attached to one another, to the same nitrogen atom, or to the same carbon atom. Thus it is given by

$CONH_2$	$CONH_2$	$CONH_2$
$\vert$	$\vert$	$\vert$
$CONH_2$	NH	CH_2
	$\vert$	$\vert$
	$CONH_2$	$CONH_2$
Oxamide.	Biuret (See Ex. 253).	Malonamide.

The cause of the reaction with proteins is the presence of one or more groupings of the last type, formed by the condensation of the carboxylic group of an amino-acid with the amino group of another amino-acid. Thus it would be given by the polypeptide, glycyl-alanyl-tyrosine

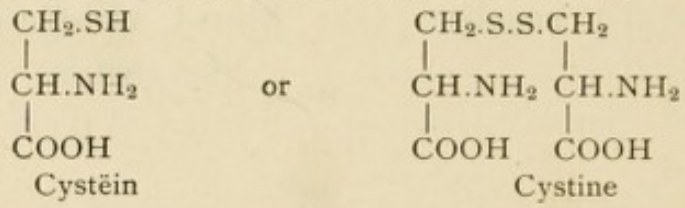
		$C_6H_4.OH$
		$\vert$
	CH_3	CH_2
	$\vert$	$\vert$
$NH_2.CH_2.CO.$	$NH.CH.CO$	$NH.CH.COOH.$
Glycyl-	-alanyl-	-tyrosine.

5. **The sulphur reaction.** Boil a little undiluted egg-white or serum with some 40 per cent. sodium hydrate for two minutes, and then add a drop or two of lead acetate. The solution turns deep black.

NOTES.—1. This reaction is due to the fact that the sulphur of the protein is liberated as sodium sulphide when boiled with the strong alkali. The sulphide gives a black colour or precipitate of lead sulphide when the solution is subsequently treated with lead acetate.

2. The reaction does not succeed with caseinogen, peptones, and certain other proteins.

3. The sulphur in the protein is mainly combined as



Cystëin Cystine

6. **Molisch's reaction.** Treat 5 c.c. of the diluted solution with three or four drops of a 1% solution of alpha-naphthol in alcohol. Mix, and run about 5 c.c. of concentrated sulphuric acid under the fluid. A violet ring is formed at the junction of the two liquids.

NOTES.—1. The reaction is due to the presence of a carbohydrate group (glucosamine) in the protein. This is converted by the acid to furfurol, which condenses with alpha-naphthol to give the purple colour. (See notes to Ex. 76.)

D. The Albumins and Globulins of Blood Serum.

Ox blood is collected into pans and is allowed to stand till clotting is complete. The serum that excludes is pipetted off and kept in the ice chest till required.

7. Take the specific gravity by floating a clean, dry urinometer in a cylinder containing the serum, and noting the graduation where the stem of the urinometer is level with the surface of the fluid. It is usually about 1030 (water being taken as 1000).

8. Take the reaction of the serum to litmus paper. It is alkaline.

Heat-coagulation of albumins and globulins.

When a solution of albumin or globulin is heated under certain conditions, the protein separates from

solution in a form that is insoluble in water, salt-solutions, dilute acids and alkalies. This change is known as "heat-coagulation."

It seems to consist of two processes:

(1) The interaction of protein and water ("denaturation").

(2) The subsequent agglutination and separation of the product.

The first process may take place without the second.

Both processes are much affected by the reaction of the sclution and by the presence of neutral salts.

In general it might be stated that an increase in acidity or alkalinity up to a certain point favours denaturation but decreases the tendency to agglutination. The reverse is true for neutral salts.

The best medium for obtaining heat-coagulation is one very slightly acid and containing a small amount of a neutral salt, preferably that of one of the alkaline earths, *e.g.* calcium chloride.

The material produced by heating the protein with water can be regarded as a hydrolytic product, meta-protein. If there be a sufficient amount of acid or alkali present there is no agglutination of this unless a certain amount of neutral salt be present. In general it can be stated that the smaller the amount of neutral salt present, the smaller is the amount of alkali or acid necessary to inhibit agglutination.

When a protein is treated with a dilute acid, *e.g.* HCl, a salt is formed. This is hydrolysed by water into protein and free HCl, which can be completely removed by prolonged dialysis. But if such a solution of protein in weak acid be boiled, the coagulum that forms consists of the salt, that is, the HCl is partly removed from the solution on coagulation.

As regards the condition of the protein in "solution," it has been shewn that the particles are really suspended in the "solvent" and that they carry an electrical charge. This charge determines the stability of the system, and any factor tending to reduce the charge promotes precipitation or coagulation. The sign of the charge on the particle is determined by the chemical nature of the particle, and may also depend on the nature of the solvent. Hardy has shewn that in the case of the proteins, which have amphoteric characters, the sign of the particle is positive when the fluid is acid and negative when the fluid is alkaline. When a salt is added to such a colloidal solution it exerts a coagulative effect which depends upon one of its ions, the coagulating ion being that which carries a charge opposite in sign to that of the particle. The coagulative power increases rapidly with the valency. Thus in acid solution the protein has a positive charge and so is precipitated by negative ions, and it is found that the potassium salt of citric acid (trivalent) is much more effective than the potassium salt of sulphuric acid (divalent), and this more than the potassium salt of hydrochloric acid. In alkaline solutions on the other hand the cation is the coagulative ion and cerium chloride ($CeCl_3$) is more efficient than barium chloride ($BaCl_2$) and this more than sodium chloride (Na Cl).

In the following two exercises, the explanations offered in the notes are sufficient for elementary students.

9. Dilute 5 c.c. of serum with 45 c.c. distilled water.

(*a*) Boil 5 c.c. in a test-tube. The solution sometimes becomes opalescent, but no definite coagulum is formed. Cool the tube and add 1 per cent. acetic acid drop by drop. A precipitate of metaprotein is formed, soluble in excess of acid.

(*b*) Boil 5 c.c. with two drops of 1 per cent. acetic acid. A white flocculent precipitate is formed. Cool the tube and add two

or three drops of strong nitric acid. The precipitate does not dissolve.

(*c*) Treat 5 c.c. of serum with ·4 per cent. hydrochloric acid, drop by drop, till the solution is clear (about 5 drops are usually necessary). Boil. A precipitate is not formed. Cool the tube and add 2 per cent. sodium carbonate, drop by drop. A precipitate is formed, which redissolves in excess.

(*d*) Boil 5 c.c. with two drops of 2 per cent. Na_2CO_3. A coagulum is not formed. Cool the tube and add 1 per cent. acetic acid. A precipitate of metaprotein is formed, soluble in excess of acid.

NOTES.—1. These reactions are of very great importance, and are to be explained as follows: Serum contains two varieties of proteins, known as globulins and albumins, which are coagulated by boiling, provided that the reaction of the fluid is neutral or very faintly acid. If the solution is alkaline (and it must be remembered that serum is alkaline) the proteins are acted on by the alkali as the temperature rises and are converted into a substance known as metaprotein, which is not coagulated by heat *when in solution*. If the reaction be markedly acid, as in (*c*), the proteins are, similarly, converted to metaprotein. But if the reaction be neutral or only faintly acid, as in (*b*), a coagulum is formed on boiling. This coagulum consists of the whole of the albumin and globulin, and is insoluble in water, dilute acids, and dilute alkalies.

2. The addition of the nitric acid in (*b*) is to ensure that the precipitate that appears on boiling does not consist of calcium or magnesium phosphate, which is soluble in dilute nitric acid. That such a phosphatic precipitate can be formed on boiling certain solutions is shown by the following experiment. Treat a solution of calcium chloride with sodium phosphate and then with excess of sodium carbonate. A precipitate of $Ca_3(PO_4)_2$ appears. Add acetic acid drop by drop till the precipitate just dissolves owing to the formation of the acid phosphate. Boil the solution for half a minute. A white precipitate appears. Add a drop or two of nitric acid. The precipitate dissolves. The appearance of the precipitate of $Ca_3(PO_4)_2$ on boiling is due to the alteration of reaction as the CO_2 is evolved.

10. Take 5 c.c. of the diluted serum in a test-tube: add two drops of 1 per cent. acetic acid and place the test-tube in a beaker of water, supporting it by a clamp so that it does not touch the bottom of the beaker. Heat the water with a Bunsen flame and note the temperature at which coagulation begins. It usually commences at about 70° C., and is complete at 82° C. It takes place chiefly between 73° and 75° C.

NOTE.—The various albumins and globulins have different coagulating points, but since this point varies with the concentration of the electrolytes in the solution it can only be used for separating and distinguishing proteins when the conditions are similar. Nevertheless the coagulation temperature serves to distinguish myosin (56°C.) and fibrinogen (56°C.) from serum-globulin (73°C.).

11. Place about 4 c.c. of serum in a test-tube and cool to 0° C. by means of a freezing mixture. Fill the tube with strong alcohol that has previously been cooled to about 8° C., and mix. A white precipitate of the proteins is formed. Filter at once and treat the precipitate with water. It dissolves.

12. Allow a few drops of serum to fall into about 10 c.c. of strong alcohol at room temperature. A white precipitate is formed. Shake well and allow to stand for half an hour. Filter and treat the precipitate with water. It does not dissolve.

NOTE.—These two exercises show that the serum proteins are first precipitated and then coagulated by strong alcohol.

13. To about 5 c.c. of diluted serum add a few drops of strong acetic acid. A precipitate is not formed. Now add four or five drops of strong nitric acid: a white precipitate is formed.

NOTE.—The serum-proteins are not precipitated by acetic acid (thus differing from mucin, caseinogen and nucleoprotein). The action of nitric acid on serum-proteins is to produce metaprotein, which is insoluble in *strong* mineral acids. This precipitability of albumins and globulins is the basis of Heller's test for these proteins in urine (Ex. 278).

Precipitation by Alkaloidal Reagents.

14. Treat 5 c.c. of diluted serum with two or three drops of strong acetic acid and two drops of potassium ferrocyanide. A white precipitate is formed. Boil. The precipitate does not dissolve.

NOTES.—1. Primary albumoses also are precipitated by potassium ferrocyanide and acetic acid, but the precipitate produced dissolves on warming and reappears on cooling. (See Ex. 52.)

2. The precipitate and fluid often become coloured blue-green on boiling. This is due to a decomposition of the hydro-ferrocyanic acid on boiling it with certain organic substances, such as proteins.

15. To some serum diluted about ten times and acidified with dilute hydrochloric acid add a few drops of tannic acid. A white or brown precipitate is formed.

16. Treat 5 c.c. of diluted serum with an equal volume of Esbach's solution. A yellowish precipitate is formed.

NOTE.—Esbach's solution is prepared by dissolving 10 grms. of picric acid and 10 grms. of citric acid in water and making the solution up to 1 litre.

17. Acidify some diluted serum with dilute hydrochloric acid and add a few drops of potassio-mercuric iodide (Brücke's reagent). A white precipitate is formed.

NOTE.—Brücke's reagent is prepared by dissolving 50 grm. of potassium iodide in 500 c.c. water, saturating with mercuric iodide (120 grm.), and making up to 1 litre.

18. Acidify 5 c.c. of serum with dilute hydrochloric acid and add a solution of phosphotungstic acid. A white precipitate is produced.

Precipitation by the salts of the Heavy Metals.

19. To diluted serum add a few drops of mercuric nitrate solution. A white precipitate is formed, soluble in saturated sodium chloride solution, and reprecipitated from this by the addition of dilute hydrochloric acid.

20. To diluted serum add ferric chloride solution. A precipitate is formed soluble in excess.

21. To diluted serum add copper sulphate solution drop by drop. A bluish-grey precipitate is formed.

22. To diluted serum add a solution of lead acetate or basic lead acetate. A white precipitate is formed.

The remaining exercises of this section deal with the special physical properties of the globulins and albumins of serum.

Globulins are insoluble in distilled water, but soluble in dilute acids and alkalies, and in weak solutions of neutral salts.

A neutral solution in a dilute salt is coagulated on boiling.

A solution in dilute acid or alkali is converted into a solution of metaprotein on boiling.

If the globulin be dissolved in a minimum amount of a neutral salt solution and the solution be diluted with

several volumes of distilled water, the globulin is partially precipitated, for a certain *concentration* of salt is necessary to keep the globulin in solution. If the globulin be dissolved in dilute acid or alkali, there is no precipitation on dilution.

The globulins are completely precipitated by full saturation with magnesium sulphate or by half-saturation with ammonium sulphate, *i.e.* by treatment of the solution with an equal volume of a saturated solution of ammonium sulphate.

Albumins are soluble in distilled water, dilute salt solutions, dilute acids and alkalies.

A neutral solution in water or salt is coagulated on boiling.

A solution in dilute acid or alkali is converted to a solution of metaprotein on boiling.

Solutions of albumins are only partially precipitated by saturation with magnesium sulphate or by half-saturation with ammonium sulphate if the reaction of the solution be neutral or alkaline.

They are more completely precipitated by solutions of these substances in the presence of acid.

They are completely precipitated by full saturation with ammonium sulphate from a neutral, acid, or alkaline solution.

NOTE.—There is considerable confusion as to the exact differences between globulins and albumins. Some writers prefer to call globulins all those heat-coagulable proteins that are soluble in neutral salts and are precipitated by half-saturation with ammonium sulphate. Others (including the author) would limit the term globulin to those heat-coagulable proteins that are insoluble in water and soluble in neutral salt solutions. There is no doubt but that the precipitate obtained by half saturation of serum with ammonium sulphate contains at least two substances, one insoluble in water (precipitated by dialysis), the other soluble in water.

Some call the insoluble body "eu-globulin" and the soluble substance "pseudo-globulin." But it is by no means certain that this latter differs at all

from the albumin that is only precipitated by full saturation with ammonium sulphate. Therefore it seems better at present to restrict the term globulin to that portion of the serum protein that is insoluble in water.

23. Dilute 5 c.c. of serum with 50 c.c. of distilled water. A faint cloud of serum-globulin is formed. Add ·4 p.c. hydrochloric or 1 p.c. acetic acid, drop by drop. The cloud becomes denser and then clears up.

NOTE.—The globulin in the serum is held in solution both by salts and dilute alkalies. Dilution alone produces a very small precipitate, but if the solution be now treated with just sufficient acid to neutralise the alkali, a much larger fraction of the globulin is thrown down. When an excess of acid is added the globulin dissolves.

24. Prepare a suspension of globulin by the following method. To 15 c.c. of serum in a beaker add 2 c.c. (about 30 drops) of 1 p.c. acetic acid and 100 c.c. distilled water. Stir and allow the mixture to stand for about 20 minutes. A precipitate of globulin settles down. Very carefully pour off the supernatant fluid and divide the suspended globulin into two equal portions in clean test-tubes. With these perform the two following exercises.

25. Add a 5 p.c. solution of sodium chloride, drop by drop, till the globulin has just dissolved. Divide the solution into three portions, A, B and C.

(*a*) Boil. The protein is coagulated.

(*b*) Dilute with about five volumes of distilled water. The globulin is partially reprecipitated.

(*c*) Treat with an equal volume of saturated ammonium sulphate solution. The globulin is reprecipitated.

26. Add ·4 p.c. H Cl, drop by drop, till the globulin has *just* dissolved. Divide the solution into three portions, D, E and F.

(*d*) Add 2 p.c. sodium carbonate solution till the globulin is partially reprecipitated (one or two drops only are necessary). Now add a few drops of 5 p.c. sodium chloride. The precipitate of globulin redissolves.

(*e*) Boil the solution. The protein is not coagulated. Cool under the tap and add enough 2 p.c. sodium carbonate

to precipitate the metaprotein that has been formed by boiling. Now add a few drops of 5 p.c. sodium chloride. The precipitate of metaprotein does not dissolve.

(*f*) Dilute with about five volumes of distilled water. The globulin is not thrown out of solution.

27. Mix about 10 c.c. of undiluted serum with an exactly equal quantity of a saturated solution of ammonium sulphate. A thick white precipitate is formed consisting of the whole of the globulin and a portion of the albumin. Filter through a dry filter paper into a dry test-tube. Label the filtrate A. Scrape the precipitate off the paper and treat it with distilled water. The precipitate dissolves, the ammonium sulphate adhering to it forming a dilute salt solution which allows the globulin to go into solution. Boil a portion of this solution. A heat-coagulum is formed.

28. Filtrate A contains serum-albumin in the presence of half-saturated ammonium sulphate. Apply the following tests:

(*a*) Boil a portion. A heat-coagulum is formed.

(*b*) To another add one drop of strong acetic acid. A white precipitate of serum-albumin is formed.

(*c*) Grind the remainder in a mortar with solid $(NH_4)_2SO_4$ till the fluid is saturated. A white precipitate of serum-albumin is formed. Filter off the precipitate and test the filtrate for proteins either by boiling or by the glyoxylic or xanthoproteic reactions. Proteins are absent, showing that all the proteins of serum are precipitated by complete saturation with $(NH_4)_2SO_4$.

NOTE.—A certain test for albumin in a solution is to half-saturate it with ammonium sulphate, filter off any precipitate that may be present and boil the filtrate. A heat-coagulum indicates albumin.

29. Serum has been dialysed in parchment tubes for two or three days against repeated changes of distilled water. Note the heavy precipitate of serum-globulin that has fallen to the bottom of the tube.

30. Dilute 5 c.c. of serum with five times its volume of tap water, add a drop or two of 2 per cent. calcium chloride and a drop or two of neutral litmus. Boil the mixture in a porcelain dish, and whilst boiling cautiously add 1 per cent. acetic acid till the reaction is *faintly* acid. Filter, and test the filtrate for proteins by the usual colour tests. If the operation has been carried out successfully the filtrate will be found to be nearly free from proteins.

NOTE.—This is the method usually employed for removing albumins and globulins from solution. It is obvious from the note to Ex. 9 that a certain amount of metaprotein is formed when the fluid is first boiled, but this metaprotein is precipitated by the acetic acid, and the precipitate is coagulated at boiling temperature so that it does not re-dissolve in the very slight excess of acid that is subsequently added. (See Chapter I., H.)

It is most important that the acid should be added slowly and not in any excess. The small amount of calcium chloride added aids in the aggregation of the protein on boiling.

E. The Chemistry of Egg-white.

31. In egg-white which has been well beaten with a whisk (to break up the containing membranes), and diluted with four times its volume of ~~distilled water~~ NaCl Soln., note a precipitate of ovo-mucin and globulin. Perform the following tests:

(*a*) Take the reaction to litmus. It is alkaline.

(*b*) Cautiously neutralise with dilute acetic acid. A slight increase in the precipitate of ovo-mucin and globulin is noticed. Remove this by filtration if necessary, and with the filtrate perform the following reactions:

(*c*) Boil a portion. A coagulum is formed, indicating the presence of either a globulin or an albumin.

(*d*) Make another portion very faintly alkaline by the addition of a drop or two of 2 per cent. Na_2CO_3. Now add an equal bulk of saturated $(NH_4)_2SO_4$. A slight precipitate of globulin or albumin is formed. Filter this off, and boil a portion

of the filtrate with a drop of 1 per cent. acetic acid. A coagulum of albumin is formed. Saturate the remainder of this filtrate with $(NH_4)_2SO_4$ by grinding with the solid in a mortar. A precipitate of albumin is formed.

(*e*) Completely remove the globulin and albumin by boiling. Filter and apply Millon's or the xanthoproteic protein test to the filtrate. Protein is found in small quantities. This protein is known as ovo-mucoid. It is not coagulated by boiling, or precipitated by acetic acid. It is precipitated by saturation with $(NH_4)_2SO_4$, and also by strong alcohol.

32. **The crystallisation of egg-albumin.** (Hopkins' method.) Separate the white from a number of new-laid eggs, taking care not to allow any of the yolk to mix with the white. Measure the egg-white and churn it up with an exactly equal volume of a neutral fully-saturated solution of ammonium sulphate by means of a whisk, adding the sulphate in portions and mixing thoroughly after every addition. Notice the strong smell of ammonia that is evolved. Filter the mixture through a large pleated filter-paper. Measure the filtrate. Take 100 c.c. of it and cautiously treat it with 10 per cent. acetic acid from a burette, noting the original level of the acid in the burette. Add the acid a drop or two at a time, shaking gently the whole time, until the precipitate produced at each addition no longer dissolves on shaking, and the whole mixture is rather opalescent. This point is usually somewhat difficult to determine, owing to the large number of air-bubbles that become suspended in the fluid and closely resemble a fine precipitate. When you are satisfied that a permanent precipitate has been produced, run in 1 c.c. of the acid in addition to the amount already added; a heavy white precipitate is thus produced. Note the amount of acid that has been used for the portion of 100 c.c., and treat the remainder of the filtrate with a corresponding amount of acid. Mix the two portions thoroughly and allow to stand overnight. Note that the precipitate has increased somewhat in amount. Mount a drop of the suspension

on a slide, cover with a slip, but do not press. Examine under the high power of the microscope, and note the aggregation of very fine needles.

The albumin can be recrystallised by filtering, dissolving in as small an amount of water as possible, filtering again, and cautiously adding to the filtrate saturated ammonium sulphate till a faint permanent precipitate is produced. If the mixture be allowed to stand for some hours the albumin will separate out as fine needles.

NOTES.—1. For the experiment to succeed it is absolutely essential that all the eggs employed be perfectly fresh. One rather stale egg may interfere with the crystallisation of a large number of fresh eggs.

2. It is important to add exactly the amount of acetic acid mentioned, that is, one per mille above the amount required to give a faint permanent precipitate.

3. The same method can be employed for the crystallisation of serum-albumin from the perfectly fresh serum of a horse, ass or mule.

F. The Gluco-proteins.

These bodies are conjugated proteins, the protein being united to a carbohydrate group.

They consist of the mucins and mucinoids or mucoids. The mucins are found in connective tissue and are secreted by certain of the salivary glands and various parts of the alimentary canal, notably the large intestine. Their solutions are viscous. They are soluble in dilute alkalies and are precipitated from solution by acetic acid the precipitate being insoluble in excess of acetic acid. They are also soluble in 0·1 per cent. hydrochloric acid. On hydrolysis with acids the sugar group is split off and will reduce Fehling's solution.

The mucoids are not so viscous and not so readily precipitated by acetic acid, the precipitate being soluble in excess. They are found in ovarian cysts and in white of egg (See Ex. 31 (*e*)).

Preparation of Mucin. Mince the submaxillary gland of an ox, grind with sand and add ·1 per cent. NaOH (1 litre to 50 grams of the moist gland). Shake well in a large bottle from time to time and leave for about half an hour. Strain through muslin and filter through coarse filter-paper. (This crude solution should not be prepared too long before use, as mucin loses its characteristic properties if left standing with alkalies.)

33. Add acetic acid drop by drop. A stringy precipitate is formed, insoluble in excess of the acid.

34. Remove the precipitate on a glass rod, wash with water, and apply the usual colour reactions for proteins, *e.g.* xanthoproteic, glyoxylic, and Millon's. They are all given by mucin.

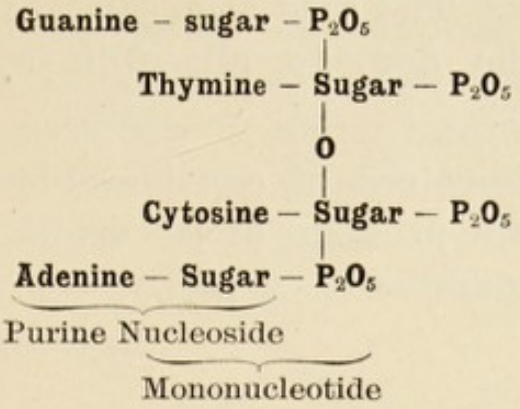
35. Treat some of the precipitate with ·1 per cent. HCl. The mucin dissolves.

36. Treat some of the precipitate with 2 per cent. Na_2CO_3. The mucin dissolves.

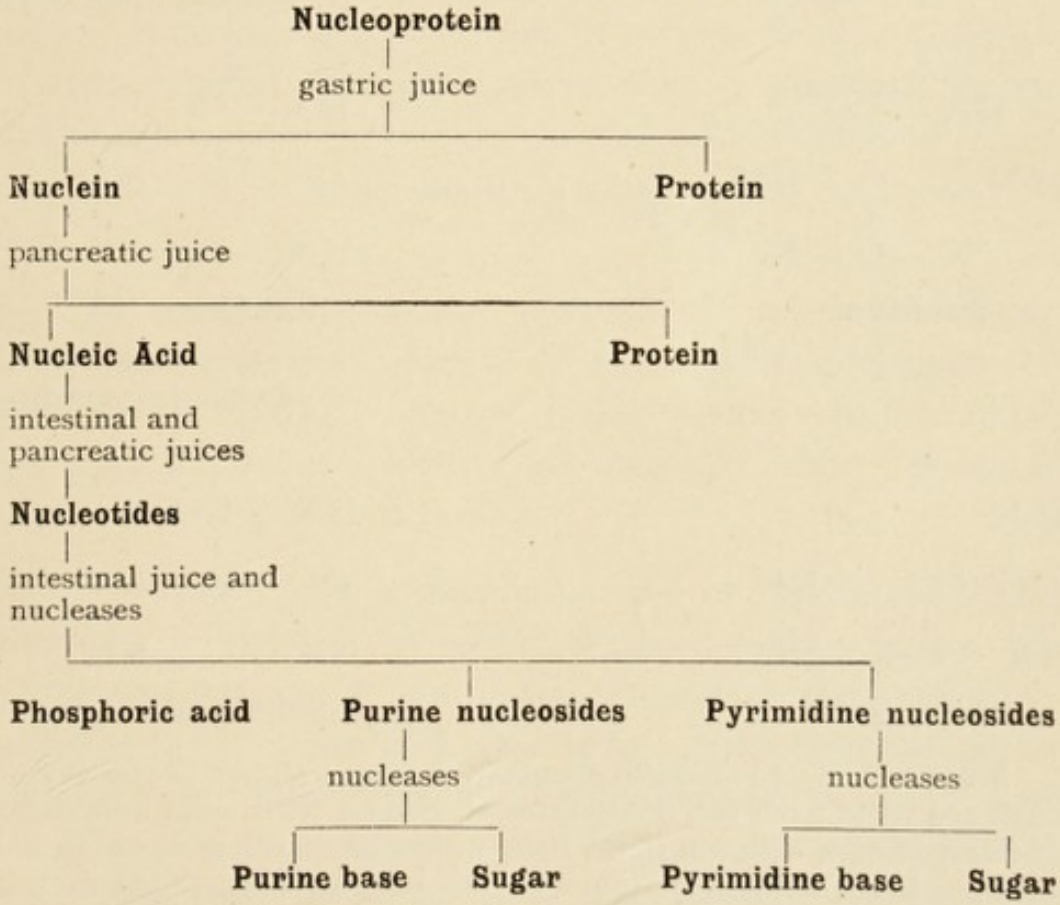
G. The Nucleoproteins and Nucleohistones.

These substances are conjugated proteins, the protein being in combination with nuclein. Nuclein is a protein combined with nucleic acid, a complex body rich in phosphorus. The nucleoproteins and nucleohistones are found in most tissues of the body, notably in those rich in cells, as the thymus, lymphatic glands, testes, pancreas, etc. They differ in the nature of the protein combined with nuclein. In the nucleoproteins it is of the nature of a peptone: in nucleohistone it is a histone. (See page 1.) The nucleic acids are polynucleotides, formed by the condensation of a certain number of *nucleotides*, which have the composition of a simple nucleic acid. The mononucleotides consist of phosphoric acid in combination with a *nucleoside*, a compound formed by the union of a sugar with a purine or a pyrimidine group. In many cases the sugar is a pentose (*d*-ribose), but in others it is a hexose which has not yet been identified.

The composition of the nucleic acid obtained from the thymus can be represented as follows:



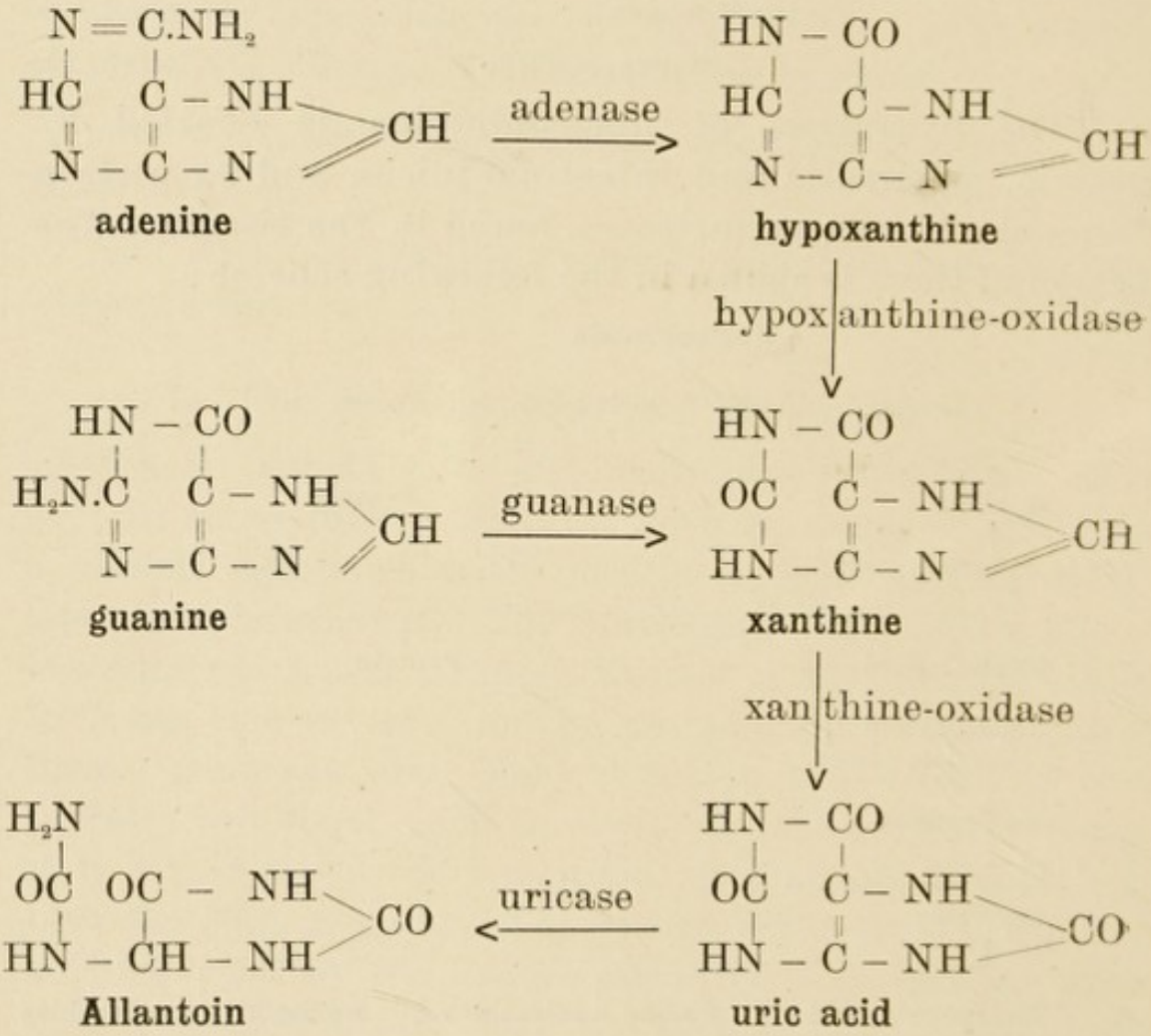
The hydrolysis of nucleoproteins is effected by gastric, pancreatic and intestinal juices, and by certain ferments, known as nucleases, found in the tissues. The action of these is shewn in the following scheme:—



The purine bases found are guanine and adenine, which are converted by tissue ferments called guanase and adenase to xanthine and hypoxanthine respectively.

Hypoxanthine can be oxidised in the tissues by hypoxanthine oxidase to xanthine, and this to uric acid by xanthine oxidase. The uric acid can be further oxidised (especially in the dog) to allantoin by the ferment uricase.

These reactions are of considerable importance in connexion with the problem of the origin of the uric acid excreted by the mammal.



Preparation. Lymphatic glands of the ox or sheep, or the thymus of a calf are freed from fat, finely minced, ground with sand and extracted for twelve hours with ten times their weight of distilled water in a large bottle, a small amount of toluol or chloroform being added to prevent decomposition. The bottle should be shaken vigorously at frequent intervals to break up the gelatinous masses that sometimes form. The fluid is strained and centrifugalised to remove all débris (filtration being very slow). This fluid contains both nucleoprotein and nucleo-histone.

Physical Properties. Nucleoproteins are acidic bodies which dissolve in dilute alkalies. The salt-like bodies thus formed are precipitated as the free acid by addition of dilute acetic acid. They dissolve to an opalescent solution in excess of strong acetic acid (distinction from mucin). Nucleohistone is precipitated as a calcium compound by ·2 per cent. calcium chloride solution. Solutions are precipitated by half saturation with ammonium sulphate.

37. To a portion add dilute acetic acid till no more precipitate is produced, and place on the water-bath at 37° C. for a few minutes. A heavy precipitate of nucleoprotein and nucleohistone is formed. Allow this to settle in a cylinder: pour or pipette off as much of the supernatant fluid as possible, and filter the remainder. Note that the precipitate is soluble in dilute alkalies and is reprecipitated by acidification; that it dissolves to an opalescent solution in excess of acetic acid (difference from mucin); and that it gives all the usual colour reactions for proteins.

38. To another portion add one-tenth of its volume of 2 per cent. calcium chloride and warm to 37° C. A white precipitate of nucleohistone is formed. Pour off the supernatant fluid, and to this fluid add dilute acetic acid drop by drop; a white precipitate of nucleoprotein is produced.

39. Precipitate the nucleoprotein and nucleohistone from the remainder of the fluid by means of acetic acid as in Ex. 37. Collect the precipitate on a filter paper, allow it to drain well, and then transfer it by means of a spatula to a small thimble-shaped porcelain capsule. Heat carefully, first to drive off the water, and then to carbonise the residue. Add one-third of a crucible full of fusion mixture (K_2CO_3 two parts, KNO_3 one part), and heat as strongly as possible till the mass fuses. Allow the melt to cool, and extract it with nitric acid (diluted with an equal quantity of distilled water) till the mixture no longer effervesces. Filter:

treat the filtrate with about one-tenth its volume of strong nitric acid and one-third its volume of ammonium molybdate: boil for two minutes. The yellow crystalline precipitate separating out on the sides of the tube shows that nucleoproteins and nucleohistone contain phosphorus, that has been oxidised to a phosphate by the fusing.

H. The Metaproteins.

The metaproteins are derived from the albumins and globulins by hydrolysis. This can be effected rapidly by dilute acids and alkalies at temperatures over 60° C. (see notes to Ex. 9): more slowly at body temperature. They are formed immediately by the action of strong mineral acids at room temperature. (See Exs. 1 and 13.) They are insoluble in water, *strong* mineral acids, and all solutions of neutral salts, but are soluble in dilute acids or alkalies in the absence of any large amount of neutral salts. They are not thrown out of solution (in acid or alkali) by boiling. But if such a solution be neutralised or precipitated by the addition of an excess of a neutral salt, the suspended metaprotein is coagulated on boiling, so that it will no longer dissolve in acid or alkali.

Preparation. Egg white or serum is diluted with ten times its volume of either ·4 per cent. hydrochloric acid or ·1 per cent. sodium hydrate and the mixture placed in a water bath or incubator at 40° C. for about twenty-four hours. The albumins and globulins are hydrolysed to metaprotein.

40. To about twenty-five c.c. add a few drops of litmus and carefully neutralise with 2 p.c. Na_2CO_3 or ·4 p.c. HCl. A bulky precipitate of metaprotein separates out. Filter. Scrape the precipitate off the paper and suspend it in a test-tube about half-full of water. Divide the suspension into six equal portions and with them perform the following six exercises :

41. Add some ·4 p.c. HCl. The precipitate dissolves. Neutralise with Na_2CO_3: the precipitate reappears.

42. Add concentrated HCl drop by drop. The precipitate dissolves with the first drop, and reappears when an excess is added.

43. Dissolve in a little ·4 p.c. HCl. Boil the solution: a coagulum is not formed. Cool under the tap and neutralise with ·2 p.c. Na_2CO_3. A precipitate is formed which is soluble in an excess.

44. Boil. Cool and add some ·4 p.c. HCl. The precipitate does not dissolve, *i.e.* metaprotein is coagulated when boiled in suspension.

45. Add a saturated solution of ammonium sulphate drop by drop. The precipitate does not dissolve in any dilution of the salt.

46. Dissolve in a little ·4 p.c. HCl. Treat the solution with an equal volume of saturated ammonium sulphate solution. The protein is precipitated.

I. The Albumoses and Peptones.

These hydrolysed proteins are obtained by the further action of acids or alkalies on globulins, albumins and metaproteins. They are best formed by the action of pepsin and hydrochloric acid on these proteins. Peptone is the end product of gastric digestion.

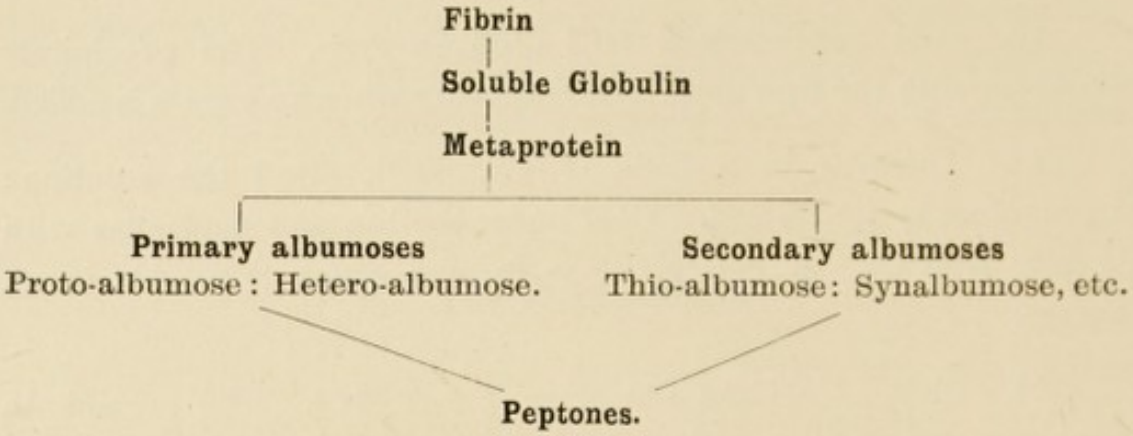
They are prepared on a commercial scale and sold as—

(i.) Witte's peptone, which is prepared from fibrin and consists of a mixture of albumoses and peptone.

(ii.) Savory and Moore's peptone, which is prepared from meat, and only contains traces of albumoses.

The following scheme indicates the successive steps

in the digestion of fibrin by pepsin and 0·2 per cent. hydrochloric acid :—



The following scheme shews the method adopted for the isolation of certain of the albumoses :—

Neutral Witte's peptone, treated with equal volume of saturated ammonium sulphate solution.

<table>
<tr><td colspan="2">Precipitate : dissolved in water. Treated with 2 volumes of strong alcohol.</td><td colspan="3" rowspan="2">Filtrate, treated with half its volume of saturated ammonium sulphate.</td></tr>
<tr><td>Ppt.
Hetero-albumose.</td><td>Filtrate.
Proto-albumose</td></tr>
<tr><td rowspan="4"></td><td rowspan="4"></td><td rowspan="4">Ppt. dissolved in water. Treated with 2 volumes of alcohol.
Ppt.
Thio-albumose.</td><td colspan="2">Filtrate. Saturated with ammonium sulphate.</td></tr>
<tr><td colspan="2">Ppt. dissolved in water. Treated with 2 volumes of strong alcohol.</td><td rowspan="3">Filtrate.
Peptones.</td></tr>
<tr><td>Ppt.
Neglect.</td><td>Filtrate.
Treated with ⅓ vols. of alcohol.</td></tr>
<tr><td></td><td>Ppt.
Synalbumose.</td></tr>
</table>

The **primary albumoses** are soluble in water, dilute acids, alkalies and salt solutions. Their solutions are not coagulated on heating. They are precipitated by half-

saturation with ammonium sulphate. They give a precipitate, that disappears on warming and reappears on cooling, either with nitric acid or potassium ferrocyanide and acetic acid. They also give a precipitate in the cold with copper sulphate.

They give all the ordinary protein colour reactions, with the exception of Molisch's.

The **secondary albumoses** have somewhat similar properties to those of the primary albumoses: but they are not precipitated by nitric acid, hydro-ferrocyanic acid, or copper sulphate.

They require more than half-saturation with ammonium sulphate to precipitate them, but are completely precipitated by full saturation. Thio-albumose gives all the protein colour reactions and is particularly rich in sulphur (hence its name).

Synalbumose gives the protein reactions, with the exception of the glyoxylic test.

The **peptones** are very soluble proteins of rather a low molecular weight, so that they slowly diffuse through parchment membranes. They are the only proteins not precipitated by full saturation with ammonium sulphate. They fail to give precipitates with Esbach's or Brücke's reagents or hydro-ferrocyanic acid, but are precipitated by other protein precipitants, as tannic acid, phosphotungstic acid and lead acetate.

For the following reactions make a 5 per cent. solution of "Witte's peptone" in hot water, just acidify with acetic acid and filter from a small amount of insoluble material (nuclein?). The solution contains all the albumoses and peptones.

47. Dilute a small amount with three or four times its bulk of water, and to portions of this apply the usual colour reactions for protein. They are all obtained. Note, in particular, that the biuret test gives a rose colour.

48. Boil the solution with a trace of acetic acid: it does not form a coagulum.

49. Add a little tannic acid: a white precipitate is formed.

50. Add a little Esbach's or Brücke's solution: a yellow or white precipitate is formed.

51. Add a little lead acetate solution: a white precipitate is formed.

52. To 10 c.c. of the 5 per cent. solution in a small beaker add 10 c.c. of a saturated solution of ammonium sulphate. A white precipitate of the primary albumoses is formed. Stir the mixture vigorously for a short time with a glass rod that has one end covered with a small piece of rubber tubing: allow to stand for a few minutes. The precipitate will usually gather together and can be almost completely collected as a gummy mass on the end of the rod. Transfer it to about 5 c.c. of hot water. The precipitate dissolves. Cool the solution and divide it into three portions.

(*a*) Add a drop of strong acetic acid and two drops of potassium ferrocyanide. A white precipitate is formed, which disappears on heating and reappears on cooling.

(*b*) To another portion add a few drops of strong nitric acid. A white precipitate is formed, which disappears on heating and reappears on cooling.

(*c*) To the third portion add a drop of copper sulphate solution. A white precipitate is formed.

53. The fluid from which the main mass of primary albumoses has been removed is filtered and treated in a beaker with a single drop of sulphuric acid, and then with ammonium sulphate that has been finely powdered in a mortar. The mixture is stirred vigorously till the fluid is saturated with the salt. A flocculent precipitate of the secondary albumoses (deutero-albumoses) is formed. Collect this on the rod as before, dissolve in a little water, divide the

solution into three parts, and repeat the three tests already performed with the primary albumoses. A precipitate is not formed by any of the reagents.

54. The fluid from which the secondary albumoses have been removed contains peptone. Filter it, and treat a portion of the filtrate with twice its volume of 40 per cent. sodium hydroxide and a drop of 1 per cent. copper sulphate. A pink colour appears, due to the presence of peptone.

Important Note.—This large excess of strong NaOH **must** be added in order to decompose the $(NH_4)_2SO_4$ with which the solution is saturated. The characteristic rose colour is only obtained when the alkalinity is due to NaOH, ammonia being quite inefficient.

5 c.c. of saturated $(NH_4)_2SO_4$ solution contains about 3·75 grms. of the salt. This requires 2·27 grms. of NaOH. 10 c.c. of 40% NaOH, containing 4 grms. of NaOH, is thus sufficient.

55. Evaporate a small portion of the original fluid to complete dryness, finishing the process on a water bath in order to prevent charring. Rub up the residue with successive small quantities of strong alcohol (95 per cent.). Add the extracts together, filter and evaporate them to dryness on a water bath. Dissolve the residue from this evaporation in a little water and test for proteins by the various colour tests. Only insignificant traces are present, showing that albumoses and peptones are insoluble in strong alcohol.

NOTE.—It is frequently desirable to remove all proteins from a solution before testing for certain substances, *e.g.* sugars, bile-salts, urea, etc. In the case of albumoses and peptones this can only be effected by the method described above, advantage being taken of the solubility of sugars, etc., in alcohol, and the insolubility of all proteins in the same. The aqueous solution prepared in this way will be spoken of as "an alcoholic extract."

Peptones. Use a 2 per cent. solution of Savory and Moore's peptone, which is usually free from albumoses.

56. Apply the usual colour reactions for proteins. They are all obtained.

NOTE.—The glyoxylic reaction may not be very intense, owing to the presence of chlorides in the preparation. Pure peptone, when freed from chloride by appropriate means, gives a very good glyoxylic reaction.

57. Add a drop or two of strong acetic acid and a drop of potassium ferrocyanide. No precipitate is produced, showing that the primary albumoses are absent.

58. Add a little Esbach's or Brücke's solution. A very slight or no precipitate is formed, if the solution be free from albumoses.

59. Saturate a portion with $(NH_4)_2SO_4$. No precipitate is produced, showing that all albumoses are absent.

60. Treat 5 c.c. of the filtrate from Ex. 59 with two volumes of 40 per cent. NaOH and a drop of copper sulphate. A pink colour is obtained.

61. Add a few drops of a solution of tannic acid. A white precipitate is formed.

62. Add a few drops of a solution of lead acetate. A white precipitate is formed.

J. The reactions of certain Sclero-proteins.

Gelatin is found in the body in the form of its anhydride, collagen. This occurs in white fibrous tissue and in the organic substance of bones, and can be converted into gelatin by boiling with a dilute acid. Dried gelatin swells in cold water, but is quite insoluble in it. On warming, a more or less viscid solution is obtained, which solidifies to a jelly on cooling provided the concentration be greater than 1 per cent. This process is reversible on warming and cooling. It is precipitated by half-saturation with ammonium sulphate, by tannic acid, phospho-tungstic acid, Esbach's and Brücke's reagents, but not by normal lead acetate. On complete hydrolysis it yields a high percentage of its nitrogen in the form of glycine, but very little in the form of the aromatic amino-acids, tyrosine or tryptophane, and none as the

sulphur-containing compound, cystine. Therefore its solutions fail to give the glyoxylic, Millon's and sulphur colour tests for proteins, and only give a slight xantho-proteic test, which is due, either to an impurity or to a small amount of phenyl-alanine.

63. Break gelatin up into small pieces and add a small amount of cold water. The gelatin does not dissolve. Immerse the test tube in a beaker of boiling water and leave it for a short time. The gelatin dissolves. Cool the tube under the tap: the gelatin sets to a jelly. Perform the following tests with an approximately 1 per cent. solution of gelatin:

(*a*) Xanthoproteic reaction: slight.

(*b*) Millon's reaction: very slight, showing absence of tyrosine from gelatin molecule. (See Notes to Ex. 2.)

(*c*) Glyoxylic reaction: not obtained, showing absence of ryptophane. (Ex. 3.)

(*d*) Biuret reaction: violet colour.

(*e*) Sulphur reaction: not obtained, showing absence of cystine. (Ex. 5.)

(*f*) Add acetic acid: no precipitate.

(*g*) Add acetic acid and potassium ferrocyanide: very slight or no precipitate.

(*h*) Add tannic acid: white precipitate.

(*i*) Add lead acetate: very slight or no precipitate.

(*j*) Half saturate with ammonium sulphate. The whole of the gelatin is precipitated, as shown by a negative biuret test in the filtrate (distinction from peptones).

(*k*) Add Esbach's or Brücke's solution: yellow or white precipitate (distinction from peptones).

Keratin. An insoluble body found in the hair, skin, nails, and horns. Remarkable for the high percentage of cystine it yields on acid hydrolysis.

64. Perform the following tests by using horn shavings, or hair. Note insolubility in hot or cold water, dilute acids, and dilute alkalies.

(*a*) Xanthoproteic reaction: well marked.

(*b*) Millon's reaction: well marked.

(*c*) Glyoxylic reaction: well marked.

(*d*) Biuret reaction: not obtained, owing to insolubility.

(*e*) Sulphur reaction: well marked.

CHAPTER II.

THE CARBOHYDRATES.

These compounds contain the elements carbon, hydrogen and oxygen, the general formula being $C_x(H_2O)_y$. They can be sub-divided into several groups.

A. The Monosaccharides.

B. The Disaccharides.

C. The Polysaccharides.

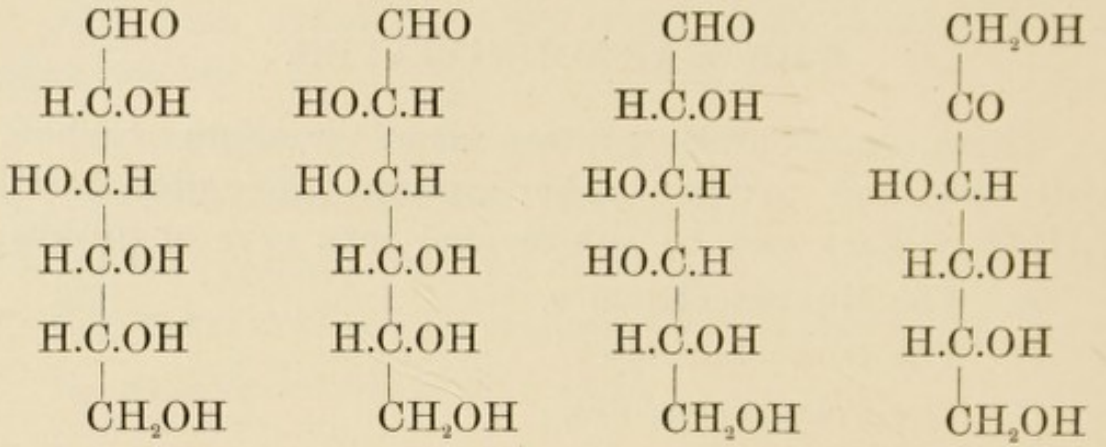
A. The Monosaccharides.

The **monosaccharides** are the simplest carbohydrates, and all the others can be hydrolysed to two or more molecules of monosaccharide by means of acids or certain ferments.

They consist of primary alcoholic ($-CH_2OH$) or secondary alcoholic ($=CH.OH$) groups linked to an aldehyde ($-CHO$) or ketone ($=C=O$) group. Those with an aldehyde group are called aldoses; those with a ketone group, ketoses. They contain from two to nine carbon atoms and are called bioses, trioses, tetroses, pentoses, hexoses, etc., depending on the number of carbon atoms in the molecule.

The lower members of the series are not important physiologically. The *pentoses* $C_5H_{10}O_5$ are found in the urine in certain pathological conditions. They form a constituent part of the molecule of nucleic acid. (See page 18.) The most important pentoses are the aldoses arabinose and xylose, obtained from gum arabic and pine-wood or straw respectively and ribose, obtained by the hydrolysis of the nucleoproteins.

The *hexoses*, $C_6H_{12}O_6$, are of great physiological importance. Of the many that have been synthesised in the laboratory only the following are found in nature and are of physiological interest:—



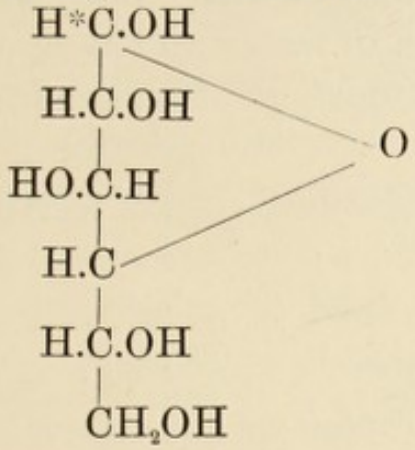
d-glucose : (dextrose, grape-sugar) | d-mannose | d-galactose | d-fructose (laevulose, fruit sugar)

It will be noticed that the first three are aldoses, whilst fructose is a ketose.

The first three are stereo-isomers, differing only in the arrangement of the H and OH groups in space round the four central carbon atoms, all of which are asymmetric. (See page 79.) It therefore follows that these compounds are optically active, that is, their solutions can rotate the plane of polarised light.

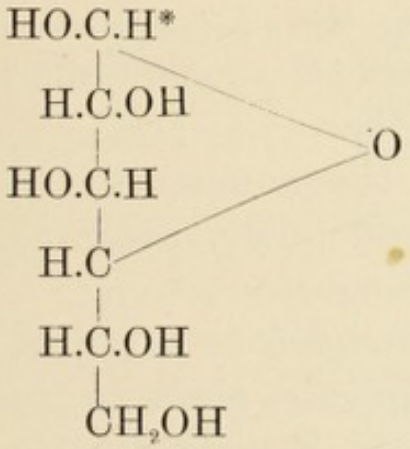
In the above formulæ they are represented as being aldehydes, but certain facts seem to indicate that they can exist in another form. Thus if glucose be dissolved in water it is found that the solution at first has a much higher rotatory power than when it has been kept for some hours or has been boiled with a trace of alkali. This phenomenon is known as mutarotation. Also it is very much less active chemically than the above formula warrants.

These properties are explained by assuming that when first dissolved in water, glucose exists as a γ-lactone, having the formula.

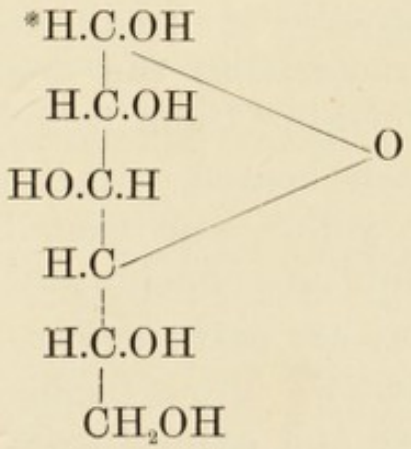


In this state the *C atom is asymmetric, so that two forms of glucose are possible, called α- and β-glucose.

Under certain conditions two forms of glucose can be isolated, one with a rotary power of 110°, the other with a rotation of 19°. When kept in solution both finally attain a rotation of 52·5°.



α-glucose.



β-glucose.

In solution both forms slowly pass into the aldelhyde form (tautomerism). If the *H atom be replaced by some other group (generally aromatic), the compound formed is called an α- or β-glucoside, which can be converted into glucose and another compound by hydrolysis with acids or certain ferments.

The natural glucosides (phloridzin, salicin, etc.) are β-glucosides.

Physical properties of the monosaccharides. They are white crystalline solids, very soluble in water and alcohol. Insoluble in ether, acetone and most of the organic solvents.

They are optically active, the natural sugars having the following rotatory powers—

Glucose = + 52·5°. Galactose = + 82°.
Fructose = − 93·8°.

Chemical properties. Being adehydes or ketones, they are susceptible of being oxidised to various acids, thus reducing certain oxidising reagents. This reaction only takes place in hot alkaline solutions, and is of great value as a test for these sugars, and especially as a basis of various methods of estimation.

They react with phenyl hydrazine in excess to give insoluble crystalline bodies called osazones. These are of the greatest value in determining the presence of and in characterising the monosaccharides, though not in distinguishing them from one another.

When heated with an alkali the monosaccharides become yellow and then brown, and finally decompose into a mixture of acids and resinous substances.

They are reduced by sodium amalgam to hexahydric alcohols. Sorbite is formed from glucose, mannite from mannose and dulcite from galactose. Fructose yields a mixture of sorbite and mannite.

On oxidation glucose gives rise to three acids—

CO_2H	CHO	CO_2H
$(CHOH)_4$	$(CHOH)_4$	$(CHOH)_4$
CH_2OH	CO_2H	CO_2H
Gluconic acid.	Glycuronic acid.	Saccharic acid.

Glycuronic acid is interesting physiologically, as it is frequently found in the urine in combination with various drugs, such as chloral, camphor, phenol, etc., in the form of a glucoside. These compounds protect the organism from the injurious effects of the drugs.

Glucose (dextrose or grape-sugar). Use a ·2 per cent. solution for the following reactions.

65. Boil with a little sodium hydroxide. The solution turns yellow. **(Moore's test.)**

NOTE.—The yellow colour is due to the formation of caramel (a condensation product) by the hot alkali. It must be noted here that glucose is completely destroyed by prolonged boiling with alkali.

66. Treat two or three c.c. of 5 per cent. caustic soda with four or five drops of a 1 per cent. solution of copper sulphate. A blue precipitate of cupric hydroxide, $Cu(OH)_2$, is formed. Add to the mixture an equal bulk of the sugar solution. The precipitate dissolves. Boil the solution for a short time. The blue colour disappears, and is replaced by a yellow or red precipitate of cuprous oxide, Cu_2O. **(Trommer's test.)**

NOTES—1. The amount of copper necessary depends on the percentage of sugar present. If only a small amount of sugar be present a mere disappearance of the blue colour is all that may happen, or possibly the fluid may assume a faint yellowish-red tint. If excess of copper be added, the reduction is obscured by the blue cupric hydrate in solution, or the black precipitate of cupric oxide that is formed on heating this in the alkaline solution. It is always best to add the copper sulphate a few drops at a time, boiling between each addition.

2. The reaction is a type of several that have been introduced for the detection of glucose, all of which depend on the fact that in alkaline solution it has reducing properties when boiled. For this reason, glucose, and all sugars that have this property are sometimes spoken of as "reducing sugars."

3. The property that glucose and other sugars have of dissolving cupric hydrate is common to a large number of organic compounds.

67. Boil about 3 c.c. of Fehling's solution (see Note 1) in a test tube. No change occurs. Add about 3 c.c. of the glucose solution and boil again. A red precipitate of cuprous oxide is formed. **(Fehling's test.)**

NOTES—1. Fehling's fluid is prepared as follows:

(*a*) Dissolve 103·92 grams. of pure copper sulphate in warm water and dilute to one litre.

(*b*) Dissolve 320 grams. of potassium sodium tartrate (Rochelle salt) in warm water, add a little carbolic acid to prevent the growth of fungi, dilute to exactly a litre and filter.

(*c*) Dissolve 150 grams. of sodium hydoxide in distilled water and dilute to 1 litre.

For use take exactly equal quantities of *a*, *b*, and *c*, and mix. Though the individual constituents keep indefinitely, the fluid when prepared suffers decomposition, so that a reduction occurs on boiling. For this reason the fluid should be prepared just before use, and must always be tested by boiling before being used.

The fluid is of such a strength that the copper sulphate in 10 c.c. is just reduced by ·05 grams of dextrose.

2. The addition of the Rochelle salt is for the purpose of dissolving the cupric hydoxide that would otherwise be precipitated by mixing (*a*) and (*c*).

3. The test is much more delicate and certain than Trommer's test, and should always be used in preference to it.

4. If the fluid that is being tested is acid, it should be neutralised.

5. Ammonium salts considerably interfere with Fehling's test. If they are present a little extra alkali should be added, and the mixture boiled for two or three minutes to allow of the evolution of the ammonia.

6. In testing for small amounts of glucose it is advisable to avoid an excess of Fehling's solution, owing to the excess of alkali tending to destroy the glucose before the latter can exert its reducing reaction on the copper. The neutral solution should be made faintly blue with Fehling's solution, and then boiled.

68. To 5 c.c. of Benedict's solution in a test tube, add about eight drops of the sugar solution. Boil vigorously for one or two minutes and allow the tube to cool spontaneously. The entire body of solution will be filled with a precipitate, red, yellow, or green in colour depending on the concentration of the sugar. **(Benedict's test.)**

NOTES—1. Preparation of Benedict's solution for qualitative test. Dissolve 173 grams. of sodium citrate and 100 grams. of anhydrous sodium carbonate in about 600 c.c. of distilled water by the aid of heat. Pour through a folded filter and make up to 850 c.c. Dissolve 17·3 grams. of crystallised copper sulphate in 100 c.c. of water and make up to 150 c.c. Pour the carbonate-citrate solution into a large beaker and add the copper solution slowly, with constant stirring. The mixed solution is ready for use and does not deteriorate on long standing.

2. Benedict's solution has certain advantages over Fehling's. For example, it is not so readily reduced by uric acid or urates, nor by creatinine. It is not reduced by chloroform, which is sometimes added to urine as a preservative. It does not destroy a small amount of sugar, as Fehling's does

(see note 6 to previous exercise). Also it can be used for testing urines for sugar in artificial light, since it is the bulk and not the colour of the precipitate that is of importance.

69. Boil some freshly prepared Barfoed's reagent and add to it the sugar solution, drop by drop, boiling the whole time. A red precipitate of cuprous oxide is formed, either at once, or on standing for a few minutes. **(Barfoed's test.)**

NOTES.—1. The reagent is prepared by dissolving 66 gm. cupric acetate and 10 c.c. of glacial acetic acid in water and making up to 1 litre.

2. This test is only given by the monosaccharides, not by maltose and lactose.

3. The reagent must be freshly prepared, otherwise it is reduced by maltose and lactose.

4. Chlorides interfere with the test, causing the appearance of a greenish white precipitate.

70. Boil 1 part of Nylander's solution with 5 parts of the sugar for about three minutes and allow to cool. A black precipitate of metallic bismuth settles out. **(Nylander's test.)**

NOTES.—1. Nylander's reagent is prepared by dissolving 50 gms. of Rochelle salt and 20 gms. of bismuth subnitrate in 1 litre of 8 per cent. caustic soda.

2. The reaction is of importance in detecting small quantities of glucose in urine. The uric acid and creatinine of concentrated urine reduce Fehling's solution, but have no action on Nylander's solution.

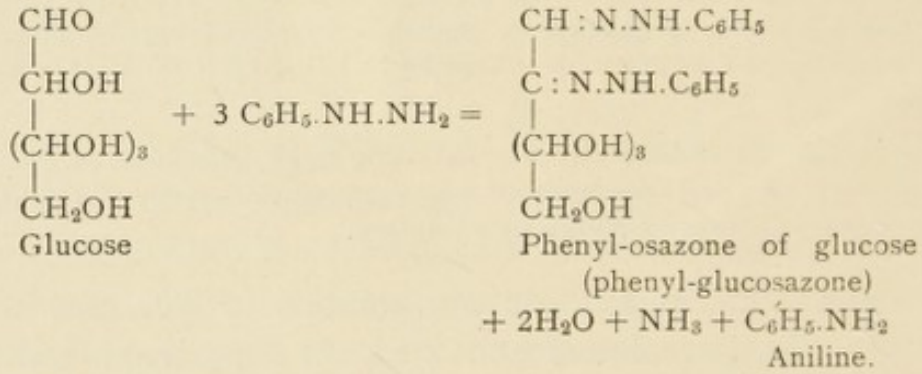
71. Treat 2 c.c. of a ·1 per cent. solution of safranine with 2 c.c. of the glucose solution and 2 c.c. of 5 per cent. sodium hydroxide. Mix and boil, avoiding any shaking. The opaque red colour gives place to a light yellow, owing to the reduction of the safranine to a "leuco-base."

72. Add to the solution of glucose some sulphindigotate of soda and some Na_2CO_3 and boil. The blue colour turns green, purplish, red, and finally yellow. Shake with air: the blue colour reappears. **(Mulder's test.)**

NOTE.—These two experiments illustrate the reducing properties of glucose in hot alkaline solution. The avidity of the reduced leuco-bases for oxygen is shown by the reappearance of the colour on cooling and shaking with air.

73. Take 10 c.c. of a 1·5 per cent. solution of glucose in a test tube. Add as much solid phenyl-hydrazine hydrochloride as will lie on a sixpenny piece, and at least twice this amount of solid sodium acetate.* Dissolve by warming, mix thoroughly, and filter into a clean test tube. Place this in a beaker of boiling water for at least half-an-hour, keeping the water boiling the whole time. Set the tube aside to cool (do not cool under the tap). A fine yellow crystalline precipitate of **phenyl-glucosazone** appears. Collect some of this by means of a pipette, transfer to a slide, cover with a glass and examine under both powers of the microscope. Note the characteristic arrangement of the fine yellow needles in fan-shaped aggregates, sheaves or crosses. Make a drawing of the crystals in the space provided at the end of the book.

NOTES.—Glucose is an aldehyde, and, like all aldehydes and ketones, forms a compound with phenyl-hydrazine. But this phenyl-hydrazone of glucose is very soluble, and cannot be readily separated. However, in the presence of an excess of phenyl-hydrazine at 100°C. an insoluble osazone is formed.



2. Phenyl-hydrazine is a yellow basic liquid, insoluble in water, but soluble in dilute acids to form salts. If the base itself is used, two or three drops should be dissolved in a few drops of strong acetic acid, and added to the sugar solution.

3. Phenyl-hydrazine hydrochloride, $C_6H_5.NH.NH_2.HCl$ does not give an osazone when boiled with glucose, unless an excess of sodium acetate be added. This acts on the hydrochloride to form phenyl-hydrazine acetate and sodium chloride.

B. The Disaccharides.

These carbohydrates have the empirical formula, $C_{12}H_{22}O_{11}$. They are hydrolysed by boiling with dilute

* It is advisable to add 1 c.c. of strong acetic acid.

acids or by the action of certain specific enzymes into two molecules of monosaccharide.

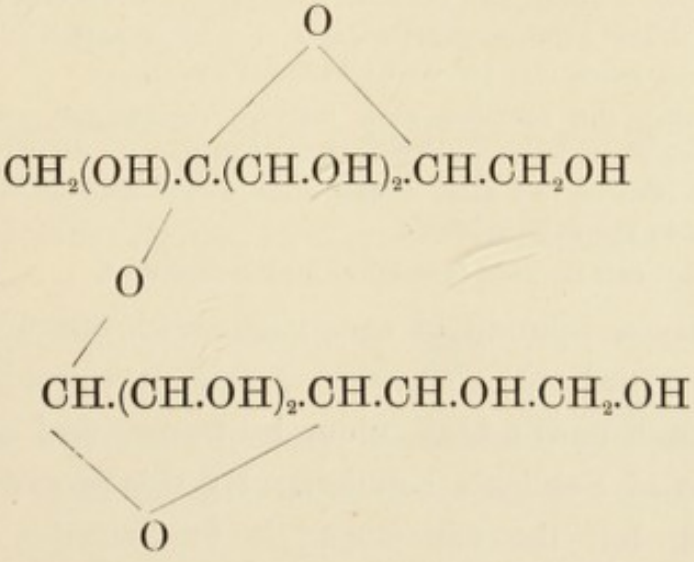
$$C_{12}H_{22}O_{11} + H_2O = C_6H_{12}O_6 + C_6H_{12}O_6.$$

The three disaccharides of physiological interest are cane-sugar, maltose and lactose (milk-sugar).

Cane-sugar (sucrose) is widely distributed in the vegetable kingdom, where it functions as a reserve material. It crystallises well, is very soluble in water, and has a much sweeter taste than glucose.

It does not reduce Fehling's solution, does not form an osazone, and does not behave as an aldelyde or ketone. It is hydrolysed very readily by boiling acids to a mixture of glucose and fructose. Cane-sugar is dextrorotatory, but since fructose is more laevorotatory than glucose is dextrorotatory, a mixture of the two in equal parts is laevorotatory. So the sign of rotation being inverted by hydrolysis, the process is known as inversion, and the product as "invert sugar." This hydrolysis is also effected by the enzyme invertase (sucrase), which is found in the small intestine and in certain yeasts.

The constitution of cane-sugar is not yet definitely established, but in all probability it is formed by the condensation of glucose and fructose in such a way as to destroy both the aldehyde and the ketone groups.



74. Repeat experiments 65, 67 and 68, with a freshly prepared 1 per cent. solution of pure white crystalline cane-sugar ("coffee sugar"). Note that it is unaffected by alkali and exerts no reducing reaction on Fehling's solution.

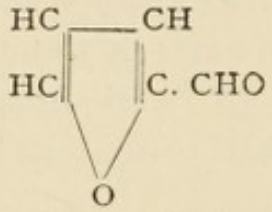
75. Treat 3 c.c. of the solution with one drop of strong sulphuric acid and boil for a minute. Add a drop of litmus solution and neutralise with caustic soda. Apply Trommer's or Fehling's test to portions of this fluid. A well marked reduction is obtained in both cases.

NOTE.—This reaction depends on the fact that although cane-sugar is a non-reducing sugar, it is converted to equal parts of glucose and laevulose by boiling with dilute mineral acids.

$$\underset{\text{Cane-sugar.}}{C_{12}H_{22}O_{11}} + H_2O = \underset{\text{Glucose.}}{C_6H_{12}O_6} + \underset{\text{Laevulose.}}{C_6H_{12}O_6}$$

76. Treat four drops of the solution of cane-sugar with four drops of 2 per cent. solution of alpha-naphthol in alcohol and 5 c.c. of fuming hydrochloric acid. Heat to boiling point. The fluid immediately begins to assume a rich purple tint.

NOTES.—1. This reaction depends on the fact that the laevulose, which is formed by the action of the acid on the cane-sugar, yields furfurol (furfuraldehyde)



which in its turn reacts with the alpha-naphthol to give a purple colour.

2. Glucose, lactose, and maltose only give this reaction very feebly. The polysaccharides and especially cellulose give a fair reaction. It is also given by certain proteins, when it is known as Molisch's rection.

3. In using the reaction as a test for cane-sugar, great care must be taken to remove proteins and dextrins from solution by the method described in Ex. 55. The residue left after evaporation of the alcohol will contain all the sugars present in the original fluid.

4. Thymol can be used instead of alpha-naphthol.

77. Mix a solution of cane-sugar with one of glucose. Boil the mixture with Fehling's solution, adding the Fehling's solution to the boiling fluid until a blue colour by transmitted light indicates a slight excess of Fehling's solution. By this procedure the glucose is destroyed, but the cane-sugar is unaffected. Filter off the

precipitate of cuprous oxide. Make the filtrate acid with sulphuric and boil. Neutralise the solution, add a ltttle more Fehling's and boil again. A well-marked reduction is obtained due to the production of glucose and laevulose by "inversion" of the cane-sugar by the acid.

NOTE.—In using this as a test for cane-sugar in the presence of glucose, the presence of the polysaccharides must be excluded by alcoholic extraction if necessary: and the solution must give a well-marked alpha-naphthol test, as lactose and maltose, after boiling with Fehling's solution, give a reducing substance by acid hydrolysis.

78. **Seliwanoff's test** for laevulose. Obtain a neutral solution containing laevulose as in Ex. 75. To 5 c.c. of Seliwanoff's reagent add a few drops of the sugar and heat the solution to boiling. A red colouration and a red precipitate are formed. The precipitate dissolves in alcohol, to which it imparts a striking red colour.

NOTES.—The reagent is prepared by dissolving 0·05 grm. of resorcin in 100 c.c. of 1 in 2 hydrochloric acid.

The test is also given by the monosaccharides after long boiling, but a precipitate is not usually formed.

Maltose is the disaccharide formed as the final product of the hydrolysis of starch by the enzyme ptyalin. It is hydrolysed by boiling acids, and by the enzyme maltose of the small intestine, to two molecules of glucose. It exhibits well-marked reducing properties towards Fehling's and Nylander's solutions, but not towards Barfoed's. It forms an osazone with phenyl-hydrazine acetate, which is more soluble than glucosazone and which melts at 206°C. Constitutionally it is glucose *a*-glucoside.

79. Repeat experiments 65 and 67 with a ·2 per cent. solution of maltose. It behaves like glucose.

80. Boil with Barfoed's reagent. No reduction. (Distinction from glucose.)

81. Examine microscopically and draw the crystals of phenyl-maltosazone that have been prepared by the demonstrator. Note that they are much broader than the crystals of glucosazone. Make a drawing of the crystals in the space provided at the end of the book.

Lactose is the sugar found in milk, and often in the urine of women during lactation. It has reactions very similar to those of maltose. It is hydrolysed by boiling acids, and by the ferment lactase into a molecule of glucose and one of galactose.

Constitutionally it is glucose-β-galactoside.

It is not fermented by ordinary yeast.

The osazone melts at 200°C.

82. Repeat Exs. 65 and 67 with a ·2 per cent. solution of lactose. It behaves like glucose.

83. Boil with Barfoed's reagent. No reduction. (Distinction from glucose.)

84. Examine microscopically and draw the crystals of phenyl-lactosazone that have been prepared by the demonstrator. Notice that they differ considerably from glucosazone, separating, usually, as ovoid or spherical clusters of fine needles. Make a drawing of the crystals in the space provided at the end of the book.

C. The Polysaccharides.

These compounds are formed by the condensation of more than two molecules of monosaccharides. Their general formula is $(C_6H_{10}O_5)n$.

Starch is widely found in the vegetable kingdom as a reserve carbohydrate. It occurs in the form of grains, the form of which is characteristic for a particular plant. These grains may consist of two materials, starch "granulose" and starch "cellulose", the latter forming a dense envelope to the grain; owing perhaps to this the grains are insoluble in cold water, and are only slowly attacked by enzymes. But on being boiled they absorb water and swell up to form a paste that is readily attacked by certain enzymes.

Starch has a very high molecular weight, and on being boiled with water forms an opalescent "solution" that is really a colloidal suspension. It does not diffuse through

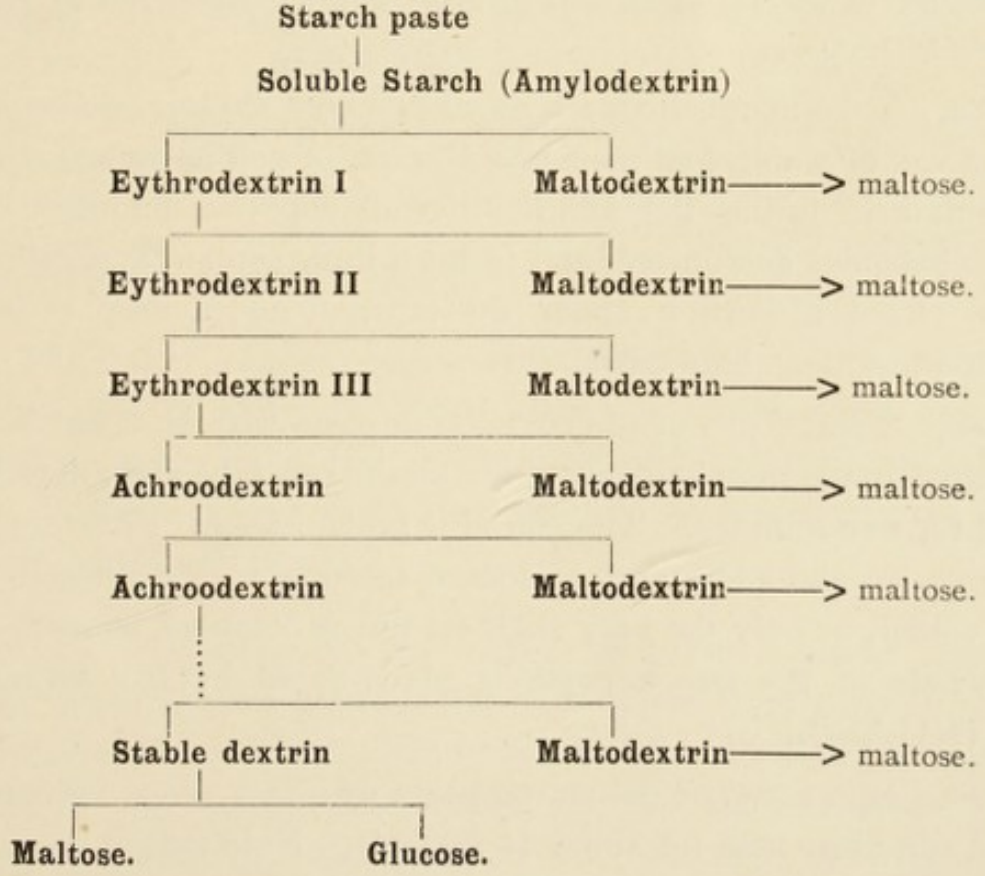
membranes and does not depress the freezing point of water.

This so-called "starch paste" is completely precipitated by half-saturation with ammonium sulphate and by the addition of an equal volume of strong alcohol.

The most characteristic reaction of starch is the blue colour it gives with free iodine solution. It does not reduce Fehling's solution and is only slowly affected by boiling alkalies.

Starch paste is hydrolysed by boiling acids and by certain enzymes, which are therefore called the amylolytic enzymes. These are ptyalin of saliva, amylopsin of pancreatic juice and the diastases found in malt and certain yeasts and moulds.

The products of hydrolysis of starch by such a ferment as ptyalin are very numerous. The following scheme indicates the probable course of the hydrolysis, but it is not claimed that it is yet finally established.



85. Place a small amount of dry potato-starch on a slide, add a drop of water, cover with a slip and examine under the microscope. Note the characteristic oval starch grains, the concentric markings and the hilum, usually eccentric. Make a drawing of the grains. Run a drop of iodine under the slip; note that the grains take on a blue colour.

86. Shake a small amount of potato starch with cold water. The starch does not dissolve. Filter, and add a drop of iodine solution to the filtrate. The characteristic blue reaction is not obtained.

87. Shake some dry starch with a little sodium carbonate solution. No change is effected. Repeat, with a little sodium hydroxide. The starch is immediately gelatinised. Add a few drops of iodine solution, a blue colour is not obtained. Treat with strong acetic acid. A deep blue colour appears.

NOTE. Free iodine is necessary to give the blue adsorption compound with starch. Sodium hydoxide removes free iodine, converting it into iodide and iodate. The action of the acid on the latter causes the appearance of free iodine and the blue colour. *Always neutralise an alkaline solution before testing for the polysaccharides.*

88. Take as much starch as will lie on a shilling, shake it up with 5 c.c. of water, and pour into 100 c.c. of boiling water, stirring the mixture during the addition. Boil for two minutes. The starch becomes gelatinised, and forms a thin, somewhat opalescent paste. Cool a portion under the tap and add a drop of iodine solution. A deep blue colour is formed.

89. Treat 5 c.c. of the cold starch paste with an equal bulk of saturated ammonium sulphate. Shake the test tube and allow it to stand for five minutes. The starch is precipitated. Filter through a dry paper, and add a drop of iodine solution to the filtrate. No blue colour, or only the very slightest tint is obtained, showing that the whole of the starch paste is precipitated by half-saturation with $(NH_4)_2SO_4$.

90. Boil 5 c.c. of the starch paste with two drops of concentrated sulphuric acid for about 15 seconds. Note that the solution

becomes perfectly clear and translucent. Add two drops of strong ammonia to neutralise the acid, cool under the tap, add an exactly equal bulk of saturated $(NH_4)_2SO_4$, shake the tube vigorously and allow it to stand for five minutes. Filter through a dry filter-paper and add two drops of iodine solution to the filtrate. A deep blue colour is obtained.

NOTE.—Starch paste is rapidly converted into "soluble starch" on boiling with dilute mineral acids. Soluble starch differs from starch paste in that it is not completely precipitated by half-saturation with $(NH_4)_2SO_4$ in the course of five minutes. If it be allowed to stand for twenty-four hours, however, it is completely precipitated.

91. Take 10 c.c. of the starch paste in a small beaker. Add five drops of concentrated sulphuric acid, bring the mixture to the boiling point, and keep it boiling for seven minutes. Add a drop of litmus solution and neutralise with sodium hydroxide, keeping the reaction on the acid rather than on the alkaline side. Cool one portion under the tap and add a drop of iodine solution. A purple, red or brown reaction of erythro-dextrin is obtained, instead of the original blue reaction of starch. To the other portion add 3 c.c. of Fehling's solution and boil. A well marked reduction is obtained.

NOTE.—Starch is converted to erythro-dextrin and glucose by boiling with dilute mineral acids. If the boiling is prolonged the erythro-dextrin is converted into glucose. The extent of boiling required to destroy the whole of the starch, and yet to leave some erythro-dextrin varies with the concentrations of the starch paste and of the acid employed.

The Dextrins are polysaccharides formed by the partial hydrolysis of starch. They differ a great deal in complexity, and, with the exception of the erythrodextrins, are characterised and separated as individuals with considerable difficulty.

They all dissolve in water to form a clear solution (distinction from glycogen). They are insoluble in strong alcohol and in ether. They all reduce Fehling's solution with the exception of amylodextrin. This indicates that they contain an aldehyde or a ketone group in the

molecule. But owing to the large size of the molecule the reducing power of the higher dextrins is very slight.

Only the higher members yield a colour with iodine.

Amylodextrin gives a pure blue with iodine. It is slowly precipitated by half-saturation with ammonium sulphate: immediately by full saturation.

Erythrodextrin I. gives a purplish colour with iodine: is precipitated by full saturation with ammonium or magnesium sulphates.

Erythrodextrin II. gives a red colour with iodine. It is precipitated by full saturation with ammonium, but not by magnesium sulphate.

Erythrodextrin III. gives a red brown colour, and is not precipitated by any mixture of salts.

Achroodextrins give no colour with iodine, and are not precipitated by salts.

Maltodextrin is the name given to a substance that was separated from the mixed products obtained by the hydrolysis of starch paste by malt diastase. It consists of three molecules of maltose united together with the elimination of two molecules of water, and retaining a terminal aldehyde group. It reduces Fehling's solution, but does not ferment with yeast or give an osazone. It is hydrolysed by ferments very rapidly to maltose: by acids to glucose.

Stable dextrin is also formed by the action of amylolytic enzymes on starch paste. It is rather resistant to the action of the enzymes, but is slowly converted into a mixture of equal parts of maltose and glucose. It is formed by the condensation of forty molecules of glucose with the elimination of thirty-nine molecules of water. In the hydrolysis of starch by enzymes, about 80 per cent.

of the starch is converted into maltose, the remaining 20 per cent. being stable dextrin.

The Dextrins.

92. Shake a little commercial dextrin with some cold water. An opalescent solution is formed. Boil the solution. It becomes perfectly translucent. (Distinction from glycogen.)

Use a 3 per cent. solution of commercial dextrin for the following reactions:

93. To about 5 c.c. of the dextrin solution add iodine solution, drop by drop, noting the colour at every addition. The colour is at first almost a pure blue but it changes through a rich purple-red to a red-brown as the iodine is added.

94. Repeat the above experiment, but boil and then cool the tube after each addition. The colour disappears on boiling, but does not reappear on cooling until several drops of iodine have been added.

95. Add a drop or two of the starch paste prepared in Ex. 88 to about 5 c.c. of the dextrin solution. To this mixture add diluted iodine solution, drop by drop. The first additions produce a pure blue colour, and it is not till a considerable amount of iodine has been added that the solution acquires a purplish tint.

NOTE.—The affinity of starch for iodine is so much greater than that of dextrin that the characteristic colour reactions of erythro-dextrin are not obtained until all the starch has been saturated with iodine. Even then it is sometimes difficult to detect, owing to the deep blue starch reaction.

96. Treat 5 c.c. of the dextrin solution with about 10 drops of the starch paste: to the mixture add an equal bulk of saturated $(NH_4)_2SO_4$, shake vigorously, and allow to stand for five minutes. The starch is precipitated. Filter through a dry paper, and to a portion of the filtrate add a drop or two of iodine solution. The purple red reaction of erythro-dextrin is obtained.

97. Saturate 5 c.c. of the dextrin solution by boiling with an excess of finely powdered ammonium sulphate. Note the precipitate of erythro-dextrin produced. Cool under the tap and filter. To the filtrate add a drop of iodine. A red-brown colour is produced.

NOTE.—This colour is due to the fact that erythro-dextrin III. is not precipitated by ammonium sulphate. This is the method employed for the identification of erythro-dextrin in the presence of glycogen, which is completely precipitated by saturation with ammonium sulphate.

98. Boil a few c.c. of the dextrin solution with a small amount of Fehling's fluid. A well-marked reduction is usually obtained.

NOTE.—Commercial dextrin is generally prepared by the action of dilute acids on starch (See Exercises 90 and 91), the action being stopped as soon as a portion fails to give a blue colour with iodine, and the products then being precipitated by alcohol. Such preparations contain some dextrose, and often a little soluble starch. At the same time it must be noted that the achroo-dextrins have a reducing action themselves even when thoroughly separated from the dextrose.

99. Take 10 c.c. of the dextrin solution in a small flask; add 30 c.c. of strong (95 per cent.) alcohol, place the thumb over the mouth of the flask and shake vigorously for some seconds. Note that a portion of the dextrin is precipitated as a gummy mass which sticks to the sides of the flask.

Pour off the alcohol, filter it and label the filtrate A. Rinse the flask out with a few c.c. of alcohol, shake off as much of this alcohol as possible, and add 10 c.c. of hot water. Shake this round the flask till the whole of the gummy precipitate dissolves. Divide the solution into three portions, B, C, and D. To B add a drop of iodine: a purple colour is produced. Boil C with a little Fehling's solution: only a slight reduction takes place. Boil D with two drops of concentrated sulphuric acid for two minutes, neutralise with NaOH, and boil with a little Fehling's solution: a well-marked reduction occurs.

100. To a portion of filtrate A, add a drop of iodine solution. No colour is produced. To another portion of about 5 c.c. add an equal bulk of strong alcohol. A white precipitate of achroo-dextrin is formed.

Glycogen is a reserve polysaccharide found in the liver and muscles. It forms a white amorphous powder, soluble in water to form an opalescent solution. It is

precipitated from solution by the addition of an equal volume of strong alcohol or by full saturation with ammonium sulphate. It does not reduce Fehling's solution, form an osazone or ferment with yeast. It gives a reddish colour with iodine. By boiling acids it is hydrolysed to glucose: by most of the diastatic enzymes to maltose, but by the diastase found in the liver to glucose. It is not affected by boiling alkalies. It is dextro-rotatory.

Estimation. Pflüger's method is undoubtedly the best. 20 to 100gm. of the tissue is cut into small pieces and placed in an Erlenmeyer flask of Jena glass. 100 c.c. of 60% potash ("pure by alcohol"—sp. gr. 1. 438) is added, a reflux condenser fitted, and the flask immersed for three hours in a boiling water bath. The alkali destroys the proteins without attacking the glycogen.

After cooling 200 c.c. of water and 800 c.c. of 96% alcohol are added and the mixture left to stand over night. The glycogen is thus precipitated free from protein. The supernatant fluid is carefully decanted and filtered. The precipitate is washed with ten times its volume of 66% alcohol, containing 1 c.c. per litre of saturated sodium chloride. After settling, the fluid is filtered through the original filter paper. This process is repeated once more, and then the precipitate is shaken with ten times its volume of 96% alcohol and filtered through the same paper. The precipitate is washed with ether, dissolved in boiling water and the solution made up to one litre. 200 c.c. of this are treated with 10 c.c. of concentrated HCl and heated in a flask on a boiling water bath for three hours, to convert the glycogen into glucose. After cooling, the solution is neutralised with 20% potash and filtered through a small paper into a 250 c.c. measuring flask. The washings from the flask used for inversion are filtered through the same paper to remove the last traces of glucose, and the solution brought up to 250 c.c. The percentage of glucose in the solution is determined by analysis. This multiplied by ·927 gives the amount of glycogen in the 200 c.c. of the solution used for inversion, and so the percentage in the tissue can be readily calculated.

Preparation. A rabbit, which has had a full meal of carrots some five or six hours previously, is killed by decapitation. The liver is cut out as quickly as possible, and the gall-bladder removed. The liver is rapidly chopped into small pieces, a small portion being reserved for Ex. 106, and the remainder immediately thrown into boiling water. After about two minutes boiling the larger morsels are strained off, pounded to a paste with sand in a mortar, and replaced in the boiling water. The proteins in solution are then coagulated by making the boiling fluid just acid with acetic acid. The fluid is filtered through coarse filter paper. In this way a crude solution of glycogen is obtained.

101. Boil 5 c.c. in a test tube. The characteristic opalescence does not disappear. (Distinction from erythro-dextrin.)

102. To a small amount of the cooled solution add iodine, drop by drop. A red colour is formed, which disappears on shaking, until with a certain amount of iodine added it is permanent. Now heat the solution. The colour disappears, to reappear on cooling.

NOTE.—If much protein is present in solution the colour will not reappear on cooling unless a considerable amount of iodine be added. This is due to the fact that proteins combine with iodine to form an iodo-protein.

103. Saturate 10 c.c. of the solution with finely-powdered $(NH_4)_2SO_4$. The glycogen is precipitated. Filter, and add a drop or two of iodine to the filtrate. No red colour is produced. Scrape the precipitate off the paper, boil with a small amount of water. The solution is markedly opalescent. Cool the solution, and add iodine. A port-wine red colour is obtained.

104. Boil 5 c.c. of the solution with a little Fehling's fluid. A very slight or no reduction is obtained.

NOTE.—If the liver has been rapidly boiled, no sugar will be present. If delay has occurred during the initial stages of the preparation, some of the glycogen will have been converted into glucose. (See Exercise 106.)

105. To 10 c.c. of the solution add 20 c.c. of strong alcohol, shake vigorously and filter. To a portion of the filtrate add iodine solution. No colour is obtained, showing that the whole of the glycogen is precipitated. Dissolve the precipitate in a little hot water: note that it is opalescent. Add three drops of strong sulphuric acid and boil for about three minutes: the opalescence disappears. Neutralise with sodium hydroxide and apply Fehling's test: A marked reduction occurs, due to the conversion of the glycogen into glucose by the boiling acid.

106. The portion of rabbit's liver that was reserved has been kept in a warm place for about six hours and extracted with boiling water as before. (Or a decoction of the liver of a sheep obtained from a butcher may be used.) Note that the solution is almost

translucent. To a portion add iodine: only a very slight or no red colour at all is produced. To another portion apply Fehling's test: a well-marked reduction occurs.

107. Prepare a solution which contains equal quantities of 1 per cent. starch paste (freshly prepared), of a strong solution of glycogen and of a 3 per cent. solution of commercial dextrin. Note that the mixture is markedly opalescent.

To a small portion add diluted iodine, and note that a pure blue *starch* reaction is obtained.

To another portion of about 5 c.c. add an equal bulk of saturated $(NH_4)_2SO_4$, shake vigorously, leave for five minutes, and filter. Note that a portion of the filtrate gives a reddish colour with iodine, and that it is distinctly opalescent. Indication of the presence of *glycogen*.

Saturate the remainder of the fluid with finely-powdered $(NH_4)_2SO_4$ and filter. The filtrate gives a reddish-brown colour with iodine. Indication of the presence of *erythro-dextrin*.

D. The Quantitative Estimation of Sugar.

The basis of nearly all the modern methods for the volumetric estimation of the sugars is the determination of the amount of the sugar solution necessary to reduce a given volume of Fehling's solution. The chief difficulty of the original method lies in deciding the exact point when the copper is reduced, as indicated by the complete disappearance of the blue colour. This is obscured by the red precipitate of cuprous oxide that is deposited.

In Ling's method an indicator is used to determine this point. In Pavy's method strong ammonia is added to form a soluble cuprous compound. In Benedict's method potassium sulphocyanide is employed for the same purpose.

Of the methods given below, Bang's is undoubtedly the most accurate, and is to be preferred when a very reliable estimation of sugar is required.

As a standard method for general work I can strongly recommend Benedict's.*

Standardisation of the Solutions. Owing to the fact that individual workers go to rather a different end point, it is advisable to perform estimations of a standard solution of sugar. This is prepared as follows: 9·5 grams of pure cane sugar are dissolved in water and the solution accurately made up to 1000 c.c. Of this solution 100 c.c. are boiled with 30 c.c. of $\frac{N}{2}$ HCl, the mixture being kept boiling for one minute. It is then cooled, neutralised by the addition of 30 c.c. of $\frac{N}{2}$ NaOH and made up to 200 c.c. with water. Such a solution contains 0·5 gm. of invert sugar per cent.

A titration of Fehling's or Benedict's solution is performed with this, and the result noted. Suppose that 10 c.c. Fehling's solution are found to be reduced by 0·054 gm. of invert sugar, use this factor rather than the theoretical 0·05.

108. Benedict's Method.

Principle of the Method.—An alkaline solution of copper sulphate, containing thiocyanate is boiled and the sugar solution run in from a burette till the blue colour just disappears. The thiocyanate forms a white insoluble compound with the cuprous hydroxide formed by the reduction of the copper, and so there is no red cuprous oxide precipitated to obscure the blue tint. A little potassium ferrocyanide is also added to prevent any possibility of the deposition of the cuprous oxide.

Preparation of the Solution.—With the aid of heat dissolve

Sodium citrate	200 grams.
Sodium carbonate (cryst).	200 grams.
(or anhydrous sod. carb. 75 grams.)	
Potassium thiocyanate (sulphocyanide)	125 grams.

in enough distilled water to make about 800 c.c. of the mixture and filter, and cool to room temperature.

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* For recent improved methods see pp. 225-231.

Dissolve 18 grams of pure, air-dried crystalline copper sulphate in about 100 c.c. of distilled water, and pour it slowly into the other liquid with constant stirring. Add 5 c.c. of a 5% solution of potassium ferrocyanide and then distilled water to make the total volume 1000 c.c. The solution appears to keep indefinitely, without any special precaution, such as exclusion of light, etc.

Method of Analysis.—Fit a 4-oz. flask into a ring of a retort stand of such a size that it is fairly firmly held. There is no need to use a wire gauze. Arrange the flask at such a height over a Bunsen burner that the reagent can be kept briskly boiling by means of a *small* flame. In the flask place 3 to 4 grams of anhydrous sodium carbonate. This can be roughly measured by taking a depth of 1 inch in a dry test tube. Then add 25 c.c. of the reagent and heat till the carbonate is in solution. Run the sugar solution in from a burette, which is best held in the hand. Run the sugar in at a fair rate, till a bulky chalk-white precipitate is formed and the blue colour lessens perceptibly in intensity. From this point the sugar is added more and more slowly, with constant vigorous boiling, until the disappearance of the last trace of blue colour, which marks the end-point. If the volume of the sugar used is less than 5 c.c., dilute it accurately with water till about 10 c.c. are judged necessary. Repeat the titration with this as before.

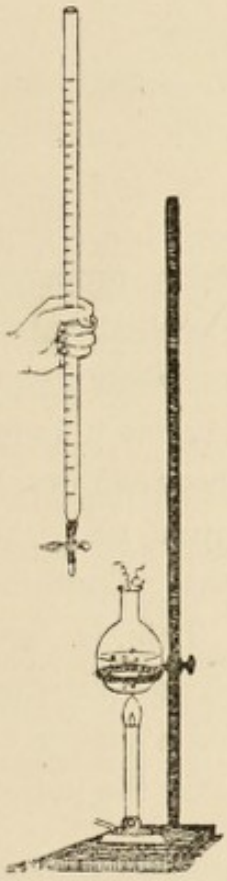


Fig. 1.

NOTES.—There is a tendency to run in an excess of the sugar, unless special care is exercised throughout the titration and particularly at the end. The solution must be kept vigorously boiling during the entire process, and towards the end the sugar must be added in portions of a drop or two, with an interval of about 30 seconds after each addition. Should the mixture become too concentrated, boiled distilled water may be added to replace that lost by evaporation.

The titration can also be carried out in a white porcelain dish of 10 to 15 cm. in diameter.

Should the solution bump excessively, a *small* amount of powdered pumice stone may be added.

Calculation of Results.

25 c.c. of Benedict's solution are reduced by 0·05 grm. of glucose.
0·053 grm. laevulose.
0·074 grm. maltose.
0·0676 grm. lactose.

Example.—First titration required 2·4 c.c.
Solution diluted 1 in 4 (10 c.c. of sugar diluted with 30 c.c. water).
Second titration required 9·7 c.c.
So 9·7 c.c. diluted solution contain 0·05 grm. glucose.

100 c.c. diluted solution contain $\frac{\cdot 05 \times 100}{9 \cdot 7}$

100 c.c. original solution contain $\frac{0 \cdot 05 \times 100 \times 4}{9 \cdot 7}$

Percentage of glucose = 2·06.

109. **Fehling's Method.**

Preparation of solution. See Ex. 67, p. 35.

Method. With a pipette measure 10 c.c. of freshly prepared Fehling's solution into a small flask. Add 40 c.c. of distilled water, heat the mixture till it boils and *keep it boiling the whole time.* Run in the sugar solution from a burette, 0·5 to 1 c.c. at a time, allowing the mixture to boil for about 15 secs. between each addition. A red precipitate of cuprous oxide forms and the intensity of the blue in the supernatant fluid decreases. Continue to add the sugar till this is completely removed. This is best determined by holding the flask by the rim at the neck and viewing it by transmitted light. If an excess of sugar be added a yellow or brown colour appears due to the formation of caramel by the action of the alkali on the sugar.

If less than 5 c.c. of the sugar are used, the solution must be diluted till about 10 c.c. are necessary. Thus if 2·5 c.c. are used in the first rough titration, the sugar should be diluted 1 in 4, by taking 25 c.c. and adding water till the volume of the solution is 100 c.c. The burette is washed out and filled with this diluted solution and the process repeated. But this time run in nearly the whole of the sugar solution judged necessary at such a rate that the mixture does not go off the boil. Then add 0·1 to 0·2 c.c. at a time till the reduction is complete. This titration should be repeated at least once more.

Calculation. 10 c.c. of Fehling's solution are reduced by 0·5 gm. glucose.

Example. 1·5 c.c. of the original solution necessary.

Sugar diluted 1 in 7 (10 c.c. sugar made up to 70 c.c.)

10·2 c.c. diluted sugar solution required for 10 c.c. Fehling's.

10·2 c.c. dil. sugar = ·05 gm. glucose.

$$100 \text{ c.c. } ,, \quad ,, = \frac{\cdot 05 \times 100}{10 \cdot 2} \ ,,$$

$$100 \text{ c.c. original sugar} = \frac{\cdot 05 \times 100 \times 7}{10 \cdot 2}$$

$$= 3 \cdot 43 \text{ per cent.}$$

110. **Ling's Method.**

Preparation of the indicator. Dissolve 1·5 gm. ammonium thiocyanate and 1 gm. ferrous ammonium sulphate in 10 c.c. water at about 45° C. and cool at once. Add 2·5 c.c. of concentrated hydrochloric acid. The solution thus obtained has invariably a brownish-red colour, due to the presence of some ferric salt. Add zinc dust, in small portions at a time, till the fluid is just colourless. On standing for some time the red colour reappears, and must be removed again by a trace of zinc dust. But the delicacy of the indicator is impaired by being decolourised several times. When this indicator is treated with a cupric salt, the colourless ferrous thiocyanate is oxidised to the red ferric thiocyanate.

Method of analysis. 10 c.c. of Fehling's solution and about 30 c.c. of water are boiled in a flask and the sugar solution is run in from a burette as described above in Fehling's method. *The indicator is not used till the blue colour has nearly disappeared.*

Then place a drop of the indicator on a white slab. Transfer a drop of the mixture from the flask to the middle of the drop of the indicator as rapidly as possible by means of a glass tube. If a red colour appears immediately on touching the drop the reduction is not completed. More sugar must be added and a fresh drop of the indicator used as before till no colour or only a faint tinge of red is obtained. If less than 5 c.c. of the sugar solution are necessary to complete the reaction, the solution must be diluted till about 10 c.c. are required, as described above in Fehling's method.

Special precautions. Use a glass tube, not a rod, for transferring the drop.

Do not put your finger on the top of the tube. Dip it in the flask and transfer it immediately to the indicator. The flask may be taken off the boil for an instant while this is done.

Do not stir the drops on the slab.

Wash the tube before using it again.

Calculation of results. This is the same as in Fehling's method.

111. Bang's Method.*

Principle. A known volume of copper thiocyanate in potassium carbonate is boiled for three minutes with a given volume of the glucose solution, that is not sufficient to reduce it completely. The copper in excess is determined by titration with hydroxylamine solution. Both the sugar and the hydroxylamine reduce the copper to colourless cuprous thiocyanate, so the end point is readily observed.

Preparation of Solutions.

1. 12·5 grams of copper sulphate are dissolved by heat in 75 c.c. of water and the solution cooled to 25°C. In a large porcelain basin 250 grams potassium carbonate, 200 grams potassium thiocyanate and 50 grams potassium bicarbonate are dissolved by stirring in 600 c.c. water. If the potassium bicarbonate does not dissolve it must be heated on the water bath to 40°C., but no higher. It is then cooled to 15°C. and the copper solution mixed with it in small quantities at a time with frequent shaking, to prevent any large amount of precipitate forming. The solution is then made up to 1 litre.

2. 6·55 grams of hydroxylamine sulphate or 5·56 grams hydroxylamine chloride are dissolved in water and the solution added to one of 200 grams potassium thiocyanate in 1500 c.c. water. The volume is made up to 2 litres.

Method of estimation. The amount of glucose added must be less than 0·06 gm. If, therefore, the solution contain less than 0·6 per cent., 10 c.c. of it are taken for the estimation. If it contain more than this, then such an amount must be taken as will yield a total amount less than 0·06 gm. In all cases the sugar solution must be made up to 10 c.c. Where there is no previous knowledge as to the strength of the sugar solution a preliminary titration should be made by boiling 10 c.c. of the sugar with 50 c.c. of the copper solution for three minutes. If the blue colour disappears,

* This method is superseded by that on p. 225.

repeat with 5 c.c., and so on until the amount is found that does not discharge the blue.

Mix the 10 c.c. sugar solution with 50 c.c. of the copper solution in an Erlenmeyer flask. Place on a wire gauze over a Bunsen burner and bring it to the boil. Maintain the boiling for exactly three minutes. Cool the solution quickly by holding the flask under the cold water tap. Titrate with the hydroxylamine solution from a burette, running it in rather slowly with frequent shaking so as to prevent any precipitate forming, which spoils the result. Add the hydroxylamine until the blue colour is discharged.

Calculation of result. The greater the excess of copper present, the greater is the reduction caused by a given weight of glucose. The reduction is therefore not proportional to the amount of sugar employed in the determination. A table has been prepared showing the weight of glucose corresponding to the amount of hydroxylamine solution that is necessary to decolourise the unreduced copper.

Example. 2 c.c. of the sugar solution and 8 c.c. of water were used. Volume of hydroxylamine required was 14·2 c.c. The table shews that 37·5 mg. of glucose were present.

So percentage of glucose is $\cdot 0375 \times \frac{100}{2} = 1 \cdot 875$.

Table for calculation of amount of glucose from hydroxylamine used in Bang's method.

Hydroxylamine solution c.c.	Glucose mgm.	Hydroxylamine solution c.c.	Glucose mgm.	Hydroxylamine solution c.c.	Glucose mgm.
43·85	5	25·10	24	10·20	43
42·75	6	24·20	25	9·50	44
41·65	7	23·40	26	8·80	45
40·60	8	22·60	27	8·20	46
39·50	9	21·75	28	7·65	47
38·40	10	21·00	29	7·05	48
37·40	11	20·15	30	6·50	49
36·40	12	19·35	31	5·90	50
35·40	13	18·55	32	5·35	51
34·40	14	17·75	33	4·75	52
33·40	15	16·95	34	4·20	53
32·45	16	16·15	35	3·60	54
31·50	17	15 35	36	3·05	55
30·55	18	14·60	37	2·60	56
29·60	19	13·80	38	2·15	57
28·65	20	13·05	39	1·65	58
27·75	21	12·30	40	1·20	59
26·85	22	11·60	41	0·75	60
26·00	23	10·90	42		

112. **The estimation of Cane-sugar.**

Boil 40 c.c. of the solution with 30 c.c. of $\frac{N}{2}$ hydrochloric acid keeping the mixture boiling for 1 minute. Cool, neutralise by adding 30 c.c. of $\frac{N}{2}$ sodium hydroxide, cool to 15° C. and make the volume up to 100 c.c. Estimate the amount of invert sugar in this solution by either of the methods given in the previous exercises.

Calculation of results.

25 c.c. Benedict's solution = ·0475 gm. cane sugar.
10 c.c. Fehling's „ = ·0475 gm. „ „

In Bang's method calculate as glucose and multiplv by 0·95.

Estimation of Maltose and Lactose.

These are estimated by the same methods as glucose, different factors being employed for the calculation.

10 c.c. Fehling's solution } = 0·0676 gm. lactose.
25 c.c. Benedict's „ } = 0·074 gm. maltose.

An accurate method for the Estimation of Lactose will be found on p. 231.

CHAPTER III.

THE FATS AND THEIR DECOMPOSITION PRODUCTS.

The fats are glycerine esters of the higher fatty acids.

An ester is a compound formed by the condensation of an alcohol with an acid.

$$C_2H_5.OH + HOOC.CH_3 = C_2H_5.OOC.CH_3 + H_2O$$

Ethyl alcohol. Acetic acid. Ethyl acetate.
(Ethyl ester of acetic acid).

Glycerine is a trivalent alcohol

$$\begin{array}{l} CH_2.OH \\ | \\ CH.OH \\ | \\ CH_2.OH \end{array} \text{ or } C_3H_5(OH)_3.$$

It can therefore condense with three molecules of a fatty acid.

The fatty acids found combined with glycerine are mostly palmitic acid ($C_{15}H_{31}.COOH$), stearic acid ($C_{17}H_{35}.COOH$) and oleic acid ($C_{17}H_{33}.COOH$). The fats formed by the condensation of glycerine with these acids are known as palmitin, stearin and olein (or tri-palmitin, etc.)

$$\begin{array}{l} CH_2OH \quad HOOC.C_{15}H_{31} \\ | \\ CHOH + HOOC.C_{15}H_{31} \\ | \\ CH_2OH \quad HOOC.C_{15}H_{31} \end{array} = \begin{array}{l} CH_2.OOC.C_{15}H_{31} \\ | \\ CH.OOC.C_{15}H_{31} + 3H_2O \\ | \\ CH_2.OOC.C_{15}H_{31} \end{array}$$

or $C_3H_5(OOC.C_{15}H_{31})_3$

Palmitin.

Properties of the fats.

The fats are solids with a low melting point, triolëin melting at -5° C., tripalmitin at 65° C., and tristearin at 71° C. In the body they are found mixed in different proportions, and the melting point of the mixture is lower the greater the percentage of triolëin. They are insoluble in water, salt solutions and dilute acids and alkalies. They are soluble in ether, alcohol, chloroform and a variety of organic solvents.

They are hydrolysed by boiling acids and alkalies, by superheated steam and by certain enzymes, called lipases or steapsins. By this means they are split into their constituents, glycerine and fatty acid. If an alkali is used as the hydrolytic reagent, the fatty acid combines with it to form a soap. This special form of hydrolysis is therefore called saponification.

Various methods have been devised for the identification of the fats, amongst them being:

1. The melting point.

2. The saponification figure. A known weight of the fat is hydrolysed by means of a known amount of standard potash. The excess of alkali is then titrated, and the number of decigrams required for the hydrolysis of 100 grams of the fat is calculated.

3. The iodine number. Oleic acid is an unsaturated acid, and can combine with two atoms of iodine. The amount of iodine that combines with 100 grams of fat can be determined, and thus the percentage of unsaturated acids in the mixture calculated.

The emulsification of the fats.

Fats can be emulsified, *i.e.* broken up into droplets, either mechanically by agitation, or "spontaneously."

The mechanical emulsification is only permanent if the droplets are surrounded by a film of protein (as in milk), or by a film of soap or other more or less colloidal substance.

"Spontaneous" emulsification takes place when a melted oil or fat that contains a certain percentage of free fatty acid is brought into contact with an alkali. The fatty acid dissolves in the alkali to form a soluble soap, and the diffusion currents thus set up break the globule of fat into small particles, the process being maintained by the continual exposure of fatty acid to the alkali. The fat in the small intestine is thus emulsified as a preliminary to complete hydrolysis by the pancreatic lipase.

The digestion of fats.

The fats are hydrolysed to a small extent in the stomach by gastric lipase. This action is greater if the fat be given in an emulsified form, as in milk.

In the duodenum, the fat mixed with the fatty acid is spontaneously emulsified by the alkaline bile, succus entericus and pancreatic juice. The emulsified fat is then completely hydrolysed to glycerine and fatty acids by the pancreatic lipase. The fatty acids are converted into soluble soaps by the alkalies present. The soaps and glycerin are absorbed into the epithelial cells bordering the villi, where they are resynthesised into fats. These are passed into the lacteals and reach the blood stream by way of the thoracic duct.

113. (*a*) Carefully allow a drop of neutral olive oil to fall gently on to the surface of some ·25 per cent. Na_2CO_3 contained in a watch-glass. The drop of oil remains quite clear and forms a thin circular film on the surface.

(*b*) Shake 5 c.c. of neutral oil with 3 drops (only) of oleic acid in a dry test tube. With a drop of this mixture repeat (*a*) using a

fresh watch-glass full of Na_2CO_3. The rancid oil slowly spreads out in an amoeboid fashion and becomes converted into a milky emulsion.

(*c*) To the remainder of the mixture of oil and oleic acid add 12 more drops of oleic acid, shake well and repeat the experiment. The drop becomes white and opaque, but does not become emulsified.

NOTES—1. It is absolutely essential that the oil be quite neutral, and this can best be tested by dropping it on to ·25 per cent. Na_2CO_3. If a spontaneous emulsion is formed, a fresh sample must be obtained, or melted fresh butter substituted.

2. The spontaneous emulsion in (*b*) is caused by the trace of oleic acid dissolving in the alkali to form a soap, diffusion currents being thus set up which divide the fat into microscopic droplets.

3. In (*c*) the large excess of oleic acid leads to the opaque ring of soap being formed round the oil, and this soap, being only slightly soluble in water, prevents the formation of an emulsion.

114. Shake a few drops of olive oil with 5 c.c. of ether in a dry tube. The oil completely dissolves. Repeat the experiment with alcohol instead of ether. The oil dissolves partially, but is not so soluble in alcohol as in ether. Pour the alcoholic solution into water. The fat is precipitated as an emulsion.

115. Touch a piece of writing paper with a glass rod that has been dipped in olive oil. The paper is rendered translucent.

Preparation of pancreatic lipase.—A perfectly fresh pig's pancreas is freed from fat, weighed, finely minced and ground with sand. It is then treated with three times its weight of water and its own weight of strong alcohol. It is allowed to stand for three days at room temperature and strained through muslin. It must not be filtered. When not in use it should be kept in a refrigerator. It will remain active for a considerable time.

NOTE. Pancreative lipase is a ferment that only acts with the co-operation of a co-ferment, which is soluble in water and not destroyed by boiling. Bile salts and certain other substances can act as the co-ferment. The ferment proper is practically insoluble in water, and is destroyed by boiling. If the pancreatic extract be filtered, neither the precipitate nor the filtrate has any appreciable action on fats; but when the two are mixed the original lipolytic action is recovered. The precipitate is the ferment; the filtrate contains the co-ferment.

Preparation of an Emulsion of Fat.—Commercial olive oil (which contains some free olëic acid) is treated in a flask with 1 drop of a 1 per

cent. alcoholic solution of phenolphthalein for every 10 c.c. of oil. Decinormal sodium hydroxide is added, with frequent shaking, till the mixture is neutral. A very stable emulsion is thus formed, and thus a considerable surface of fat is exposed to the action of the ferment.

Fat-splitting action of lipase (steapsin).

116. Label three test tubes A, B and C.

To A add 2 c.c. of pancreatic extract and 1 c.c. of water.
„ B „ 2 c.c. „ „ boil, and add 1 c.c. of water.
„ C „ 2 c.c. „ „ and 1 c.c. of 1% bile salts.

To each add 5 c.c. of the emulsion of oil, shake thoroughly and place in a water bath at 40° C. for 1 hour.

Titrate each tube with $\frac{N}{10}$ NaOH from a burette and note the volume required to make the solution neutral. The amount required for B is a measure of the acidity of the 2 c.c. of pancreatic extract. This deducted from the amount required for A and C is a measure of the amount of fatty acid formed by the action of the ferment. It is greater in C than A, indicating the adjuvant action of bile salts on the lipolytic action.

117. Shake 5 c.c. of neutral olive oil in a test tube with 2 c.c. of the extract of the pancreas and place the tube in a water bath at 37° C. At the end of every ten minutes pipette off a little of the oil that rises to the surface, allow a drop of it to fall gently on to some ·25 per cent. Na_2CO_3 contained in a watch-glass, return the rest to the tube, shake vigorously and return it to the warm bath. As the action of the ferment proceeds spontaneous emulsion will occur, showing that some of the neutral oil has been converted into a fatty acid. If the action is allowed to proceed considerably further no emulsion will be produced, for the reasons stated in the notes to Ex. 113.

NOTE.—This is one of the methods employed for demonstrating the fat-splitting power of steapsin; but, naturally, it can only be used when perfectly neutral oil can be obtained.

118. Repeat the above experiment, but boil and then cool the 2 c.c. of pancreatic extract before adding the olive oil. A

spontaneous emulsion is not formed at any stage, showing that the ferment is destroyed.

NOTES.—1. This or a similar control experiment should always be performed side by side with the actual experiment when investigating the action of ferments.

2.—Be particularly careful to cool the extract after boiling, otherwise the alkali may exert a slight saponifying action at the higher temperature.

119. Boil 10 c.c. of fresh milk, cool it under the tap, add 2 c.c. of the pancreatic extract, 3 c.c. of litmus solution and 2 c.c. of 2 per cent. sodium carbonate. Shake well and divide into two portions, A and B. Boil A to destroy the ferment. Place both tubes in the water bath at 37° C. In the course of ten minutes or so the blue colour in tube B will change to red, indicating that some of the neutral fat in the milk has been hydrolysed to a fatty acid.

NOTE.—This is the most convenient method for the recognition of the action of lipase. The fat of milk being finely emulsified offers a very large surface for the action of the steapsin. The milk should be boiled first to destroy any bacilli present that might form lactic acid from the lactose.

Glycerine (Glycerol).

120. Treat a drop or two of glycerine in a test-tube with a solution of copper sulphate and then with sodium hydroxide. A blue solution is obtained, glycerine preventing the precipitation of cupric hydroxide.

121. Boil the solution thus obtained. Reduction does not occur.

122. Heat strongly a drop or two of pure glycerine with solid potassium hydrogen sulphate in a dry test tube. The pungent odour of acrolëin (acrylic aldehyde) is noticed.

$$\underset{\text{Glycerine.}}{CH_2OH.CHOH.CH_2OH} = \underset{\text{Acrolëin.}}{CH_2:CH.CHO} + 2H_2O$$

123. Treat about 5 c.c. of a 0·5 per cent. solution of borax with sufficient of a 1 per cent. alcoholic solution of phenolphthalein to produce a well-marked red colour. Add a 20 per cent. aqueous solution of glycerine, drop by drop, until the red colour is just

discharged. Boil the solution: the colour returns, provided that an excess of glycerine has not been added **(Dunstan's test for glycerine).**

NOTES.—1. Any ammonium salt will discharge the colour, but in this case it does not return on heating.

2. Any polyhydric alcohol is likely to give the same reaction. The sugars are all polyhydric alcohols, but are distinguished from glycerine by their reducing properties, etc., and by the fact that they are not volatile when distilled by steam.

3. The probable explanation of the reaction is as follows. Sodium borate is partially hydrolysed in aqueous solution to boric acid and sodium hydroxide. Boric acid being a weak acid is only feebly ionised and therefore the solution reacts alkaline. On adding glycerine, glyceroboric acid is formed. This is a strong acid and hence the reaction of the solution changes from alkaline to acid. On heating, unless a large excess of glycerine be present, the glyceroboric acid is hydrolysed to glycerine and boric acid and the solution again becomes alkaline.

The Higher fatty acids and their salts, the soaps.

124. Shake a few drops of oleic acid with 5 c.c. of water, ether, and alcohol respectively in separate tubes. The acid is insoluble in water, but soluble in alcohol or ether.

125. Place a drop of oleic acid on writing paper: a greasy stain results.

126. Shake the alcoholic solution of oleic acid with dilute bromine water. The colour of the bromine is discharged, owing to the unsaturated acid absorbing the halogen till it is saturated.

127. Repeat the experiment with an alcoholic solution of stearic acid or commercial "stearine" (a mixture of stearic and palmitic acids). The colour of the bromine persists, since these acids are members of the saturated series.

128. Heat about 10 drops of oleic acid with 10 c.c. of water and to the hot mixture add 40 per cent. NaOH drop by drop till the solution is clear. If an excess be added the excess of sodium ions causes a precipitate (see note below). A clear solution of a soap, sodium oleate, is formed. Divide this into three portions.

To A add a few drops of strong HCl or H_2SO_4 till the reaction is distinctly acid. Oleic acid separates out and rises to the surface of the tube.

To B add finely-powdered sodium chloride and shake. The soap is rendered insoluble and rises to the surface.

To C add some calcium chloride. A precipitate of an insoluble soap, calcium oleate, is produced.

NOTE.—B illustrates the principle of "salting out," which is used in the manufacture of soaps. The excess of sodium ions in the solution, produced by the addition of the sodium chloride, lowers the solubility of the sodium oleate, which is therefore precipitated.

129. Boil 2 c.c. of olive oil with 5 c.c. of a 20 per cent. alcoholic solution of sodium hydroxide in a basin over a *small* flame for five minutes or until the alcohol has all evaporated away. Add about 5 c.c. of alcohol and heat again to dryness, stirring the whole time. Add about 30 c.c. of distilled water and boil till dissolved. Add solid sodium chloride and stir. The soap formed is precipitated. Filter some off, dissolve in boiling water and repeat the experiments described in the previous exercise.

CHAPTER IV.

THE CHEMISTRY OF SOME FOODS.

A. Milk.

The composition of milk differs considerably in different animals.

The percentage composition of average samples of human and cow's milk is as follows:—

	Protein.	Fat.	Carbohydrate.	Salts.
Human.....	1·5	3·1	5·0	0·2
Cow's.....	3·4	3·7	4·8	0·7

Other differences are that in cow's milk the proportion of caseinogen to lactalbumin is about 6 to 1 compared with 2 to 1 in human milk.

Caseinogen, the chief protein of milk, is a phosphoprotein. It is insoluble in water, dilute acids and salts, but dissolves in alkalies to form a salt-like body. It also dissolves in strong acids. It is salted out of solution by half-saturation with ammonium sulphate.

It does not coagulate on boiling. But when milk is boiled a skin forms on the surface. A similar skin forms whenever a protein solution mixed with an emulsion of a fat is heated. The skin contains protein mixed with fat. If it be removed, another skin immediately forms.

130. Examine a drop of fresh cow's milk under the microscope with a high power. Notice the highly-refractive fat globules of varying size, the smallest globules exhibiting the peculiar vibration known as Brownian movement.

131. Take the specific gravity of milk with a lactometer. It varies between 1028 and 1034.

NOTE.—When the milk is skimmed the specific gravity rises from 1033 to 1037, owing to the removal of the fat which has a low specific gravity. The specific gravity is also lowered by dilution with water.

132. Place a drop of fresh milk on pieces of blue and red litmus paper and wash off with distilled water. The blue paper is turned red and the red paper blue, *i.e.* the milk is amphoteric in reaction, due to the mixture of acid and alkaline salts.

133. Take 5 c.c. of milk in a test tube and dilute with distilled water till the test tube is nearly full. Add three drops of strong acetic acid and mix thoroughly. A flocculent precipitate of caseinogen is formed, which mechanically carries the fat down with it. Filter this off and label the filtrate A. Precipitate two more portions of 5 c.c. each, adding the filtrates to A, and reserving the precipitate.

134. Take 5 c.c. of milk, add water as before, and then an excess of strong acetic acid. A precipitate is not produced, owing to the solubility of caseinogen in an excess of acid.

135. Treat a portion of the precipitate from Ex. 133 with some 2 per cent. Na_2CO_3 solution. The caseinogen dissolves, leaving the fat in suspension. Apply the protein colour reactions to the solution: all, except the sulphur test, are given.

136. Treat 5 c.c. of milk with 5 c.c. of saturated ammonium sulphate solution. The caseinogen is precipitated, entangling the fat with it. Filter and boil the filtrate. A heat coagulum of lactalbumin is obtained. Treat the precipitate of caseinogen and fat on the paper with water. The caseinogen dissolves.

NOTE.—The caseinogen dissolves in water because it is precipitated as a salt by ammonium sulphate. On the addition of dilute acetic acid to this solution, a precipitate of caseinogen is again obtained.

137. Treat a considerable portion of the precipitate obtained in Ex. 133 as directed in Ex. 39. Phosphorus is found to be present in the caseinogen.

138. Allow another portion of the precipitate obtained in Ex. 133 to drain thoroughly, press it with dry filter paper and transfer it to a dry tube. Shake it vigorously with 5 c.c. of ether, pipette off the ether, and evaporate the etheral solution in a basin over a boiling water bath, turning out the flame before putting on the dish containing the ether. A small amount of fat is left in the dish. Wipe the dish round with a piece of writing-paper. A translucent grease spot is formed.

139. Examine filtrate A. Add a drop of litmus, and note that it is markedly acid. Boil, and whilst boiling add 2 per cent. Na_2CO_3, drop by drop, until the reaction is only faintly acid. If the reaction should, by accident, be made alkaline, dilute acetic acid must be added till the reaction is faintly acid. A coagulum of lactalbumin is formed. Filter this off and reserve the filtrate (B).

140. Boil a small portion of filtrate B with a little Fehling's solution. A well-marked reduction is obtained, due to the presence of lactose.

141. Try Barfoed's reaction with this filtrate. A reduction is not usually obtained. (See Ex. 69.) Sometimes the lactose is slightly hydrolysed by the boiling in Ex. 139.

142. Treat the remainder of filtrate B with two or three drops of strong ammonia and boil. A slight precipitate of calcium phosphate is produced. Filter this off, dissolve it in a little strong acetic acid, and add a solution of potassium oxalate. A white precipitate of calcium oxalate is formed. Treat with 2 c.c. of nitric acid and 5 c.c. of ammonium molybdate solution. Boil for two minutes. A yellow crystalline precipitate is formed, showing the presence of phosphates in milk.

B. The Clotting of Milk.

When milk is treated with a neutral or faintly acid extract of the mucous membrane of the stomach, a clot forms after a certain time. This is due to the conversion of the caseinogen of the milk into an insoluble protein

called casein. This entangles the greater portion of the fat, the whole being known as the curd. The fluid portion that separates from the curd is called the whey, and contains the salts, lactose and lactalbumin.

The conversion of caseinogen into casein was believed at one time to be due to a special enzyme called rennet or rennin. But it is probable that the action is one common to all proteolytic enzymes, as trypsin and erepsin can cause milk to clot.

Soluble ionised calcium salts participate in the clotting action, their rôle being to convert a soluble product of ferment action into an insoluble one. The mechanism of clothing is shewn in the following scheme:—

Caseinogen. Proteolytic enzyme. Soluble calcium salt.

(Rennet)

Soluble protein + Soluble casein
resembling albumose.

Insoluble casein.

The following experiments can be performed with a commercial preparation of rennet:

143. Treat 5 c.c. of milk with about 2 c.c. of an active solution of rennet-ferment. Place the tube in the warm bath, and observe it from time to time. Note that the milk soon forms a clot so firm that the tube can safely be inverted: on standing longer the clot contracts and exudes a nearly clear fluid (whey).

144. Perform a control test by boiling and then cooling the rennet before adding it to the milk. Clotting does not take place.

145. Treat 5 c.c. of milk with 2 c.c. of 2 per cent. Na_2CO_3 and the same amount of rennet: place the tube in the warm bath. Clotting does not take place.

NOTE.—Commercial rennin is prepared from the fourth stomach of a sucking calf, or from the mucous membrane of the stomach of a pig. The pepsin, and so also the rennetic action, is destroyed by alkalies.

146. Take 10 c.c. of milk, add one-third of its volume of 1 per cent. potassium oxalate (to remove all soluble calcium salts) and divide into three equal portions which are placed in three test-tubes, labelled A, B and C.

To A add 1 c.c. of 2 per cent. calcium chloride and 2 c.c. of rennet.

To B add 2 c.c. of rennet.

To C add 2 c.c. of boiled rennet.

Place the three tubes in the warm bath for about ten minutes. Note that A clots and that B and C do not.

Boil B (to destroy the rennet) and cool the tube.

To B and C add 1 c.c. of 2 per cent. calcium chloride.

A flocculent precipitate of insoluble casein is immediately formed in B: in C there is no precipitate.

NOTE.—In A there is caseinogen, rennet and $CaCl_2$
In B ,, ,, and rennet.
In C ,, ,, and $CaCl_2$
After ten minutes, B contains soluble casein, which is precipitated by the subsequent addition of $CaCl_2$.

C. Cheese.

147. Shake some grated cheese in a dry test tube with ether, and examine the ethereal solution for fat as in Exercise 138. Fat is present in considerable quantity.

148. Pound the residue from the above in a mortar with a 2 per cent. solution of sodium carbonate and filter. Acidify a portion of the filtrate. A precipitate of casein is formed, which is soluble in excess of acid. To the remainder of the filtrate apply the usual protein colour reactions: they are all obtained.

D. Potatoes.

149. Scrape the clean surface of half a potato with a penknife, keeping the scrapings as fine as possible. Place the

scrapings in a beaker of water, stir well, and strain through fine muslin into another beaker. Allow this to stand for a few minutes and then note the white deposit of starch. Pour off the supernatant fluid and reserve it for the next exercise. Fill the beaker containing the starch with water, stir well, and again allow the starch to settle. By repeating this process of lixiviation the starch can be obtained quite pure. Examine a little microscopically and note the characteristic form of the grains (See Ex. 85). Heat a little with water, cool, and add iodine. A deep blue colour is obtained.

150. Filter the fluid A, and test portions of the filtrate for proteins by the usual colour tests. Only small quantities of protein are found to be present, the most marked reaction being Millon's.

E. Flour.

White flour from the endosperm of wheat grains contains 70 to 75 per cent. of starch, about 8 per cent. of protein and about 1 per cent. of fat. The proteins are gliadin (soluble in 70 to 80 per cent. alcohol), and glutelin (soluble in alkali). When treated with water these two proteins form a sticky mass called gluten, the viscidity being due to the gliadin. Thus grains poor in gliadin, as rice and oats, do not form dough when mixed with water.

Flour only contains glucose if germination has taken place before milling.

Whole flour is obtained from the whole of the grain, except the outer husk and outer part of the bran. It is possible that it contains something essential to growth and general nourishment. It is not quite so digestible as white flour. The bran in it stimulates the intestine and so acts as a mild laxative.

151. Mix some wheat flour with a *little* water to form a *stiff* dough. Allow this to stand for a short while, preferably at 37° C.

Wrap a piece, the size of a chestnut, in muslin, and knead it for a few minutes in a basin of water; pour the suspension into a

beaker, and note the white deposit of starch grains that settles down on standing. Examine this microscopically, noting that the grains differ considerably from those of potato-starch in being smaller, circular, and with a central hilum. Make a drawing of the grains. Boil a little with water, cool, and add a drop of iodine. The deep blue starch reaction is obtained.

152. Knead the dough thoroughly under the tap until no more starch comes through the muslin. A yellowish, sticky mass, known as gluten, is left behind. Test portions of this by the usual protein colour reactions: they are all obtained, gluten being a protein.

F. Bread.

The dough formed by adding water to flour is impervious to the digestive juices. Before it can be used it has to be aerated and the gluten rendered porous.

A pure culture of yeast is mixed with warm water, flour and salt. The dough thus formed is thoroughly kneaded, and the mass kept warm for some hours. During this time the yeast cells multiply and convert some of the starch into glucose and this into alcohol and CO_2. Also the ferment of the flour called diastase becomes active and converts some of the starch into glucose. More flour is added and the process allowed to proceed for some hours longer. The gas formed causes the mass to rise. The dough is weighed out into loaves, which after being allowed to rise once more for a certain time are heated to about 232° C. for an hour and a half. The heat kills the yeast, expands the gas bubbles, and causes the outer part of the dough to become hardened by coagulating the proteins. It also converts starch into soluble starch and dextrin, thus forming the crust. The brown appearance of this is due to the conversion of glucose into caramel.

153. Take a piece of the crumb of a stale white loaf, rub it up finely and pound with cold water in a mortar. Strain and squeeze through muslin. A white fluid is obtained containing wheat starch grains. Filter the fluid. To a portion of the filtrate add a little Fehling's solution and boil: a well-marked reduction occurs due to the presence of glucose. To another portion add iodine: a purple colour is produced, showing the presence of erythro-dextrin. If very dilute iodine be cautiously added, a blue colour is produced at first, showing that a small amount of soluble starch is present.

Boil a small amount of the residue of the bread with water in a beaker, strain through muslin and filter. Cool and test the filtrat for starch and dextrin. (Ex. 96 and 97.)

154. Repeat the above exercise, using the crust of bread instead of the crumb. Note that glucose is absent or present in traces only: dextrin and starch are present, a considerable portion of the latter existing as soluble starch and being present in the cold water extract.

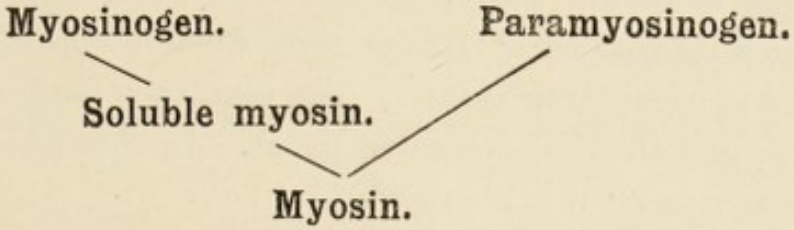
G. Meat (Muscle).

The most important constituents of living striated muscle are—

Proteins.	Myosinogen and Paramyosinogen.
Pigment.	Myohaematin.
Fat.	
Nitrogenous extractives.	Creatine. Hypoxanthine. Xanthine.
Non-nitrogenous extractives.	Glycogen. Sarcolactic acid.
Inorganic.	Water. Salts, chiefly potassium and magnesium phosphates.

The **proteins** of living muscle are mainly **myosinogen** (80 per cent.) and **paramyosinogen** (20 per cent.). The former is an albumin, coagulating at 57° C. The latter is a globulin, coagulating at 47° C.

On standing or on treatment with dilute acids they are converted into **myosin** the protein of dead muscle. In this transformation, myosinogen passes through an intermediate stage of **soluble myosin** which coagulates at 40° C.



155. **Preparation of fresh muscle extract.** A rabbit is killed, a cannula fixed into the aorta and an opening made in the right auricle. The vessels are then washed free from blood with 0·9 per cent. sodium chloride. The muscles of the limbs are removed, rapidly minced and treated with ice-cold 5 per cent. magnesium sulphate, and the mixture left in the ice chest for about 24 hours. The extract is filtered and the following tests performed with it:

156. Take the reaction to litmus. It is generally neutral.

157. Dilute a small portion with four volumes of distilled water and leave the tube in the water bath at 37° C. for some time. A clot of myosin forms, leaving muscle serum.

158. Take the reaction of the muscle serum to litmus. It is distinctly acid, due to the production of sarcolactic acid.

159. Add some acetic acid to another portion of the extract. A precipitate of myosin occurs immediately.

160. Take 5 c.c. of the extract in a test-tube: place the tube in a beaker of water, supporting it by a clamp so that it does not touch the bottom of the beaker. Heat the water with a Bunsen flame and note the temperature in the tube at which distinct

coagulation occurs. It is usually at about 47° C. Filter off the coagulum of paramyosinogen and heat again. Another and larger coagulum of myosinogen occurs at 57° C.

161. **Preparation of Myosin.** Fresh veal is finely minced in a machine, stirred with a large volume of water for a quarter of an hour, strained through muslin, and the washing process repeated once more. In this way certain proteins and other substances soluble in water are removed. The veal is now collected on muslin, squeezed to remove the water, ground with sand, and extracted with five times its volume of 10 per cent. ammonium chloride for several hours at room temperature. The extract is filtered through muslin, linen, and then coarse filter paper. In this way a crude, viscid solution of myosin is obtained.

162. Boil a portion of the solution. A heavy coagulum is formed. Wash the coagulum and on it perform the protein colour reactions. They are all obtained.

163. Pour 100 c.c. into a litre of water contained in a tall cylinder; mix well, and note the precipitation of myosin, due to the reduction in the concentration of salts.

Allow this to settle and then pour or pipette off as much of the supernatant fluid as possible. A suspension of myosin in dilute ammonium chloride is thus obtained for the next three experiments.

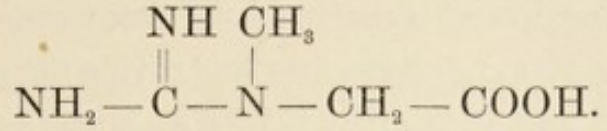
NOTE.—If this suspension be allowed to stand it slowly becomes converted into an insoluble variety.

164. To a portion add a saturated solution of common salt, drop by drop. The precipitate dissolves. Add solid NaCl to saturation: the myosin is reprecipitated.

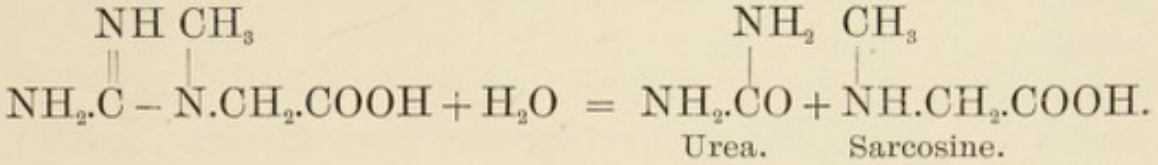
165. To a portion add saturated $(NH_4)_2SO_4$ till the precipitate just dissolves. Now add an equal bulk of saturated $(NH_4)_2SO_4$. The myosin is reprecipitated.

166. Dissolve in a little $(NH_4)_2SO_4$ and take the temperature at which the myosin coagulates. It coagulates at about 57° C. (See Ex. 160.)

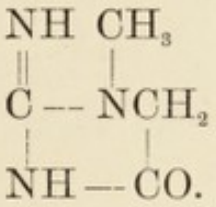
Creatine.—This is the most abundant nitrogenous extractive in muscle, being present to the extent of about 0·4 per cent. Chemically it is methyl-guanidine-acetic acid.



On hydrolysis with baryta water it is converted into urea and sarcosine (methyl glycine).



On being boiled with mineral acids it is dehydrated to creatinine.



Creatinine is found in normal human urine, but creatine only under abnormal conditions.

167. **Separation of creatine from meat extract.** Dissolve 10 grams of commercial meat extract in 200 c.c. of water. Add slowly a saturated solution of lead acetate till no further precipitate is formed, carefully avoiding an excess. This is best done by filtering samples and testing them with lead acetate. Filter off the precipitate of proteins and phosphates. Warm the filtrate and decompose the soluble lead compounds by means of a stream of sulphuretted hydrogen. Warm and filter off the precipitate of lead sulphide. Evaporate the filtrate, filtering off any sulphur or sulphide that may be deposited. Continue the evaporation till a syrup is obtained. Allow this to stand in the ice chest for two or three days. Creatine separates out, mostly as oblique rhombic crystals. Examine a few under the microscope. Treat the syrup

with 200 c.c. of 88 per cent. alcohol, stir thoroughly with a glass rod and filter through a small paper. The creatine remains on the paper, the alcoholic filtrate containing the purine bases.

168. **Conversion of creatine into creatinine.** Dissolve the creatine in about 30 c.c. of hot water and divide the solution into two equal portions, A and B. Treat B with an equal volume of normal HCl and heat on a boiling water bath in a flask fitted with a cork and long glass tube (to act as an air condenser) for three to five hours. The creatine is converted into creatinine. Neutralise the solution with caustic soda.

Test A and B for creatinine by the following tests:

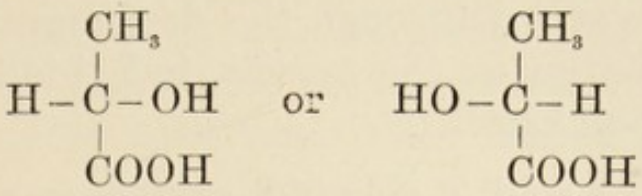
169. **Jaffé's test for creatinine.** Treat 10 c.c. of the solution with 15 c.c. of saturated picric acid solution and 5 c.c. of 10 per cent. caustic soda. Allow the mixture to stand for 5 minutes and dilute to 200 c.c. A deep orange colour appears in B due to the formation of picramic acid from creatinine. The creatine in A gives no colour.

170. **Weyl's test for creatinine.** Treat 5 c.c. with a few drops of a freshly prepared solution of sodium nitroprusside and make the solution alkaline with sodium hydroxide. A ruby-red colour appears, which soon turns yellow. Acidify with an excess of acetic acid and heat. A green tint appears, and a blue deposit of Prussian blue may result on standing.

Purine bases. These compounds are interesting because of their chemical relationship to uric acid. This relationship is shown by the formulae given on page 20.

The purine bases found in meat extracts are chiefly hypoxanthine and xanthine. They can be obtained from the alcoholic solution obtained in Ex. 166, by evaporating off the alcohol, adding ammonia and precipitating with ammoniacal silver nitrate.

Sarcolactic acid is dextro-rotatory *a*-oxy-propionic acid.



The middle carbon atom of this compound is attached to four different groups, $-CH_3$, $-H$, $-OH$ and $-COOH$. Solutions of such asymmetric compounds have the power of rotating the plane of polarised light, either to the right or to the left.

If the carbon atom be represented as a regular tetrahedron, and the four different groups placed at the apices, then any arrangement of the groups round the tetrahedron will show a figure which is reversed by its image in a mirror. Projected on to a plane surface the above formulae are obtained. The first of these is dextro-rotatory, and the other is laevo-rotatory.

If an asymmetric compound be prepared by artificial synthesis, it consists of equal amounts of *d*- and *l*-forms and is therefore optically inactive (racemic or *dl*-).

The lactic acid found in muscle is *d*-lactic. That formed by the fermentation of lactose and other carbohydrates is generally *dl*-lactic. Certain bacteria, however produce *l*-lactic acid.

Sarcolactic acid is present to a very small extent in fresh living muscle. The amount increases rapidly in fatigue, especially in the absence of a proper supply of oxygen. On leaving a fatigued muscle in an atmosphere of oxygen, the amount of lactic acid decreases.

There is a marked production of lactic acid at the onset of rigor mortis. But if a fresh muscle be suddenly coagulated by dropping it into boiling water, there is no such marked production of the acid.

It is probable that the lactic acid appearing in fatigue and in rigor arises through the decomposition of some complex material in the muscle, but this has not been definitely established.

Sarcolactic acid is a liquid, soluble in water, alcohol and ether. It forms a characteristic zinc salt, which is obtained by boiling a solution with excess of zinc carbonate, filtering and evaporating slowly. The crystals contain two molecules of water of crystallisation, the zinc salt of ordinary fermentation lactic acid containing three.

171. **Hopkins' reaction for lactic acid.** To 3 drops of a 1 per cent. alcoholic solution of lactic acid in a clean, *dry* test tube add 5 c.c. of concentrated sulphuric acid and 3 drops of a saturated solution of copper sulphate. Mix and place the tube in a beaker of boiling water for about five minutes. Cool thoroughly under the tap, add two drops of a ·2 per cent. alcoholic solution of thiophene, and shake. Replace the tube in the boiling water bath. As the mixture gets warm a fine cherry-red colour develops.

NOTE.—Lactic acid is oxidised in sulphuric acid solution to some substance which gives a red colour with thiophene. The copper sulphate aids this oxidation, which is inhibited by water.

172. **Uffelmann's reaction for lactic acid.** Treat a few c.c. of Uffelmann's reagent with a few c.c. of a dilute (0·4 per cent.) solution of lactic acid. The violet colour is instantly turned to a yellow.

NOTES.—1. Uffelmann's reagent is prepared by treating a 1 per cent. solution of phenol (carbolic acid) with very dilute ferric chloride till the solution becomes coloured an amethyst-violet.

2. The reaction is not very reliable, since other acids as tartaric, oxalic and citric give it.

173. **The Formation of Lactic Acid in Fatigue.** A pithed frog is kept on ice for about half-an-hour. Remove one hind limb and replace it on the ice. Expose the lumbar plexus of the other side and stimulate it electrically by means of a strong interrupted current for at least ten minutes. Cut off the hind limb, strip the

skin off the two limbs and treat the muscles separately as follows: Rapidly remove the muscles, grind them with ice cold 95 per cent. alcohol and sand. Transfer the mixture to a beaker, and warm for a few minutes on the water bath. Filter through a small paper and evaporate to complete dryness on a water bath. Treat the residue with about 5 c.c. of cold water and rub it up thoroughly with a glass rod. Filter and boil the filtrate with as much animal charcoal as will lie on a threepenny piece. Filter and evaporate the filtrate to complete dryness on a water bath. Allow the residue to cool and apply Hopkins' test by treating the residue with strong sulphuric acid, shaking round till solution is obtained, transferring to a dry test tube, adding three drops of saturated copper sulphate etc. A fine red colour develops in the tube containing the extract from the tetanised muscle, but none or very little in the other.

Glycogen. The percentage of glycogen in fresh muscle varies from 0·5 to 1 per cent., so that the total amount in all the muscles of the body may be greater than in the liver. The muscle glycogen decreases after muscular exercise, but not so rapidly as that in the liver.

The estimation of glycogen is described on page 49.

CHAPTER V.

THE COMPOSITION OF THE DIGESTIVE JUICES AND THE ACTION OF CERTAIN ENZYMES.

The digestive enzymes or ferments are bodies that have the power of accelerating the rate of hydrolysis of certain substances. They are often divided into groups depending on the nature of the substance on which they act (the so-called substrate or zymolyte). Thus those acting on starch are called amylolytic; on proteins, proteolytic; on fats, lipolytic, etc. The enzymes are often named in such a way as to indicate their origin and their action, the termination -ase being employed. Thus ptyalin, the amylolytic enzyme of saliva, can be termed salivary amylase, to distinguish it from pancreatic amylase (amylopsin). Gastric lipase, the lipolytic enzyme of the gastric juice, is similarly distinguished from pancreatic lipase (steapsin).

The chemical composition of the enzymes is at present uncertain, owing to the extreme difficulty of preparing them in a pure state. The proteolytic enzymes are either proteins, or compounds so readily absorbed by proteins that it is impossible to separate them. The enzymes acting on certain of the carbohydrates are possibly themselves of a carbohydrate nature.

The properties of the enzymes as a class are as follows: They are soluble in water, dilute salt solutions, dilute alcohol and glycerine. They are precipitated by saturation with ammonium sulphate and by strong

alcohol. They are colloidal and non-diffusible. They are most active at a certain temperature, called the optimum temperature, which is generally about 45° C. Their action is suspended by cooling, but is completely destroyed by raising the temperature to 100° C.

The enzymes are remarkably specific in their action, that is, they act only on a particular substance or on a group of substances having some similarity in chemical composition and configuration. A striking example of this is seen in the case of the glucosides (see page 33). The enzyme maltase (*a*-glucase) hydrolyses *a*-methyl- and *a*-ethyl-*d*-glucosides, but has no action on *β*-methyl- or *β*-ethyl-*d*-glucosides, or on any *l*-glucoside or on *d*- or *l*-galactosides. The enzyme emulsin (*β*-glucase) acts only on *β*-ethyl, methyl or phenyl-*d*-glucosides. Lactase acts only on the *β*-galactosides. It is probable that the enzyme first unites with the substrate, and to do this it must have a configuration in space corresponding with that of the substrate.

The hydrolysis is effected by the water molecules, or by the H and OH ions formed from the water. In some cases a certain concentration of H or OH ions must be present to enable the enzyme to act. Thus pepsin acts in acid solution only: trypsin requires a certain concentration of OH ions.

The action of most enzymes is retarded by the accumulation of the products of the reaction, and in certain cases the reaction is reversible.

This is well seen in the case of lipase, which induces the following reaction :—

Ethyl butyrate + water $\rightleftharpoons$ ethyl alcohol + butyric acid.

The velocity of reaction is proportional to the amount of the enzyme present, provided that the amount of the

enzyme is very small compared with that of the substrate. If the amounts of enzyme and substrate are at all comparable, the laws of mass action are followed. But complications are introduced by the fact that some of the enzyme is thrown out of action by being absorbed by the products of the action.

In certain cases enzyme action is dependent on the simultaneous presence of two substances. These are sometimes called co-ferments. It has been shewn that the zymase that is responsible for the alcoholic fermentation of sugar by yeast can only act in co-operation with phosphates and some substance that is diffusible and not destroyed by boiling. Also the lipase of the pancreas requires the presence of some soluble, heat-stable substance to allow it to act. Bile salts have this property, as has been seen in a previous chapter. The action of the enzymes can be retarded by certain substances. These are of two classes: paralysers and anti-enzymes. The paralysers are generally salts of the heavy metals, which probably alter the physical state of the colloidal enzymes. The anti-enzymes are of an organic nature. They probably combine with the enzyme and thus prevent it from acting on the substrate. Examples are seen in the case of the anti-trypsin of normal serum, of the intestinal mucous membrane and of the tissues of intestinal parasitic worms.

A. Saliva.

Saliva is of value as a lubricant in the act of deglutition, and in some animals this is its sole function. In many animals, however, it contains an enzyme, ptyalin, which acts on starch, converting it finally into maltose, with perhaps a small amount of glucose. It is claimed by certain workers that for the complete hydrolysis of starch three ferments are necessary, viz., amylase that converts

starch into the dextrins: dextrinase that converts the dextrins into maltose: and maltase that converts maltose into glucose. In the case of the action of ptyalin on starch as conducted in vitro, the final product consists of about 80% of maltose, the remaining 20% being a comparatively simple dextrin called "stable dextrin," owing to its resistance to the further action of the ferment. But if this dextrin be isolated the action of ptyalin is to hydrolyse it very slowly and incompletely to equal parts of maltose and glucose.

Ptyalin acts best in a medium that is *very* faintly acid. It is rapidly destroyed by dilute HCl, but can be protected by the presence of proteins with which the acid combines, the concentration of hydrogen ions being thus decreased. It is probable that the action of ptyalin on the carbohydrates of a mixed meal continues for about 30 minutes in the mixed gastric contents.

Inorganic salts, particularly sodium chloride, favour its action, probably by causing the appearance of hydrogen ions, by some obscure adsorption phenomenon of the colloidal starch. This effect of NaCl is best seen if the ferment preparation has previously been freed from electrolytes by alcohol precipitation and thorough dialysis against distilled water. Such preparations are almost inactive, but become active on the addition of traces of weak acids or neutral salts.

174. Collect about 5 c.c. of your own saliva in a small beaker. Test the reaction with neutral litmus paper: it is alkaline.

NOTE.—The first portion of saliva collected is very apt to be neutral or even slightly acid, probably owing to bacterial decomposition in the mouth. But if the secretion is free, that collected later is invariably alkaline.

175. Transfer the saliva to a test tube and add strong acetic acid. A stringy precipitate of mucin is formed, insoluble in excess of acid. Stir the mixture vigorously with a glass rod: the mucin

forms a clump which can be removed by the rod. To the clear fluid remaining add some Millon's reagent and boil. Only a slight red precipitate is formed, showing that the proteins of saliva consist almost entirely of mucin.

176. Obtain diluted saliva as follows: warm some distilled water in a beaker to about 40° C. With a portion of this thoroughly rinse the mouth out. Now take about 20 c.c. of the warm water into the mouth and move it about by the tongue for at least a minute. Collect the fluid thus obtained in a clean beaker, and repeat the process twice more. Thoroughly mix the diluted saliva thus obtained, shake it vigorously and filter.

177. In a clean test tube place 5 c.c. of 1 per cent. starch paste, freshly prepared with distilled water, and 5 c.c. of the diluted saliva. Mix well and place the tube in a water-bath maintained at a temperature of about 40° C. Place a series of drops of iodine solution on a white porcelain plate, and from time to time transfer, by means of a glass rod, a drop of the digesting mixture to a drop of the iodine. The blue colour produced at first will later become blue-violet, red-violet, red-brown, and light-brown yellow, as the starch, and then the erythro-dextrin are converted into other products. When a drop of the mixture no longer gives any colour with iodine, boil a few c.c. of it with a few c.c. of Fehling's solution. A well-marked reduction is obtained, showing that a ferment (ptyalin) in saliva has converted the starch into a reducing sugar, which is, however, not glucose, but maltose.

178. Perform a control test by first boiling, and then cooling the saliva before adding it to the starch. (See Ex. 118.) No action whatever takes place when the mixture is allowed to stand on the warm bath, proving that the effect in the above exercise was due to a ferment.

179. **The investigation of the activity of ptyalin under various conditions by the method of the achromic point.**

In each of a series of clean test tubes place about 1 c.c. of an

iodine solution that has been diluted to a pale straw-colour with distilled water.

Carefully measure 5 c.c. of the 1 per cent. starch paste into a perfectly clean test tube, add five drops of distilled water and place the tube in a warm bath at 40° C. for a few minutes.

To the starch paste add 5 c.c. of the diluted saliva, previously warmed to 40° C. in the warm bath. Mix the two fluids, and note the time of the addition. At intervals transfer a few drops of the digestive mixture to one of the samples of iodine by means of a small pipette made from quill tubing. The same series of colour changes will be observed as were seen in Ex. 177. Note the time when the addition of the mixture of iodine ceases to produce any colour. This point, which is the moment when the last trace of erythro-dextrin is converted into achroo-dextrin and maltose, is known as the achromic point. The time that is taken to reach this point ("chromic period") is a measure of the activity of the ferment.

Repeat the exercise, and note that the chromic period obtained agrees fairly closely with that previously found.

NOTES.—1. It will be seen that the starch and the ferment solution are separately warmed to the temperature at which the exercise is performed. Otherwise the results will not be strictly comparable with those of the following exercises.

2. A convenient chromic period is one of about five minutes. If it is less than two minutes, the saliva should be diluted with an equal volume of distilled water. If it is more than ten minutes the starch paste should be diluted with an equal bulk of water and boiled well to ensure thorough mixing. In either case the chromic period under these new conditions must be carefully noted for comparison with those obtained in the following exercises.

180. Repeat the above exercise, substituting five drops of a 5 per cent. solution of sodium chloride for five drops of water. The chromic period is considerably reduced.

NOTE.—The concentration of NaCl in the digestive mixture is between ·01 and ·02 per cent. Even lower concentrations than this have a marked effect in increasing the activity of ptyalin. A concentration of 5 per cent. of NaCl usually slightly decreases the activity.

181. Repeat the above exercise, using one drop of ·4 per cent. hydrochloric acid and four drops of water. The chromic period is considerably reduced. With two drops of ·4 per cent. HCl and three drops of water the chromic period may or may not be reduced, according to the alkalinity of the saliva.

NOTE.—One drop of ·4 per cent. HCl in the mixture gives a concentration of acid of about ·002 per cent. But the saliva contains a little alkali and also some protein which enters into a loose combination with the acid. The concentration of free HCl in the digestion mixture will therefore be less than ·002 per cent.

182. Repeat the above exercise, using five drops of ·4 per cent. hydrochloric acid instead of one drop. The chromic period is indefinitely prolonged.

NOTE.—The concentration of HCl in this experiment is under ·02 per cent. In the absence of proteins such a concentration rapidly destroys ptyalin, the activity not returning on neutralisation.

183. Repeat Exercise 179 at the temperature of the room, at 30° C. and at 55° C. The chromic period is least at 45° C.

B. Pepsin.

Pepsin is the proteolytic ferment found in the gastric juice. It acts on most proteins, finally converting them into a mixture of peptones and polypeptides. It is important to note that it does not hydrolyse them as far as free amino-acids, thus differing from trypsin and erepsin. The intermediate stages in the action are given on page 24.

Pepsin acts in an acid medium only. The optimum strength of acid is one with a concentration of hydrogen ions found in a 0·2 per cent. solution of HCl. The ferment is rapidly destroyed by alkalies. It is secreted by the peptic cells of all parts of the stomach, in which it appears as a precursor, called pepsinogen. This is relatively stable to alkalies and is converted into pepsin by the action of HCl.

For the following experiments use a 1 per cent. solution of commercial pepsin in water.

184. Place equal amounts of fresh washed fibrin in four test tubes labelled A, B, C, and D.

To A add 5 c.c. of pepsin and 5 c.c. of ·4 per cent. HCl.

To B add 5 c.c. of pepsin and 5 c.c. of water.

To C add 5 c.c. of water and 5 c.c. of ·4 per cent. HCl.

To D add 5 c.c. of pepsin that has been boiled and then cooled, and 5 c.c. of ·4 per cent. HCl.

Place the four tubes in a water bath at 40° C. for at least thirty minutes.

Note that in

A, the fibrin swells up, becomes transparent and then dissolves;

B, the fibrin is unaltered;

C, the fibrin swells up, becomes transparent, but does not dissolve;

D, the fibrin is like that in C.

NOTE.—These exercises show that neither ·2 per cent. HCl alone, nor pepsin alone, can digest fibrin, but that pepsin in the presence of ·2 per cent. HCl has this property. In D the ferment pepsin has been destroyed by boiling.

185. **The detection of pepsin.** Obtain some fibrin that has been stained with carmine (see note below). Treat the ferment solution with the same volume of 0·4 per cent. HCl. Divide this into two equal portions and label them A and B. Boil B for a minute, and cool the tube. To each tube add a few flakes of the stained fibrin. Place them on the warm bath for ten minutes. Shake and observe the colour of the fluid. In A it will be red. In B it will be almost or quite colourless.

NOTE.—The carmine solution for staining fibrin is prepared by dissolving 1 gram of carmine in about 1 c.c. of ammonia and adding 400 c.c. of water. The solution is kept in a loosely-stoppered bottle till the smell of ammonia has become faint. Fresh washed fibrin is chopped finely, placed in the carmine solution for twenty-four hours, strained off and washed in running water till the washings are colourless. If not required immediately, it should be kept under ether and washed with water before use. It cannot be used for testing for trypsin, owing to the solubility of the dye in alkalies.

186. **The estimation of Pepsin by Mett's Method.***

Preparation of the tubes. The whites of several new-laid eggs are beaten to break the membranes, strained through linen or muslin and allowed to stand till free from air bubbles. The liquid is then drawn up into lengths of glass tubing with an internal diameter of between 1 and 3 mm. Each length is laid flat on a piece of wire gauze, so arranged that it can be dropped into a saucepan of hot water, having a double bottom ("porridge saucepan"). The water in the saucepan is boiled and allowed to stand till that in the inner vessel has cooled to 85° C. The gauze with the prepared tubes is then placed in this inner vessel, and allowed to stand till the water is quite cold. The tubes can be preserved by sealing the ends with shellac.

Method of estimation. Cut off lengths of 2 cms., breaking the tubes sharply to get an even edge of coagulated egg white.

Measure 10 to 20 c.c. of the ferment into a small Erlenmeyer flask. In it place three of the tubes of egg-white, shake and cork, and place the flask in a thermostat at 40° C. for 24 hours. The mixture must not be shaken during the digestion. Measure the length of the tube (T) and of the remaining egg-white (W) by means of a millimetre scale and a magnifying glass. T − W = the amount of protein digested (D). Take the average for the three tubes. D varies as the square root of the amount of ferment present.

NOTES.—Filtered gastric contents should be diluted with $\frac{N}{30}$ HCl in the proportion of 1 c.c. of gastric contents to 15 c.c. of acid.

For practice use a 0·5 per cent. solution of Merck's pepsin in $\frac{N}{30}$ HCl.

Dilute 1, 4, and 9 c.c. to 16 c.c. with $\frac{N}{30}$ HCl. The amounts of egg-white digested should be as $\sqrt{1} : \sqrt{4} : \sqrt{9}$ *i.e.* as 1 : 2 : 3.

187. **Action of alkalies on Pepsin.** Treat 5 c.c. of the pepsin solution with half its volume of 2 per cent. sodium carbonate and place on the bath at 40° C. for half-an-hour. Neutralise with ·4 per cent. HCl, and then add an equal volume of ·4 per cent.

* An alternative method is described on p. 248.

HCl to the fluid. Add some carmine fibrin and place the tube on the warm bath. The fibrin does not dissolve, showing that pepsin is destroyed by dilute alkaline salts.

C. The Acidity of Gastric Juice.

The acidity of the gastric contents is due to three causes, viz.:

1. The free hydrochloric acid.
2. The HCl combined with proteins.
3. Acid salts.

The sum of these three is called

4. The total acidity.

The sum of 1 and 2 is called

5. The physiologically active HCl.

The estimation of these different quantities in the gastric contents is of considerable importance in many pathological conditions. A test meal of toast and tea is given, and an hour afterwards the gastric contents are removed by means of a tube.

Total acidity. Ten c.c. of the filtered contents are titrated with N/10 NaOH, using phenolphthalein as an indicator. The result is expressed in terms of grams of HCl in 100 c.c., by multiplying the number of c.c. by 0·0365.

Free HCl. The estimation of this is practically that of the concentration of hydrogen ions in the gastric contents. HCl is very freely dissociated into H and Cl ions in such dilutions as those found in the stomach. But weak acids, as lactic and butyric, are only slightly dissociated. Also the addition of proteins to a solution of HCl decreases the concentration of H ions, owing to the formation of a compound that only dissociates to a

relatively small extent. The student is advised to read the remarks on acidity in the section on the acidity of the urine.

The estimation of the free HCl is best done by the electrical method that is mentioned in the section quoted above.

The use of indicators is not to be advised. According to the latest researches it is certain that even Toepfer's reagent (dimethyl-amido-azo-benzene) reacts with an excess of butyric and lactic acids, and also with HCl in combination with protein.

The simplest clinical method that gives results at all comparable with the electrical method is that of titrating with standard NaOH until no reaction is obtained for free HCl with Gunsberg's reagent. The method is rather tedious.

187A. **Gunsberg's test for free hydrochloric acid.**

A. Place a single drop of Gunsberg's reagent in a porcelain dish on a boiling water bath. When dry, add a single drop of 0·04 per cent. HCl to the film of reagent and again take to dryness. A brilliant carmine colour develops.

B. Repeat the experiment, using a mixture of equal parts of 1 per cent. acetic acid and 1 per cent. sodium chloride in place of the HCl. Only a yellow or brown stain results.

C. To 10 c.c. of 0·04 per cent. HCl add 5 c.c. of 1 per cent. Witte's peptone. Try Gunsberg's test with a drop of this. Free HCl is absent.

D. To the remainder of the fluid C add a drop of phenolphthalein and titrate with N/10 NaOH till pink. Compare the amount used with that required to neutralise 10 c.c. of 0·04 per cent. HCl. The absence of free HCl in C is obviously not due to the presence of any alkali in the peptone. The HCl has combined with the protein to form a protein-HCl compound.

NOTE.—**Preparation of the reagent.** Dissolve 2 grammes of phloroglucin and 1 gramme of vanillin in 30 c.c. of absolute alcohol. The solution should be freshly prepared to give sharp results, but it can be preserved for a certain time in dark glass bottles.

188. **Estimation of free HCl by Gunsberg's reagent.** If free HCl is present as determined by the method given in the previous exercise, titrate 10 c.c. of the fluid with N/10 soda, performing Gunsberg's test with a drop of the mixture after every addition. The end point is reached when a drop of the mixture fails to give the test. If many drops have been used, the titration must be repeated, adding nearly the whole of the calculated amount of soda in one operation.

Calculation. Multiply the amount of N/10 soda used by 0·0365. The result is the number of grams of free HCl per 100 c.c.

189. **Prout-Winter method for the estimation of the physiologically active HCl and of mineral chlorides.**

A. 10 c.c. of the filtered gastric contents are mixed with an excess of sodium bicarbonate in a platinum crucible and evaporated to dryness over a water-bath. The crucible is then heated over a Bunsen flame and the contents incinerated. The total chlorides in the ash is determined by extracting with water and applying Volhard's method. Express the result in terms of HCl per 100 c.c.

B. Repeat the experiment without adding the bicarbonate. The free HCl and that combined with proteins is evolved, and only the mineral chlorides retained. Estimate these as before. A minus B gives the amount of physiologically active HCl.

NOTE.—Usually the "active" HCl is only slightly less than the total acidity, shewing that no abnormal acids are present. But in certain diseases there is a great difference between the two results, and it is in these cases that the estimation is of value.

The amount of mineral (sodium) chloride is of great interest in connection with carcinoma, in which condition free HCl is absent and the mineral chlorides are much increased. This may point to a neutralisation of the acid by some alkaline secretion.

In gastric ulcer the free HCl is increased above normal, and is always considerably greater than the mineral chlorides.

D. Trypsin.

Trypsin is the proteolytic ferment secreted by the pancreas. The pancreatic juice contains a precursor called trypsinogen. This is converted into trypsin on reaching the duodenum by the action of the enterokinase secreted by the mucous membrane of the small intestine.

Trypsin differs from pepsin in two important particulars. In the first place it acts in a medium that is alkaline to litmus. The optimum concentration of hydroxyl ions is not certain. Probably that concentration in which the ferment acts best is one that has a destructive action on the ferment. Consequently the optimum concentration of alkali will be greater for a short than for a long digestion. It is important to note in this connection that trypsin is not at all stable in alkaline solutions. To preserve the ferment a minute amount of acid is added.

In the second place trypsin differs from pepsin in being able to hydrolyse the protein molecule to the final products, the various amino-acids and basic substances.

Preparation of trypsin. Obtain the fresh pancreas of a pig. Free it from fat as far as possible. Weigh it. Mince it finely and add three times its weight of distilled water and its own weight of strong alcohol. Shake well in a flask and allow it to stand for three days at room temperature, shaking the flask occasionally. Strain through muslin and filter through a large folded filter. The filtrate, which comes through very slowly, is measured and treated with 1 c.c. of strong HCl for every litre. This causes the appearance of a cloudy precipitate, which settles in a week or so and can then be filtered off. The fluid keeps for an indefinite period, if stoppered, without the addition of any antiseptic, the alcohol itself acting as an antiseptic. The fluid is rich in trypsin and in amylopsin, the amylolytic ferment of the pancreas. It does not contain any lipase.

It seems to be identical with, though usually rather more active than, the commercial extract known as Benger's "liquor pancreaticus."

Detection of Trypsin.

The digestion of fibrin does not give a satisfactory method for the determination of the presence of trypsin owing to the relatively slow rate at which the action takes place.

The best method is that of Gross, who uses a solution of casein. This is precipitated by dilute acetic acid, but it is rapidly acted on by trypsin and is converted into substances that are soluble in dilute acids. We thus have a means both of detecting and of comparing the activities of tryptic solutions, by finding the time required for the disappearance of a certain amount of casein.

Preparation of the Casein Solution. Dissolve 5 grams of Hammarsten's casein in 42·5 c.c. of N/10 NaOH and 450 c.c. of boiling water. Filter whilst still warm, cool and make the volume up to 500 c.c.

190. Measure 10 c.c. of the casein solution into a test tube and place it on the warm bath for a few minutes, so that it may acquire the temperature of the bath.

Measure 5 c.c. of the pancreatic extract (previously diluted with four volumes of water) into another tube and warm. Mix the two solutions, noting the time. At intervals remove about a c.c. by means of a pipette or glass tube and run it into a similar volume of 1 per cent. acetic acid. At first a heavy white precipitate of casein is produced. But after a certain length of digestion, depending on the activity of the ferment, no precipitate is produced.

NOTE.—The disappearance of the casein cannot be due to pepsin, for free HCl is not present.

The products of the action of Trypsin on Proteins.

The final products of the action of trypsin and other powerful hydrolytic reagents on proteins consist of a number of substances which differ somewhat in nature and amount with the protein. They are mostly monamino acids, with the amino-group replacing an H atom

attached to that carbon atom which is itself attached to the -COOH group. That is, they are α-amino acids.

$CH_3.CH_2.COOH$. Propionic acid.
$CH_3CH(NH_2).COOH$. α-amino-propionic acid.
$CH_2(NH_2)CH_2.COOH$. β-amino-propionic acid.

Classification of the Products.

Mon-amino-acids.	Mono-carboxylic.	Fatty series.	Group A.
		Aromatic series.	Group B.
	Di-carboxylic.		Group C.
Di-amino-acids.			Group D.
Heterocyclic compounds.			Group E.
Carbohydrate compound.			Glucosamine, an amino-hexose.

Group A. 1. Glycine (amino-acetic acid), $CH_2.(NH_2).COOH$.

2. Alanine (α-amino-propionic acid), $CH_3.CH.(NH_2).COOH$.

3. Leucine (α-amino-isocaproic acid), $C_6H_{13}NO_2$.

4. Cystine (dicysteine, or di-β-thio-α-amino-propionic acid).

Group B. 5. Phenylalanine. $C_6H_5.CH_2.CH.(NH_2).COOH$.

6. Tyrosine (oxy-phenyl-alanine),

$$C_6H_4\begin{matrix}OH\\ CH_2CH.(NH_2).COOH\end{matrix}.$$

7. Tryptophane (indol-alanine),

$$C_8H_6N.CH_2.CH.(NH_2).COOH.$$

Group C. 8. Aspartic acid (amino-succinic acid).

9. Glutamic acid (α-amino-glutaric acid).

Group D. 10. Arginine (α-amino-δ-guanidine-valerianic acid),

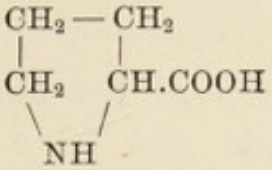
$$HN:C\begin{matrix}NH_2\\ NH.\end{matrix}\ CH_2.CH_2.CH_2.CH.(NH_2).COOH.$$

11. Lysine (α, ϵ-diamino-caproic acid).

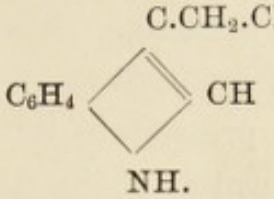
Group E. 12. Histidine (β-imidazole-alanine),

$$\begin{matrix}&&CH\\ &\diagup&\parallel\\ HN&&N\\ |&&|\\ CH=C&-&CH_2.CH.(HN_2).COOH.\end{matrix}$$

13. Proline (α-pyrrolidine-carboxylic acid),



Tryptophane (indol-alanine).



The Isolation of the Products.

The following three methods have been employed.

1. Fractional crystallisation.
2. Fractional precipitation, that is, a reagent is used which only precipitates one or two of the substances present in the mixture, *e.g.* mercuric sulphate in acid solution only precipitates tryptophane and cystine : phosphotungstic acid only precipitates Groups D and E (with the exception of proline).
3. Fractional distillation of the esters. The compounds are converted into their ethyl esters, which are dried and distilled under very low pressures. Since they have different boiling points they can be separated.

191. 150 grams. of commercial casein ("protene" or "plasmon"), 50 to 100 c.c. of the tryptic solution described on page 94, and a litre of 1 per cent. Na_2CO_3, have been digested for about ten days at 40°C. in a large flask, 1 gram. of sodium fluoride and about 30 c.c. of chloroform or tuluol being added, and the mouth of the flask securely plugged with cotton wool, soaked in chloroform, to prevent bacterial decomposition. About 100 c.c. of the mixture are given to you. Boil the mixture, and whilst boiling add strong acetic acid, drop by drop, till the reaction is acid. Cool under the tap, and filter off the undigested casein, etc.

A. Treat 5 c.c. of the filtrate with bromine water, drop by drop; a pink colour gradually develops, which deepens and then disappears as more bromine water is added. When the colour is no longer intensified by the addition of bromine, add 2 or 3 c.c. of amyl alcohol and shake. On standing, the alcohol rises to the

surface coloured a fine red or violet. This reaction is due to the presence of *tryptophane*.

B. Treat another 5 c.c. of the filtrate with ten drops of concentrated sulphuric acid and 10 c.c. of a 10 per cent. solution of mercuric sulphate in 5 per cent. H_2SO_4. Shake the tube and leave it for five minutes. Note the yellow precipitate of a mercury compound of *tryptophane*. Filter this off and label the filtrate *A*. Wash the precipitate through a hole in the paper into a clean tube, fill with water, shake and filter again, neglecting the filtrate. Wash the precipitate on the paper once more with water and then let it drain. Scrape a portion off the paper, transfer it to a tube, add 2 c.c. of "reduced oxalic acid" and then 2 c.c. of concentrated sulphuric acid. A purple colour is produced, showing that tryptophane is responsible for the glyoxylic reaction. (See Ex. 3.)

Treat another portion of the precipitate with Millon's reagent and boil. A yellow colour is produced, not the characteristic red of Millon's reaction.

To another portion of the precipitate apply the xanthoproteic test. A well-marked reaction is obtained. (See notes to Ex. 1.)

To portions of filtrate *A* apply the glyoxylic, Millon's, and the xanthoproteic reactions. Only the latter two are obtained, the tryptophane, but not the tyrosine, having been removed by the mercury reagent employed.

C. Treat the remaining 90 c.c. of the filtrate with a few drops of ammonia and evaporate to a small bulk (about 20 c.c.) either on the water-bath or by use of a small free flame. Allow the residue to stand twenty-four hours. Notice the formation of a crystalline crust. Examine a portion of this microscopically and observe the feathery masses and sheaves of fine white needles, characteristic of *tyrosine*. Filter this off and evaporate the filtrate still further. *Leucine* separates out on standing, and, examined microscopically, shows rounded cones with a radiating striation. Make a drawing of the crystals of tyrosine and leucine.

CHAPTER VI.

THE COAGULATION OF BLOOD.

Factors concerned.

1. *Fibrinogen* (Fgn.) a globulin, present in blood-plasma. It is soluble in dilute salt solutions, acids and alkalies, insoluble in distilled water. It coagulates at 57° C. It is precipitated by half-saturation with sodium chloride.

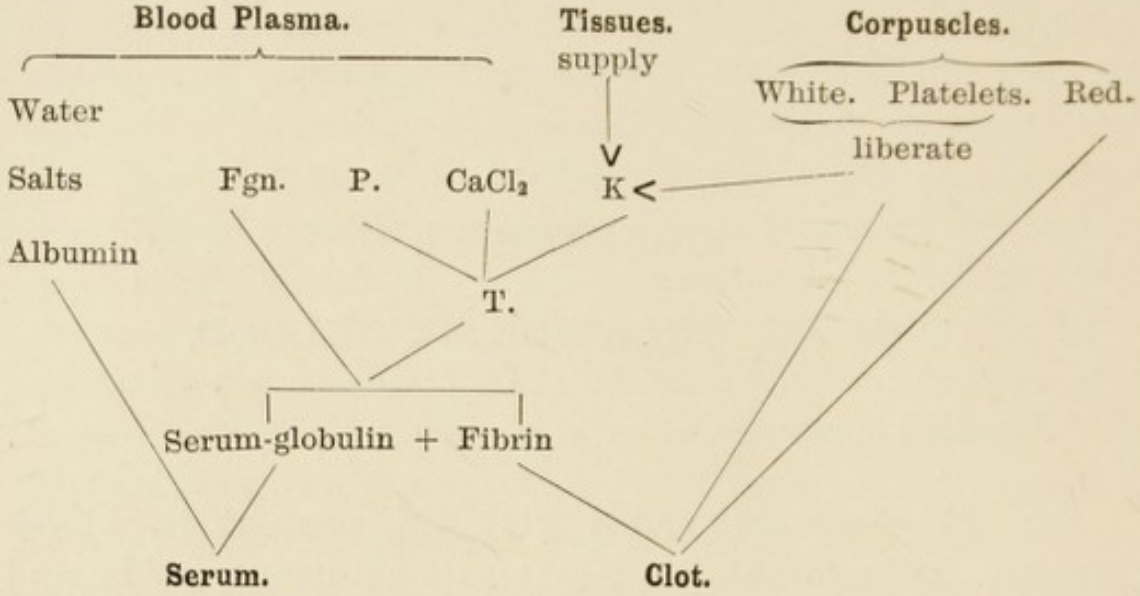
2. *Pro-thrombin* (P) a substance of unknown composition, found in plasma, attached to the fibrinogen. It is destroyed by boiling.

3. *Thrombokinase* (K) a substance found in all tissues and also liberated in the blood by the disintegration of leucocytes and blood-platelets. It converts pro-thrombin into thrombin, under certain conditions.

4. *Calcium salts*, found in plasma, and necessary for the action of thrombokinase. The calcium salts must be of such a nature that they are ionised in solution.

5. *Thrombin* (T), a ferment formed by the interaction of 2, 3 and 4. It probably splits fibrinogen into serum-globulin and fibrin. The latter, being insoluble in the constituents of normal plasma, comes out of solution and with the corpuscles forms the clot.

The phenomena of blood coagulation are represented in the following scheme:—



Coagulation is hindered by

1. Cooling.

2. Substances which precipitate calcium salts, or convert the calcium into the non-ionised condition, as oxalates, citrates and soap solutions.

3. Alkalies, which prevent the liberation of K by the corpuscles, delay the action of T, and tend to dissolve fibrin.

4. Strong salt solutions, which have a similar action.

5. Anti-thrombin, a substance found in small amounts in the plasma, and in relatively large amounts in extracts of the head of the leech. It combines with T to render it inactive.

6. Anti-kinase, found in the blood, after the slow injection into the blood stream of certain substances, as tissue-extracts, certain snake-venoms, etc.

7. Fluorides, which precipitate calcium salts and prevent the liberation of K.

Preparation of fibrin ferment (thrombin). Blood serum is treated with four or five times its volume of strong alcohol, well stirred and allowed to stand for two or three days. The precipitate is collected, dried on filter paper in the air, and extracted with water. The filtered extract contains fibrin ferment.

Preparation of "salted" plasma. Two litres of water are placed in a large bottle or jar (provided with a tightly-fitting stopper) and the level of the fluid marked by a label. The water is poured off and 400 c.c. of a saturated solution of magnesium sulphate substituted. Blood is collected in the bottle till the level is reached, care being taken to ensure thorough mixing with the salt solution by stopping the flow of blood from time to time and turning the bottle upside down. The corpuscles are removed by centrifugalisation and the plasma pipetted off. It should be kept in a refrigerator till required.

192. **The clotting of salted plasma.** Take 2 c.c. of salted plasma in a test tube, add 10 c.c. of water, and divide into two portions, A and B. To A add a few drops of fibrin ferment (or of serum). Place both tubes in the warm bath at 40° C. and examine from time to time. Clotting takes place in both tubes, but much more rapidly in A than in B.

NOTE.—Dilution with water decreases the concentration of the magnesium sulphate, so that any fibrin formed by the ferment (which can now act on the fibrinogen) becomes insoluble in this low concentration of salt.

193. **The preparation of fibrinogen.** To 20 c.c. of the salted plasma add an equal volume of a saturated solution of sodium chloride. A precipitate of fibrinogen is formed. Allow the tube to stand for a few minutes and then filter through a small paper. Scrape the precipitate off the paper and treat it with about 5 c.c. of 5 per cent. NaCl. The fibrinogen dissolves.

NOTE.—If bird's blood be drawn directly into a clean vessel in such a way that contact with the tissues is absolutely avoided, it clots very slowly. This is because the leucocytes are very stable and do not liberate thrombokinase. If this blood be centrifugalised at once, a non-clotting plasma is obtained. Fibrinogen can readily be prepared from this by the method given in Ex. 24. The suspension so obtained is dissolved in dilute salt solution.

194. Divide the solution thus obtained into two portions, C and D. To C add two drops of fibrin ferment. Place both tubes in the warm bath and observe them at intervals. C clots rapidly; D very slowly.

195. **The heat-coagulation of fibrinogen.** Heat 5 c.c. of salted plasma as in Ex. 10. Notice the coagulation of fibrinogen which occurs at 56° C. Continue heating to 60° C. and then filter. Dilute the filtrate as in Ex. 193; add fibrin ferment, and place on the warm bath. Coagulation does not occur.

Preparation of oxalate plasma. Blood is drawn as in the preparation of salted plasma into a bottle which has 200 c.c. of a 1 per cent. solution of potassium oxalate in place of the 400 c.c. of saturated magnesium sulphate. The plasma is separated, as before, by centrifugalisation.

196. **The clotting of oxalate plasma.** Dilute 5 c.c. of the plasma with 10 c.c. of distilled water and divide into three portions E, F, and G. To E add a few drops of 1 per cent. calcium chloride; to F, a few drops of fibrin ferment or serum. Place the three tubes on the water bath and observe them at intervals. E clots in a few minutes; F clots slowly; G does not clot.

Preparation of fluoride plasma. This is prepared as oxalate plasma using a 3 per cent. solution of sodium fluoride in place of the 1 per cent. potassium oxalate.

197. **The clotting of fluoride plasma.** Dilute 5 c.c. with 10 c.c. of water and divide into three portions, H, K, and L. To H add a few drops of 1 per cent. calcium chloride; to K a few drops of fibrin ferment. Place the three tubes in the warm-bath and observe them at intervals. K clots rapidly; H and L do not clot.

CHAPTER VII.

THE RED BLOOD CORPUSCLES AND THE BLOOD PIGMENTS.

A. The Laking of Blood.

The red corpuscles consist of an envelope and meshwork called the stroma, which encloses a solution of haemoglobin and various salts. The stroma consists of a protein, probably a histone, with which is associated a lipoid material, related to cholesterin and lecithin. The envelope behaves as a semi-permeable membrane to a great many solutions, readily allowing water to pass into or from the corpuscle, but preventing the passage of most salts and other dissolved substances. Thus if the corpuscles are placed in a solution which has a higher osmotic pressure than the fluid within the corpuscles, water passes out of the corpuscle, which therefore shrinks. Such fluids are called "hypertonic." If they be placed in fluids of a lower osmotic pressure ("hypotonic"), water passes into the corpuscle to equalise the pressures, but salts cannot pass out. The corpuscles swell and the expansion may be sufficient to lead to the disruption of the envelope, so that the enclosed haemoglobin passes into the body of the solution. This bursting of the corpuscles is known as laking or haemolysis. A solution of the same osmotic pressure as that of the fluid within the corpuscle is said to be "isotonic" or "normal." For mammalian blood 0·9 per cent. sodium chloride is normal; for frog's blood, 0·65 per. cent. Other physical means of inducing haemolysis are by repeatedly freezing and thawing the blood,

or by warming to 60° C. The envelope can also be ruptured by chemical means. Certain substances, such as the bile salts, ether, chloroform, acids, alkalies, and saponin are solvents for the lipoids.

Another method of inducing haemolysis is by the addition of certain organic substances developed in certain animals. Thus rabbit's corpuscles that have been washed with isotonic saline are laked when treated with the blood serum of a dog. This haemolytic power of dog's serum on rabbit's blood is very much increased by previously injecting the dog with rabbit's blood.

198. Have two burettes, one containing 1 per cent. sodium chloride, the other distilled water.

Label a series of clean, dry test-tubes, A, B, C, etc.,

In A place	4·5	c.c. NaCl and	5·5	c.c. H_2O	=	·45 % NaCl.
B „	5	„	5	„	=	·5 „
C „	5·5	„	4·5	„	=	·55 „
D „	6	„	4	„	=	·6 „
E „	6·5	„	3·5	„	=	·65 „
F „	7	„	3	„	=	·7 „

To each tube add three drops of freshly defribinated blood, mix by inverting and allow the tubes to stand for a few minutes. A will be translucent, the corpuscles being fully laked. F will be opaque. Note the dilution which just causes laking. It is generally about ·55 per cent.

NOTE.—The solution that just causes laking is hypotonic to the blood, indicating that the corpuscles can absorb a considerable quantity of fluid before the envelope is ruptured.

199. To 5 c.c. of 0·9 per cent. sodium chloride add some ether and shake vigorously. Then add three drops of blood, mix by inversion. The blood is laked.

200. To a ·2 per cent. solution of bile salts in normal saline add three drops of blood. It is laked.

201. Add some blood to a 2 per cent. solution of urea in water. The blood is laked.

202. Repeat the experiment with a 2 per cent. solution of urea in normal saline. The blood is not laked.

B. Haemoglobin and its Derivatives.

Haemoglobin (Hb) is a compound protein, being a member of the group of chromoproteins. It is formed by the union of a pigmented non-protein substance containing iron, and called haematin (Hn), with globin, a member of the histone group of proteins.

It is soluble in water and dilute salt solutions: insoluble in ether and alcohol.

It is decomposed by acids and alkalies into haematin and globin. It is decomposed and coagulated by heat.

It forms compounds with oxygen and carbon monoxide, called oxyhaemoglobin ($Hb\text{-}O_2$) and carboxyhaemoglobin (Hb-CO). Both are dissociated into Hb and the gas by exposure to a vacuum. Hb-CO is much more stable than $Hb\text{-}O_2$, and the avidity of Hb for CO is more than 130 times greater than the avidity of Hb for O_2. A small percentage of CO in the air breathed will thus result in the formation of relatively considerable amounts of Hb-CO in the blood. This can be converted into $Hb\text{-}O_2$ by exposure to a high tension of O_2, such as is obtained by breathing pure O_2.

The $Hb\text{-}O_2$ obtained from certain animals crystallises readily, but the crystals differ somewhat, according to the animal from which they are obtained. Also the volume of O_2 combining with 1 gram of Hb varies, the figure for the horse being 1·34 c.c. of O_2 per gram of Hb. The oxygen is probably united to the iron of the haematin molecule, the reaction $Fe + O_2 \rightleftharpoons FeO_2$ being the basis of the reaction $Hb + O_2 \rightleftharpoons Hb\text{-}O_2$.

The ratio $\frac{\text{volume of } O_2 \text{ evolved in c.c.}}{\text{weight of iron in grams.}}$ is called the specific oxygen capacity.

Theoretically it is

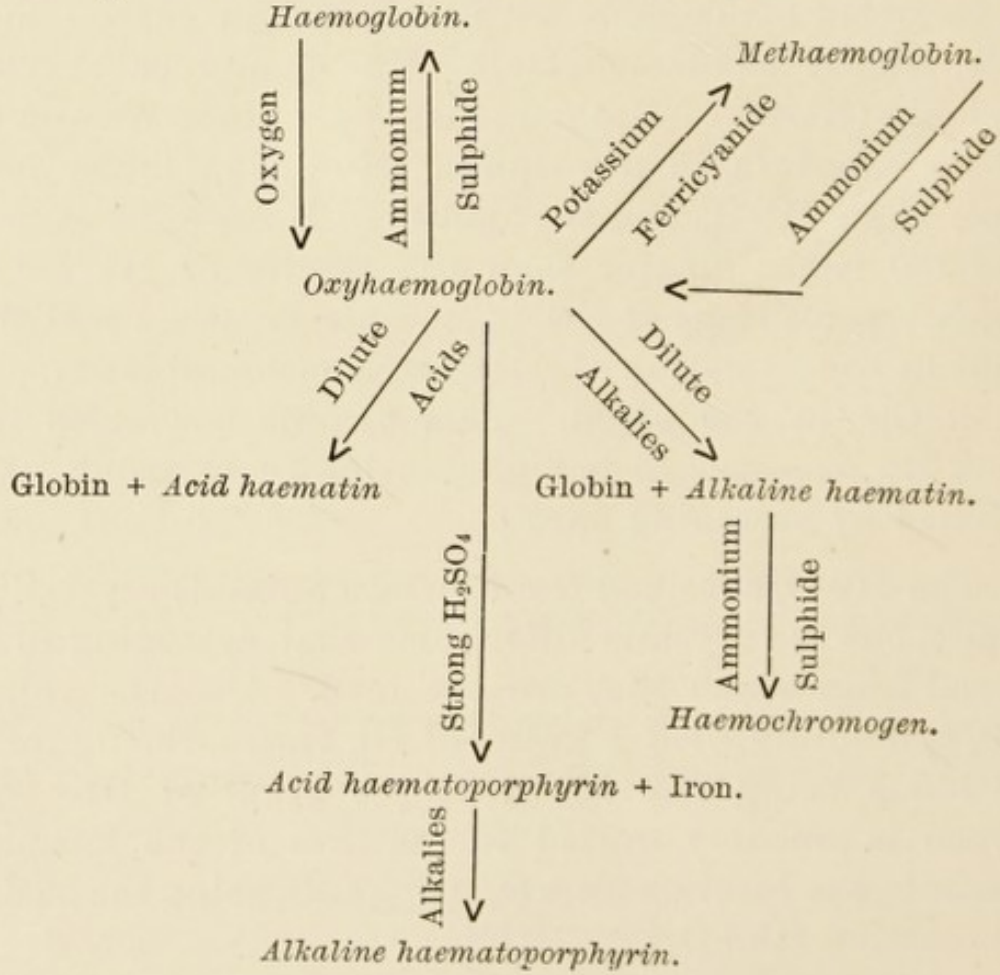
$$\frac{O_2}{Fe} = \frac{1 \text{ molecular volume } O_2}{1 \text{ gram molecule Fe}} = \frac{22{,}394}{55{\cdot}85} = 401.$$

Recent analyses of the blood of various animals have given the value 401·8, which agrees very closely with the theoretical.

The volume of oxygen loosely held by 1 gram of $Hb.O_2$ is 1·345 c.c.

So the minimum molecular weight of oxyhaemoglobin is $\frac{22{,}394}{1{\cdot}345} = 16{,}712$.

The method of formation of certain of the derivatives of haemoglobin can be represented as follows:—



203. **Crystallisation of oxyhaemoglobin (Rapid method).** To a few c.c. of defibrinated dog's blood in a test tube add ether, drop by drop, till the blood is completely laked. Add to the blood a pinch of finely powdered ammonium oxalate; allow the salt to dissolve by gentle shaking, and let the tube stand. Crystals of oxyhaemoglobin separate out, especially if the solution is cooled to 0°C. by means of ice. Examine them microscopically, and note that they are in the form of thin rhombic prisms.

Make a drawing of the crystals.

NOTE.—This experiment does not always succeed as described. If the blood fails to crystallise out in an hour, place a drop on a slide, spread it out to form a thin layer and leave it for five minutes; cover with a slip and note the crystals of oxyhaemoglobin that form at the edges.

C. The Spectroscopic Examination of the Blood Pigments.

The use of the Direct-vision Spectroscope.

The instrument described is the small pocket spectroscope, with wave-length scale attached, manufactured by Zeiss and Co. The instrument (fig. 2) consists of two tubes. The shorter tube A contains a transparent photographic scale of wave-lengths, with a mirror to project its image into the field of vision. By means of the tube D this scale can be focussed and by the screw F it can be adjusted to its proper position. The tube G contains a series of alternating prisms of crown and flint glass, arranged to allow the spectrum to be observed by the eye in the line of the tube. The tube B which slides on G has a vertical slit, the width of which can be adjusted by turning the collar E.

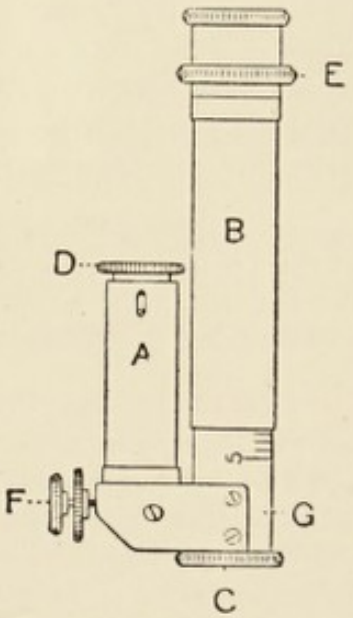


Fig. 2.—Zeiss' direct-vision spectroscope with wave-length scale (×½).

To adjust the spectroscope: see that D and B are pushed in as far as they will go. Look through C towards the light with A to your left, and turn E till the spectrum is only just visible. (It is most important to use an extremely narrow slit.) Slide B out very slowly (in most instruments for 3½ divisions as marked on the barrel G) till fine black vertical lines can be seen in the spectrum, and notice particularly a fine black line immediately to the left of the narrow strip of yellow. This line is known as the D line of Fraunhofer. The wave-length of it is ·59μ, a position indicated on the scale by the division marking it (the one to the right of 0·6) being produced further down than any other. If necessary alter the

position of the scale by turning the screw F until the D line exactly coincides with the division mentioned. If the instrument has to be adjusted at night-time, when the D line cannot be observed, set the scale by use of the emission-spectrum of sodium (obtained by placing a few crystals of common salt on the wick of a spirit lamp). The emission spectrum of sodium exactly corresponds to the D line. The scale is so drawn that, if it be set in position as described, the wave- length of light in any part of the visible spectrum can be read directly.

The numbers on the scale indicate wave-lengths in thousandths of a millimetre, the unit being 1μ. In the more recent patterns the wave-lengths are given in millionths of a millimetre, the unit being 1λ. Thus the wave-length of the D line is 589λ. The other Fraunhofer lines that can be readily observed with the instrument are C(657λ), E (527λ), b (518λ) and F ($486\ \lambda$).

To observe absorption spectra: slightly open the slit of the spectroscope, thus obtaining a better illumination. Direct the instrument to the light, and place the test tube containing the fluid to be examined directly in front of, and touching, the tube B, with its axis parallel to the slit, taking care not to interfere with the illumination of the scale. With strong solutions of certain pigments observed in this way it is often difficult to avoid illuminating the two ends of the spectrum, the light being reflected from the sides of the tubes, and not passing through the solution. To obviate this it is perhaps better to place the solution in a beaker, remembering that the absorption of light increases with the depth of layer examined, as well as with the concentration of the pigment. For accurate work the haematoscope should be employed. This is a vessel with parallel glass slides 1 cm. apart.

In handling the instrument the screw F is very liable to be turned and so the position of the scale to be shifted. From time to time, therefore, the slit should be narrowed, and an observation made to ascertain whether any shifting of the scale in reference to the D line has occurred.

Record the absorption of light of the various pigment solutions on the blank scale, to be found towards the end of the book. Fill in with black pencil marks the exact parts of the spectrum where light is absorbed, leaving the remainder blank. It will not be found advisable to use coloured pencils.

204. **Oxyhaemoglobin.** Take 5 c.c. of distilled water in a test tube, and add one drop of defibrinated blood, shake well and observe the spectrum of **dilute** oxyhaemoglobin. There are two absorption bands in the green. The one near the D line (the α band) is somewhat narrower and darker than the β band. The middle of α is about λ 578, and that of β about λ 540.

205. Add two more drops of defibrinated blood and examine again. The spectrum has become very much cut off, especially at the blue end: the absorption bands have probably merged into one, leaving a little patch of blue light and a broader belt of red light on the two sides. Record the spectrum of the solution on the chart as that of a **medium** solution of oxyhaemoglobin.

206. Add another drop or two of defibrinated blood, and note that the blue light becomes absorbed, light only coming through in the red. (**Strong** solution.) If the concentration is still further increased, the red also is absorbed.

NOTE.—It is important to observe that a *medium* solution of oxyhaemoglobin has a single band in the green.

207. **Haemoglobin (reduced haemoglobin).** Treat 5 c.c. of water with one drop of defibrinated blood and thus obtain a solution of oxyhaemoglobin of such a strength that two well-marked absorption bands can be observed. Add two drops of a solution of ammonium sulphide, mix and *warm to about 50° C.*, avoiding any unnecessary shaking: or if Stokes' fluid is obtainable, add two or three drops, in which case there is no necessity to warm. Note, in the latter case, that the bright scarlet colour of oxyhaemoglobin gives place to the less vivid colour of reduced haemoglobin. Examine the solution spectroscopically. There is a *single* broad band in the green which overlaps the space enclosed by the two bands of oxyhaemoglobin, and is fainter than either. Its centre is about λ 565.

NOTES.—Stokes' fluid is prepared as follows: dissolve 3 grams. of ferrous sulphate in cold water: add a cold aqueous solution of 2 grams. of tartaric acid and make the solution up to 100 c.c. with water. Immediately before use add strong ammonia until the precipitate first produced is redissolved. It rapidly absorbs atmospheric oxygen and must, therefore, be freshly prepared. Its great advantage over ammonium sulphide is that it can be used in the cold, whilst with the sulphide the solution must be warmed.

208. Place your thumb over the top of the test tube containing the reduced haemoglobin and shake vigorously. Examine immediately with the spectroscope, and note that the two bands of oxyhaemoglobin have reappeared owing to the oxidation of the

haemoglobin by the oxygen of the air. If the tube be allowed to stand for a short while, reduction may appear again from excess of reducing reagent present.

209. **Carboxyhaemoglobin.** Obtain some CO-haemoglobin that has been prepared by passing a stream of carbon monoxide or coal-gas through a solution of oxyhaemoglobin. Notice the peculiar bluish tinge of the solution. Examine a portion spectroscopically, and, if necessary, add water till two well-marked bands are visible. Note that they are very similar to the two bands of oxyhaemoglobin. Accurate observation, however, will show that they are both slightly nearer the violet end of the spectrum, the middle of α being λ 572 and of β λ 535.

NOTES.—1. A small amount of ether added to the blood facilitates the preparation of Hb-CO in preventing excessive frothing.

2. If the student can satisfy himself of the difference between the position of the absorption bands of Hb-O_2 and Hb-CO, he can always obtain a sample of Hb-O_2 for comparison with an unknown solution by pricking his finger.

210. Take a portion of the diluted solution of CO-haemoglobin just examined, treat it with a few drops of ammonium sulphide, warm in a bath at 50°C. for three minutes and examine with the spectroscope. No change takes place in the spectrum. (Distinction from oxyhaemoglobin.)

211. In two test tubes place 2 or 3 c.c. of solutions of oxyhaemoglobin and CO-haemoglobin of the same depth of colour. Fill the test tubes with water and mix well. Note that the CO-haemoglobin takes on a well-marked carmine tint, whilst the oxyhaemoglobin turns yellow. This simple test, which can only be seen on extreme dilution, rapidly serves to distinguish the two compounds.

212. **Katyama's test for CO-Haemoglobin in blood.** Add 5 drops of blood to 10 c.c. of water: then add 5 drops of orange-coloured ammonium sulphide. Mix and add enough strong acetic acid to make the mixture faintly acid. With blood containing

CO, a rose-red colour appears: with normal blood a dirty greenish-grey. The colour is still perceptible with one part of the CO-blood to 5 of normal blood.

213. **Methaemoglobin.** To 5 c.c. of water add four drops of defibrinated blood. To the strong solution of oxyhaemoglobin thus formed add two drops of a saturated solution of potassium ferricyanide. The colour of the solution changes to a chocolate-brown. Examine with the spectroscope: there is visible a prominent band in the red, with its centre at about λ 630. There is marked absorption of the blue end of the spectrum. Dilute with an equal bulk of water and examine again: two faint bands appear in the green in the position of the bands of oxyhaemoglobin.

213A. Dilute the solution of methaemoglobin thus obtained with another volume of water. Treat 5 c.c. of this with two or three drops of ammonium sulphide and examine immediately. The colour changes to a red: the absorption band in the red disappears, and the spectrum of oxyhaemoglobin is seen. Warm the solution to 50°C. and allow it to stand for a short time (possibly with the addition of another drop or two of the reducing reagent). The two bands give place to the single band of reduced haemoglobin. Shake with air: oxyhaemoglobin is reformed.

214. Take a few c.c. of defibrinated blood in a test tube, add an equal quantity of water and warm to 50°C. to lake the blood. To the solution thus obtained add an equal bulk of saturated potassium ferricyanide. Mix by giving one shake, and then hold the tube at rest in an oblique position for a short time. Note the bubbles of gas (oxygen) that are evolved.

NOTES.—1. Oxyhaemoglobin is converted into methaemoglobin by the action of oxidising reagents, such as ferricyanides, nitrites, chlorates, and permanganates, and in the body, by the action of many aromatic substances, such as phenol.

2. The reaction is peculiar in that an amount of oxygen is evolved equivalent to that held in combination by the oxyhaemoglobin, although methaemcglobin contains the same percentage of oxygen as oxyhaemoglobin. This reaction is the basis of the modern method of estimating the amount of oxygen in the blood.

The reaction might be represented by the following equation :—

Oxyhaemoglobin. From ferricyanide. Methaemoglobin. Gas.

The oxygen is represented as being in a different state of combination in methaemoglobin, since it cannot be removed by submitting the compound to a vacuum.

3. When methaemoglobin is treated with a reducing reagent, the first change that occurs is that the linkage of the oxygen atoms reverts to that of oxyhaemoglobin; later the oxygen is removed and reduced haemoglobin formed.

215. **Acid haematin.** To 5 c.c. of water add four drops of defibrinated blood and five drops of strong acetic acid and heat. The colour changes to brown; and the solution shows an absorption band in the red, which is further from the D line than that of methaemoglobin. Its centre is about λ 650.

216. **Acid haematin in ethereal solution.** Treat a few c.c. of defibrinated blood with one drop of strong hydrochloric acid and a few c.c. of acetic acid: extract this with about 5 c.c. of ether by gentle shaking, pour the ether into a clean tube and examine it with the spectroscope. There is a prominent band in the red (centre λ 638); on dilution with ether three other bands can be seen; a very narrow one with centre λ 582; a broad one stretching from about λ 555 to λ 530 and another from λ 512 to λ 498.

217. **Alkaline haematin.** Treat a moderately strong solution of oxyhaemoglobin with a few drops of strong sodium hydroxide and warm. The colour changes to brown. Examine with the spectroscope: a faint band is seen in the red, stretching from the D line to about λ 630. There is a considerable absorption of the blue end of the spectrum.

NOTE.—A band of alkaline methaemoglobin in the yellow-green is often seen as well.

218. **Alkaline haematin in alcohol.** Mix defibrinated blood into a thin paste with solid potassium carbonate and evaporate to complete dryness on a water bath. Powder the residue, boil

with alcohol in a flask on the water-bath and filter. The solution contains alkaline haematin free from proteins. It shows the absorption band of alkaline haematin more distinctly than the crude aqueous solution prepared in Ex. 217.

219. **Haemochromogen** (reduced alkaline haematin). Prepare a solution of alkaline haematin from dilute oxyhaemoglobin as in Ex. 217. Treat it with a few drops of ammonium sulphide. The colour of the solution changes to red. Examine with the spectroscope. Two absorption bands are seen in the green. The band nearer the D line (the α band) is very prominent and sharply defined, with its centre at about λ 558. The β band is much fainter and has its centre at λ 520.

NOTE.—In very dilute solutions only the α band can be seen. The absorption of light in this region is so intense that if a solution of oxyhaemoglobin, so dilute that its absorption bands cannot be readily seen, be converted by appropriate means into haemochromogen, the α band of this pigment is usually observable.

220. **Acid haematoporphyrin.** To a few c.c. of concentrated sulphuric acid in a test tube add two drops of defibrinated blood (see note to Ex. 221) and mix by gentle shaking. Note the rich purple colour of the solution. Examine with the spectroscope. Two bands are seen: the α band, with centre at λ 600, being fainter and narrower than the β band, centre λ 554.

221. **Alkaline haematoporphyrin.** To the solution of acid haematoporphyrin just prepared add five or six more drops of defibrinated blood, shaking gently after the addition of each drop. Pour the strong solution into about 50 c.c. of cold water in a beaker, stir well and note the precipitate that rises to the surface. Transfer this precipitate to a test tube by means of a rod; treat it with a few c.c. of alcohol and boil. And 5 c.c. of sodium hydrate. A solution of alkaline haematoporphyrin is thus obtained, which examined spectroscopically after suitable dilutions shows a four banded spectrum. The centres of the bands are at λ 622, λ 576, λ 539 and λ 504 approximately.

NOTE.—The conversion of blood pigment into haematoporphyrin involves two processes. Firstly, the acid splits off the protein constituent (globin) and forms acid haematin; secondly, the acid haematin loses its iron and becomes haematoporphyrin. The first change is effected very readily even by dilute acids, but the separation of the iron from the haematin normally requires highly concentrated mineral acids. It has, however, been shown that if the blood be first reduced the iron is split off with much greater ease by the acid. An efficient method of reducing defibrinated blood is that of "auto-reduction," in which a tightly corked vessel full of blood is allowed to stand for a few days. If exercises 220 and 221 be carried out with this reduced blood, care being taken by use of a pipette to prevent re-oxidation, the haemoglobin is entirely converted into haematoporphyrin, no trace of the brown haematin being left.

222. **Preparation of haemin crystals.*** (Teichmann's crystals.) Place a drop of defibrinated blood on a glass slide, add a speck of sodium chloride and rub with a glass rod till the salt has dissolved. Evaporate to complete dryness by supporting the slide about a foot above a *small* flame. Rub the red residue to a fine powder with a pen-knife, collect it into a little heap and add a drop of glacial acetic acid on the end of a glass rod. Rub into a paste, and place a little of this on a clean slide, add a drop of glacial acetic acid, cover with a slip and cautiously heat over a *small* flame till it just boils. Let a drop more of the acid run under the slip and then allow to cool. Examine microscopically for the brown rhombic prisms of haemin (haematin hydrochloride). Draw them in the space provided at the end of the book.

NOTES.—Care must be taken to add only a trace of sodium chloride, to dry slowly, and to observe that the acid mixture really boils and that all the acid is not evaporated off.

This test can be applied to dilute solutions of haemoglobin by acidifying with acetic acid, precipitating with freshly prepared tannic acid, and treating the dried precipitate on a slide with a trace of salt and glacial acetic acid as described above. Suspected blood stains on linen, instruments, etc., should be extracted with a little alkali, the solution evaporated to dryness and treated as above.

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* An improved method will be found on p. 252.

CHAPTER VIII.

THE CONSTITUENTS OF BILE.

Bile is secreted continuously into the hepatic ducts by the liver. During the intervals of digestion it is stored in the gall bladder, being poured into the duodenum when the acid chyme passes through the pylorus.

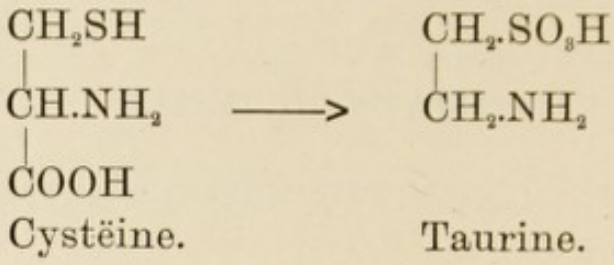
During its stay in the gall bladder there is an absorption of water and an increase in the protein content, resulting in an increase in the specific gravity from about 1010 to 1040

The percentage composition of human bile varies considerably. The following are average figures :—

	From Gall Bladder.	From Fistula.
Water	86 ...	... 98
Solids	14 ...	... 2
Bile salts	9 ...	... 0·8
Protein ..... Bile pigments	... 3 ...	... 0·3
Cholesterin	0·2 ...	... 0·06
Lecithin and fat ...	1·0 ...	... 0·04
Inorganic salts ...	0·8 ...	... 0·8

The bile salts are the sodium salts of glycocholic and taurocholic acids. They are formed by the condensation of cholalic acid ($C_{24}H_{40}O_5$) with glycine (amino-acetic acid, $CH_2.NH_2.COOH$) and taurine respectively. Glycine is one of the products obtained by the hydrolysis of proteins.

Taurine is derived from a similar product, cystëine.



Cystëine. Taurine.

The bile acids are hydrolysed into their constituents by boiling acids and also by the intestinal bacteria.

The bile salts are soluble in water and alcohol, insoluble in ether.

Their solutions have a remarkably low surface tension. (See Hay's test.)

They have the following functions:—

1. They have a marked adjuvant action on pancreatic lipase. (See Ex. 116.)

2. They are solvents for the fatty acids and thus markedly increase the absorption of fats. (See p. 65.)

3. They thus help to remove the fatty film surrounding the protein, and allow the proteolytic ferments to act. In this way, by assisting the absorption of proteins, they diminish bacterial decomposition. They are not direct antiseptics.

Preparation of Bile Salts.—Mix 40 c.c. of ox gall with enough animal charcoal (about 10 grams) to form a paste. Evaporate to dryness over a water bath, stirring at intervals. Grind the residue in a mortar, transfer it to a flask, add about 70 c.c. of 96 per cent. or absolute alcohol and boil on the water bath for 20 minutes. Cool and filter into a dry beaker. Add ether to the filtrate till there is a slight permanent cloudiness. Cover the beaker with a glass plate and allow it to stand in a cool place for 24 hours. A crystalline mass of bile salts separates out. The crystals are filtered off and allowed to dry in the air.

For the following tests use a 1 per cent. solution of bile salts or diluted ox or sheep gall:—

223. **Pettenkofer's test for bile salts.** To 5 c.c. of the solution add a *small* particle of cane-sugar and shake or warm

till this has completely dissolved. To the cooled solution add 5 c.c. of concentrated sulphuric acid, inclining the test tube so that the acid settles to the bottom. Gently shake the test tube from side to side. As the fluids gradually mix a deep purple colour develops.

NOTES.—1. This reaction depends on the production of furfurol from the cane-sugar by the strong acid. (See Ex. 76.)

2. If too much cane-sugar be taken the fluid will turn brown or black, owing to the charring produced.

3. Proteins give a very similar reaction with furfurol in the presence of strong acids. Proteins also tend to give a brown char with sulphuric acid. For these reasons it is advisable to remove the proteins from solution before attempting the test.

4. The purple colour obtained is only stable in the presence of strong sulphuric acid. It disappears on dilution with water.

5. If a small portion of the coloured fluid be diluted with 50 per cent. sulphuric acid, and examined with the spectroscope, two absorption bands will be seen, one between the lines C and D, nearer the latter; the other in the green, overlapping E and B.

6. The test cannot be applied directly to urine, owing to the presence of chromogenic substances that yield intense colours with sulphuric acid.

224. **Hay's test for bile salts.** Take 10 c.c. of the solution in a test tube. Sprinkle the surface with flowers of sulphur and note that they fall through the liquid to the bottom of the tube. Repeat the test with water, noting that the particles remain on the surface.

NOTES.—1. This test for bile salts depends on the remarkable property that they possess of lowering the surface tension of water, thus enabling the particles of sulphur to sink through the fluid.

2. The test is of great value for the detection of bile salts in urine.

3. This property of bile salts is utilised by draughtsmen in preparing tracings on oiled paper, on which ink collects in drops, and does not spread well. If the paper be first treated with a little ox-gall and allowed to dry the difficulty is removed, owing to the reduction in surface tension.

4. A method for estimating bile salts in urine has been described by Grünbaum, depending on this property. The rate of escape of the urine from standard capillary tubes is noted, the rate increasing with the concentration of bile salts.

225. **Oliver's test for bile salts.** Acidify 5 c.c. of the solution with two or three drops of strong acetic acid, filtering if necessary. To the acid solution add an equal quantity of 1 per cent. solution of Witte's peptone. A white milkiness or a decided precipitate is produced, insoluble in excess of acid.

NOTES.—1. The precipitate formed consists of a compound of protein with bile acids.

2. The test can be applied to urine. (Ex. 288.)

The Bile Pigments.

Bilirubin, $C_{32}H_{36}N_4O_6$, is a reddish-brown pigment most abundant in the bile of carnivora. It is readily oxidised by the oxygen of the air into biliverdin, $C_{32}H_{36}N_4O_8$, the green pigment found mostly in the bile of herbivora. These compounds are formed in the liver cells from the products of disintegration of haemoglobin. Haematin is $C_{32}H_{32}N_4O_4Fe$, and haematoporphyrin is isomeric with bilirubin.

They are weak acids, forming sodium and calcium salts, the latter being insoluble in water. Free bilirubin is soluble in ether and chloroform: the sodium compound is insoluble, as is free or combined biliverdin.

By oxidation bilirubin is converted, through a number of ill-defined bodies, such as bilicyanin, and bilifuscin, into choletelin, the end product of Gmelin's reaction.

By further oxidation a compound, haematinic acid ($C_8H_8O_5$), is formed, identical with the product obtained by the oxidation of haematin or haematoporphyrin.

By reduction with sodium amalgam in alcoholic solution the bile pigments are converted into hydrobilirubin, which is also formed by the action of more powerful reducing reagents on haematin or haematoporphyrin.

These facts all indicate the close relationship between haematin and the bile pigments.

In the bowel the bacteria first reduce bilirubin to hydrobilirubin. This is then attacked, two nitrogen atoms being probably removed, the result being the formation of stercobilin, which is mainly excreted in the faeces. But a small amount is absorbed and excreted in the urine as urobilinogen.

226. **Gmelin's test for bile pigments.** Take a few c.c. of fuming yellow nitric acid in a test tube and by means of a pipette, carefully place on the surface of this an equal amount of bile. Shake the tube very gently from side to side, and note the play of colours in the bile as it becomes oxidised by the acid. Proceeding from acid to bile the colours are yellow, red, violet, blue, and green.

NOTES.—This test can be modified in many ways.

1. Add a drop of yellow nitric acid to a thin film of bile on a white porcelain plate. The drop of acid becomes surrounded by rings of the various colours.

2. Filter some diluted bile repeatedly through an ordinary filter paper, and then place a drop of fuming nitric acid on the paper. The play of colours is usually well seen.

227. **Cole's test for bile pigments.*** To about 50 c.c. of diluted bile add an excess of baryta-mixture. Stir well, heat, and allow to stand for a short time. The precipitate, containing an insoluble barium compound of bilirubin, coheres together. Remove the main mass of the fluid by means of a pipette, and then filter. Open the filter paper on a tile and scrape the precipitate off the paper. Place it in a test-tube, add about 4 c.c. of strong alcohol, two drops of strong sulphuric acid, two drops of a 5 per cent. solution of potassium chlorate, and boil for a minute. Allow the precipitate of barium sulphate to settle. The supernatant alcohol is coloured a greenish-blue.

* An improved modification of this test will be found on p. 251.

NOTES.—1. If the precipitate obtained by the baryta-mixture is very slight, a small amount of sodium phosphate solution should be added to increase the bulk of the precipitate.

2. This test is especially valuable for detecting the presence of bile pigments in urine.

3. The test is a modification of one originally described by Huppert.

The Protein of Bile.

When bile is treated with acetic acid a precipitate is formed insoluble in excess. This was formerly thought to be mucin. But it has been shown that it is nucleoprotein, the bile salts present preventing the re-solution in strong acetic acid. (See Ex. 225.) In human bile, however, mucin is present as well as nucleoprotein.

The protein is secreted by the cells lining the ducts and the gall bladder, so that bile from the gall bladder contains a much greater percentage than fistula bile.

228. To a small quantity add strong acetic acid, drop by drop. A precipitate is formed, insoluble in excess of acid. This precipitate consists of a nucleoprotein, together with a considerable amount of the bile salts and bile pigments.

NOTE.—The protein precipitated was formerly supposed to be mucin, owing to the insolubility in excess of acid. It has, however, been shown that it is a nucleoprotein, the insolubility in excess of acid depending on the presence of the bile salts. (See Ex. 288.) It is generally spoken of as "pseudo-mucin."

Human bile contains both mucin and nucleoprotein.

Cholesterin. $C_{27}H_{43}OH$ or $C_{27}H_{45}OH$ is a monovalent alcohol found in the bile. It is present in nearly all the fluids and tissues of the body, notably in the central nervous system. It is found in large amounts in egg-yolk. In the blood plasma it is present as an ester, as it is in lanoline, the "fat" obtained from sheep's wool. We have already seen that it is a constituent of the envelope of red blood corpuscles (p. 103). It forms one of the varieties of gall stones, found after inflammation of the mucous membrane of the gall bladder.

It is soluble in ether, alcohol, chloroform, and acetone. It is only slightly soluble in cold, easily in hot alcohol. It is soluble in bile salts, insoluble in water, weak acids and alkalies. It crystallises from boiling alcohol in plates of a characteristic shape: from the other solvents in needles. It melts at 145°C., and in chloroform solution shows an optical activity $[\alpha]_D = -36{\cdot}6°$.

Its chemical constitution is not yet determined, but it probably belongs to the terpene series.

Preparation.—Sheep's brain is minced, ground with sand and intimately mixed with three times its weight of plaster of Paris. After some hours the hard mass is ground, and extracted three times in the mortar with acetone. The acetone extract is filtered and evaporated spontaneously. The cholesterin crystallises out, and can be recrystallised from hot alcohol.

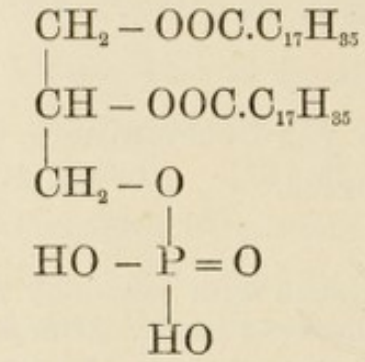
229. Mount a few crystals of cholesterin in water, examine under the microscope, and draw them. Note the rhombic plates, which are often incomplete at one corner. Irrigate the crystals with strong sulphuric acid: they turn red at the edges. Now add a drop of iodine solution: the crystals give a violet colour, changing to a green, blue, and finally a black.

230. **Salkowski's reaction for cholesterin.** Dissolve a little in a few c.c. of chloroform; to the solution add an equal quantity of strong sulphuric acid and shake. The upper layer of chloroform becomes red, the layer of sulphuric acid yellow with a green fluorescence.

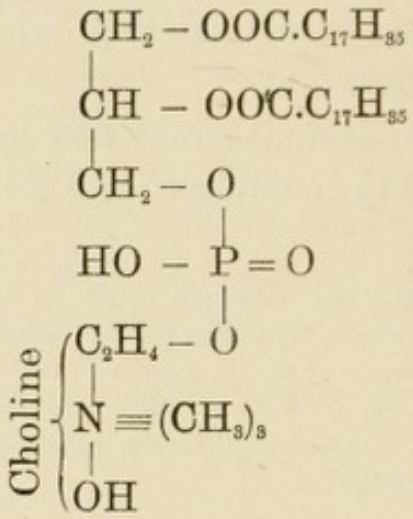
231. **Liebermann-Burchard reaction for cholesterin.** Dissolve a little cholesterin in 2 c.c. of chloroform, contained in a perfectly dry tube. Add ten drops of acetic anhydride, then two drops of strong sulphuric acid, and shake. The solution becomes coloured a deep blue.

Lecithin is a complicated fat-like body, generally found in the body and elsewhere with cholesterin. (See p. 121.)

It can be regarded as a compound of the base choline with esters of glycerophosphoric acid.



Di-stearyl glycerophosphoric acid.



Lecithin.

CHAPTER IX.

URINE AND ITS CHIEF CONSTITUENTS.

A. The average composition.

The composition of the urine varies with the individual and with the diet. Below are given the figures in grams for the daily output of

A. The average man on the average mixed diet.

B. An individual on a liberal diet.

C. The same individual on a diet deficient in proteins.

B. and C. are taken from a paper by Folin.

	A.			B.			C.		
		Nitrogen.	Per cent. of Total N. or S.		Nitrogen.	Per cent. of Total N. or S.		Nitrogen.	Per cent. of Total N. or S.
Urea	30	14	87·5	31·6	14·7	87·5	4·72	2·2	61·7
Ammonia	0·6	0·5	3·1	·6	0·49	3·0	·51	0·42	11·3
Creatinine	1·55	0·57	3·6	1·55	0·58	3·6	1·61	0·60	17·2
Uric Acid	0·8	0·23	1·4	·54	0·18	1·1	·27	0·09	2·5
Undetermined		0·7	4·4		0·85	4·8		0·27	7·3
Total—N		16·0	100·0		16·8	100·0		3·6	100·0
Inorganic SO_3	2·92		88·2	3·27		90·0	0·46		60·5
Ethereal SO_3	·22		6·6	0·19		5·2	0·10		13·2
Neutral SO_3	·17		5·2	0·18		4·8	0·20		26·3
Total SO_3	3·1		100·0	3·64		100·0	0·76		100·0

B. The Physical Chemistry of the Urine.

I. General Properties.

Normal human urine is a clear yellowish fluid, the depth of the tint depending largely on the concentration. On standing, a cloud (nubecula) of mucoid containing epithelial cells separates out. After a heavy meal urine may be passed cloudy, due to earthy phosphates and carbonates. On standing, these settle to the bottom of the vessel as a white deposit, insoluble on warming, but soluble in acids.

Also on standing a cloud of urates may settle as a reddish deposit that clears up on warming.

Fresh urine has a characteristic odour of the aromatic type, due to the presence of some substance that has not yet been recognised. On standing, an unpleasant ammoniacal odour develops as the result of bacterial decomposition.

II. The Specific Gravity.

Usually lies between 1012 and 1024 (water = 1000). With copious drinking it may fall to 1002. After excessive perspiration it may rise to 1040.

The determination of the specific gravity for clinical purposes is most conveniently made by means of a urinometer, a weighted cylinder that floats in the urine. The depth to which it sinks depends on the density of the fluid, and this can be read directly by means of a graduated scale on the stem. The instrument is calibrated for a certain temperature, usually 15° C.

The urine should be either cooled or warmed to this temperature, or a correction made by adding 1 unit for every 3 degrees above this, or subtracting 1 for every 3

degrees below the standard. Thus if the reading be 1018 at 18° C., the corrected Sp. Gr. is 1019.

To obtain the best results two separate instruments should be at hand, the one calibrated from 1000 to 1020 and the other from 1020 to 1040.

The total amount of solids in the urine can be roughly calculated from the specific gravity by Long's coefficient. The last two figures of the specific gravity × 2·6 gives total solids in 1000 c.c.

Thus specific gravity at 25° C. = 1017.

Total solids in 1000 c.c. = 17 × 2·6 = 44·2 gms.

Häser's coefficient (2·33) on a similar basis, but calculated for 15° C. is probably inaccurate.

232. Take the specific gravity of normal urine by means of a urinometer. Wipe the instrument clean, and float it in the centre of a cylinder containing the urine. Remove all froth, by means of filter paper or by placing a single drop of ether on the surface of the urine. Take care that the instrument does not touch the sides of the vessel. Place the eye level with the surface of the fluid and read the division of the scale to which the latter reaches. Read the level of the true surface of the urine, not the top of the meniscus around the stem.

Fig. 3.
Urinometer.

III. The Osmotic Pressure (Cryoscopy).

The freezing point of pure water is 0° C. That of solutions is lower than this, and the depression of the freezing point is proportional to the molecular concentration of the solution. In the case of electrolytes (salts, alkalies and acids) in aqueous solution it is proportional to the concentration of (molecules + ions), that is to the "osmotic cencentration."

Since the osmotic pressure of a solution is also proportional to the molecular or osmotic concentration of the solution, it follows that a determination of the depression of the freezing point (cryoscopy) enables us to get a measure of the osmotic pressure.

With non-electrolytes the gram-molecule in 1000 gms. of water causes a depression (Δ) of the freezing point of 1·85° C.

So that $\frac{\Delta}{1{\cdot}85}$ = molecular concentration.

With electrolytes, $\frac{\Delta}{1{\cdot}85}$ = osmotic concentration = concentration (molecules + ions).

The quantitative relationship between Δ and osmotic pressure is that a Δ of 0·001° C. = an osmotic pressure of 9·1 mm. mercury.

In urine the concentrations of certain substances, such as urea, are much greater than they are in the blood. The work done by the kidney in effecting this concentration can be calculated from a consideration of the osmotic concentration, *i.e.* Δ, of each substance in blood and urine. It is quite erroneous to imagine that the work done can be calculated from a knowledge of the total osmotic concentration of the blood and urine respectively.* But, at

* A full discussion of the subject will be found in Moore's article in "Recent Advances in Physiology" (p. 159).

the same time, the determination of Δ of the blood and of the urine secreted by each kidney in certain renal diseases may give us valuable information as to the relative activities of the two organs.

Δ of blood is about 0·55° C., the same as that of a 0·9 per cent. solution of sodium chloride.

Δ of urine varies considerably with the diet, volume of fluid taken and other conditions. For the mixed 24 hours urine of an average man it is usually about 1·2° C. The following values are of interest in this connection:—

Δ × volume of urine = molecular diuresis.

$\frac{\Delta}{\text{NaCl per cent.}}$ is of considerable pathological significance. It is fairly constant in health, varying between 1·25 and 1·6. It exceeds 1·7 in heart disease or in any condition that causes a retardation of the renal circulation. The only febrile condition in which it is less than 1·7 is malaria.

233. **The determination of the freezing point** by Beckmann's method. In the outer chamber (C) place a mixture of ice and water. Add saturated salt solution until the temperature falls to about 3° C. lower than the anticipated freezing point of the urine. During the course of the experiment the freezing mixture must be stirred occasionally by means of F, and ice or salt added to maintain its temperature within about 1° C. of the original.

In the tube A place enough distilled water to cover the bulb of the Beckmann thermometer D. This is graduated to 1/100th° C., and can be read by means of a magnifying glass to 1/1000° C. The thermometer must not touch the sides or bottom of the tube A. The tube B serves as an air jacket to A. Stir the water regularly by means of the platinum stirrer E. The temperature falls, and then after a time rises sharply, and remains steady for a con-

siderable time. The temperature to be read is the highest obtained at this rise. This is the freezing point (W) of distilled water.

Now replace the water by the urine, rinsing the tube out with it once or twice. Repeat the experiment and note the freezing point (F) as before. $W - F = \Delta$.

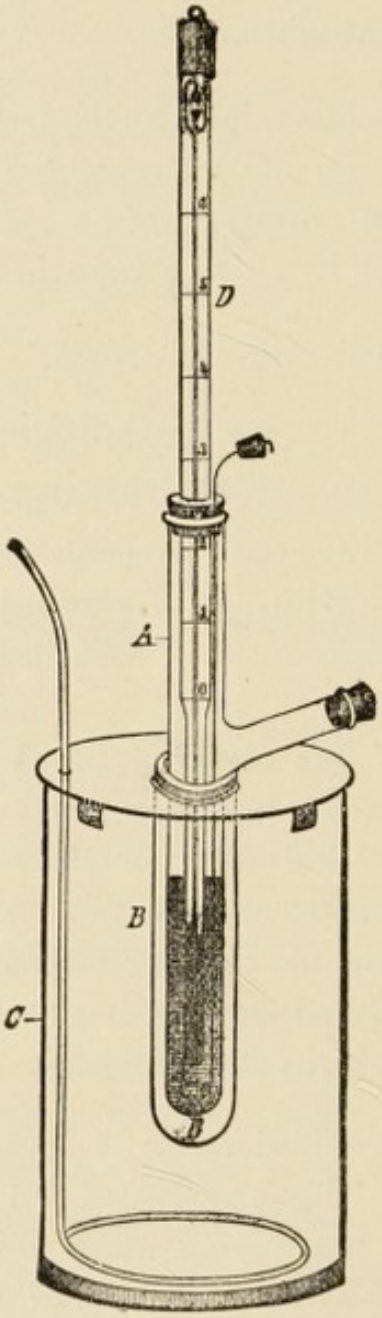


Fig. 4. Beckmann's freezing point apparatus.

Fig. 5. Beckmann's Thermometer.

NOTES.—1. It is of the utmost importance to take care to prevent too great a super-cooling of the urine. This should never exceed 1° C. If it has exceeded

this in a preliminary experiment, it must be repeated, and when the temperature has fallen 0·5° C. below the freezing point, a minute crystal of ice must be introduced through the side tube. These crystals are best prepared by taking in a dry test-tube, some hollow glass beads (that have been carefully dried) adding a small amount of urine, pouring off the excess of fluid, and immersing the tube in a freezing mixture. They should be introduced by means of a pair of cooled forceps.

2. The observed Δ is usually too great, owing to the super-cooling. The simplest correction is

$$\Delta \text{ corrected} = \Delta \text{ observed} \times \left(1 - \frac{C}{80}\right)$$

where C = the super-cooling in degrees.

3. To set the thermometer. Turn the thermometer upside down and by gentle shaking mix the mercury in the upper portion with that in the capillary tube. Then place the thermometer in water at about 2° C. Give a slight knock and thus break the mercury column. It is now ready for use.

4. When reading the thermometer during an experiment it should be tapped with a piece of indiarubber tubing.

IV. Acidity.

It has been shewn that in all aqueous solutions the product of the concentration of hydrogen ions (C_H^+) and that of the hydroxyl ions (C_{OH}^-) is constant. That is $C_H^+ \times C_{OH}^- =$ a constant.

In distilled water at 18° C. these concentrations are equal and are both $10^{-7\cdot07}$. So that the constant $= 10^{-7\cdot07} \times 10^{-7\cdot07} = 10^{-14\cdot14}$. In solutions of acids C_H^+ exceeds $10^{-7\cdot07}$ and C_{OH}^- is less than $10^{-7\cdot07}$, but the product of the two is always $10^{-14\cdot14}$.

Acids differ markedly in the degree to which they are ionised in solution. Thus in N/10 hydrochloric acid 91 per cent. of it is ionised. So C_H^+ is 0·091 N. Now $0\cdot091 = 9\cdot1 \times 10^{-2} = 10^{\cdot96-2} = 10^{-1\cdot04}$.

It is convenient to express this as $p_H = 1\cdot04$. That is, p_H is the logarithm to the base 10 of the concentration of

H ions in grams per litre, the negative sign being understood.

N/10 acetic acid is only dissociated to the extent of 1·3 per cent.

So C_H^+ is ·0013 N $= 1{\cdot}3 \times 10^{-3} = 10^{\cdot 11 - 3} = 10^{-2{\cdot}89}$

That is $p_H = 2{\cdot}89$.

An "indicator" is a substance that shows a change in colour when a certain amount of an acid or an alkali is added to it. At a certain stage of the addition there is an intermediate tint, and the solution is now said to be "neutral to that indicator." It must be clearly understood that this so-called neutrality does not necessarily correspond to an equality in the concentration of $\overset{+}{H}$ and $\overset{-}{OH}$ ions. Further, a solution that is neutral to one indicator may have a concentration of $\overset{+}{H}$ ions widely different from that in a solution that is neutral to another indicator. Thus a solution neutral to phenolphthalein has a p_H = about 9: one neutral to methyl-orange has p_H=about 4. The value p_H for any solution can be determined electrically by means of the potential set up between the solution and hydrogen. Further, it has been shown that if two solutions show the same tint with a given indicator at about the neutral point of this indicator, then these solutions have the same p_H. Sörensen has evolved a method of determining the true acidity of solutions based on this principle. The p_H is roughly found by the addition of various indicators. Then a series of solutions is prepared with known values of p_H. A certain indicator is added to each and to the solution. Those that have exactly the same tint have equal values of p_H.

For the details of the application of the method to urine, the student should consult a paper by G. S. Walpole, Bio-chemical Journal, Vol V., p. 207.

The range of certain indicators is given below.

	p_H		
Methyl violet	0·1	—	3·2
Tropaeolin OO	1·4	—	2·6
Di-methyl-amino-azo-benzene (Töpfer's reagent)	2·9	—	4·2
Methyl Orange	3·1	—	4·4
Methyl Red	4·2	—	6·3
p-Nitrophenol	5·0	—	7·0
Litmus	5·0	—	8·0
Neutral Red	6·8	—	8·0
Rosolic Acid	6·9	—	8·0
α-Naphtholphthalein	7·3	—	8·7
Phenolphthalein	8·3	—	10·0
Thymolphthalein	9·3	—	10·5
Tropaeolin O	11·1	—	12·7

Normal urine has p_H about 5, that is, it is acid to litmus and phenolphthalein, but alkaline to methyl orange.

The amount of N/10 sodium hydroxide that must be added to make the mixture neutral to phenolphthalein is sometimes called its "acidity." It would be better to call this the "titration acidity." For the method of its determination see Ex. 317.

The acidity of normal urine is due partly to the presence of acid phosphates, but largely to free organic acids.

C. The Pigments of Urine.

Urochrome is the chief pigment of normal urine. It is a yellow substance which has no definite absorption band. Nothing certain is known as to its constitution or origin, except that it is apparently not derived from the bile pigments. It has marked reducing properties.

Urobilin occurs in fresh normal urine as its chromogen, urobilinogen. This is converted into urobilin by acids or by the action of light and oxygen. The amount excreted is markedly increased in fevers, in diseases of the liver and bile passages, by destruction of the red corpuscles, especially in pernicious anaemia, and during the absorption of blood clots. In certain of these cases the urobilin itself is found in the urine, and can be identified by its characteristic absorption band, urobilinogen not giving a definite band.

Urobilinogen is a pyrrol body and is responsible for Ehrlich's reaction with p-dimethyl-amino-benzaldehyde.

The origin of urobilin from the bile pigments is discussed on page 119. It may be added that the urobilin absorbed from the bowel into the circulation is mostly excreted by the liver into the bile, so that only a small portion reaches the urine. Should the liver cells be injured there is a marked increase in the excretion of either urobilin or urobilinogen in the urine.

Uroerythrin is found in small amounts in normal urine. It is increased in fever and certain diseases of the liver.

It is soluble in amyl alcohol. Solutions have a reddish colour, but are unstable to light.

The pigment is usually associated with the urates or uric acid of the urine.

Haematoporphyrin is found in traces in normal urine. There is a certain increase in fevers, and some other diseases, but a very marked increase in certain cases of poisoning by sulphonal or trional, especially in women.

Urorosein occurs in urine as a chromogen which is converted into the pigment by the action of strong acids, such as HCl.

It is insoluble in ether and is thus distinguished from indigo blue formed in the test for indican. (Ex. 304.)

The chromogen seems to be an indol body, possibly indol-acetic acid.

234. Note the colour of normal urine and examine some in a beaker by the spectroscope. Note that there are no definite absorption bands, but a general absorption of the violet. Urochrome, the chief urinary pigment, yields no bands.

235. Saturate at least 200 c.c. of urine with ammonium sulphate. Filter off the precipitate and let it dry completely in the air. Extract it with a small amount of strong alcohol A brownish solution containing urobilinogen is obtained. Treat this with a few drops of hydrochloric acid : the urobilinogen is converted to urobilin. Examine with the spectroscope, and note a single absorption band situated at the junction of the blue and the green. Its centre is about λ 490.

D. The Inorganic Constituents.

Kations.

Sodium and potassium are found to the extent of 3·2 gm. K_2O and 5·23 gm. Na_2O per diem. The ratio $K_2O : Na_2O$ generally equals 1 : 1·54.

During starvation this can rise as high as 3 : 1, owing to the excretion of the potassium of the tissues, sodium being found in a much smaller amount than potassium. The same is found in all wasting diseases.

Calcium and magnesium are mainly excreted by the bowel. The amounts in urine are 0·33 to 0·6 gm. CaO and 0·16 to 0·24 gm. of MgO.

The amounts of these alkaline earths in the urine are increased by the administration of organic acids, or in conditions such as diabetes in which the formation of such acids is increased.

Iron also is mainly excreted by the bowel. It is found in human urine only in organic combination, and then only to the extent of 0·5 to 10 milligrams per diem.

Anions.

Chlorides form the chief part of the anions of the urine. The amount excreted is often calculated as if it all existed as NaCl, though the amount of sodium in the urine is normally not sufficient to combine with all the chlorine. The amount in the urine depends largely on the amount in the food, but since an important function of the kidney is to maintain a constant osmotic pressure of the tissue fluids, mainly by variations in the amount of NaCl excreted, it follows that anything tending to cause a change in the osmotic equilibrium in the body is liable to alter the excretion of chlorides in the urine.

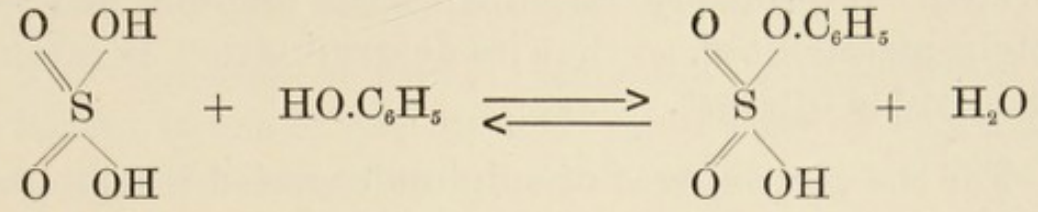
Thus during starvation and during the formation of exudates in pneumonia the chlorides may disappear from urine. The amount of Cl excreted per diem is about 7 gms. Reckoned as NaCl it is 12 grams.

For the method of estimation see Ex. 318.

Sulphates. Only a small portion of the sulphate excreted in the urine is taken in as such with the food. The greater portion is derived from the oxidation of sulphur containing substances, chiefly proteins. The amount of sulphates is thus a rough measure of the total amount of protein metabolised, the ratio $\frac{N}{SO_3}$ being usually $\frac{5}{1}$

Sulphates are excreted very rapidly after a protein meal, reaching a maximum about the third hour. This seems to indicate that cystine, the sulphur complex of proteins, is split off and absorbed very early in the digestion of proteins.

Ethereal sulphates are esters formed by the union of sulphuric acid with phenols.



Sulphuric acid. Phenol. Phenyl sulphuric acid.

The proportion of the sulphur that is present as ethereal sulphate varies considerably. Folin has shewn that in starvation and on diets relatively deficient in proteins the proportion increases, as does that of the "neutral" sulphur. There is also a marked increase after the administration of certain phenolic substances, or when such compounds are formed in the body by bacterial decomposition, as in intestinal obstruction and severe constipation. In such cases the phenols found conjugated with sulphuric acid are

$C_6H_5.OH$.....phenol
C_6H_4 $CH_3(1)$ .....p-cresol
$OH\ (4)$
} formed from tyrosine.

$C_8H_6N.OH$.....indoxyl, formed from tryptophane.

These bodies are poisonous. They unite with sulphuric acid, probably in the liver, to form the innocuous ethereal sulphates.

The ethereal sulphates form soluble barium salts, and can be separated from the inorganic sulphates by treatment with barium chloride and filtering. They are hydrolysed to the phenol and sulphuric acid by boiling with hydrochloric acid.

"Neutral" Sulphur. In urine there is always present a certain amount of sulphur in a form less oxidised than that of a sulphate. The exact nature of the compounds in urine containing sulphur in this form is not yet clear.

It is probable that the amount of "neutral" sulphur in the urine is independent of the total amount of sulphur excreted. It probably varies with the amount of tissue protein metabolised, so that its determination is often of considerable interest.

For the percentages of sulphur excreted in the three forms under different metabolic conditions see page 123.

For the methods of determination of the sulphur see Exs. 320-322.

Phosphates. The phosphates of the urine are present on the one hand as salts of the alkali metals and of ammonium; on the other, as salts of the alkaline earths, calcium and magnesium. About 3·9 grms. of P_2O_5 are excreted per diem in the urine. Phosphoric acid forms three series of salts. The formulae for that of sodium and calcium are

Normal phosphate, Na_3PO_4 : $Ca_3(PO_4)_2$.

Mono-hydrogen phosphate, Na_2HPO_4 : $CaH(PO_4)$.

Di-hydrogen phosphate, NaH_2PO_4 : $CaH_4(PO_4)_2$.

The three sodium salts and $CaH_4(PO_4)_2$ are soluble in water: the other two calcium salts are insoluble. The normal and mono-hydrogen phosphates are alkaline in reaction to litmus: the di-hydrogen phosphates are acid.

The phosphates of the urine are derived partly from the inorganic phosphates of the food, partly from the oxidation of phosphorus-containing substances of the food and tissues, such as nucleo-proteins, lecithins and phospho-proteins, and partly also from the phosphates of bone. The exact share played by these various compounds in forming the urinary phosphates is difficult to determine owing to the fact that a proportion of the phosphates, varying between 12 and 50 per cent., are excreted by the

bowel. In this connection it may be noted that alkaline phosphates of the food are more likely to be excreted in the urine than are earthy phosphates.

The excretion of varying amounts of phosphates by the kidney is one of the methods by means of which the reaction of the body fluids is maintained in equilibrium. An increased excretion is always seen in cases of acid poisoning and in the acidosis associated with diabetes.

As soon as the urine shews a certain grade of alkalinity, a precipitation of earthy phosphates takes place. This is sometimes known as phosphaturia, but it is not necessarily associated with an increase of phosphates in the urine. In the phosphaturia of juveniles it is probable that there is an excessive amount of calcium in the urine, due to a defective excretion of the large intestine.

A certain amount of phosphorus is found in the urine in an organic form, not as a phosphate. It may be present as glycero-phosphoric acid. The average daily amount is about 50 mgms.

For method of estimation see Ex. 319.

236. Test for **chlorides** by adding to about 3 c.c. of urine a few drops of pure nitric acid and 3 c.c. of a 3 per cent. solution of silver nitrate. An abundant curdy precipitate of silver chloride appears at once. If the chlorides are less in quantity, the solution merely becomes milky or opalescent.

NOTE.—If nitric acid is not added, urates might be precipitated by silver nitrate, especially if the urine be ammoniacal.

237. To a test tube nearly full of urine add a little strong ammonia and boil. A white flaky precipitate of the **phosphates of calcium and magnesium** is formed. Filter off the precipitate, wash with water, and dissolve in 5 c.c. of dilute acetic acid. Divide the solution into two parts. To one part add a solution of potassium

oxalate. A white precipitate is produced, showing the presence of **calcium** in the urine.

238. To the other portion of the solution add an equal bulk of strong nitric acid and about 5 c.c. of ammonium molybdate. Boil: a yellow crystalline precipitate is produced, showing the presence of **phosphates.**

NOTE.—Neutral urine is very apt to yield a precipitate of earthy phosphates on boiling, owing to the change of reaction due to the evolution of CO_2 (See notes to Ex. 9).

239. **To demonstrate the presence of acid-phosphates in urine.** Treat 5 c.c. of urine with an equal volume of 5 per cent. solution of barium chloride. Filter repeatedly through a small filter paper till the filtrate is clear. Treat the filtrate with a little baryta mixture and boil. Filter; dissolve the precipitate in nitric acid and boil the solution obtained with ammonium molybdate. The yellow precipitate shows the presence in the urine of acid phosphates, such as NaH_2PO_4.

NOTE.—Anyalkaline phosphate, Na_2HPO_4, present in the urine is precipitated by $BaCl_2$ as $BaHPO_4$. The acid phosphates remain in solution as $Ba(H_2PO_4)_2$. On the addition of the alkaline baryta mixture, the acid phosphate is converted into the insoluble alkaline phosphates of barium. If no precipitate is produced when the baryta-mixture is added, there are no acid phosphates present in the sample of urine.

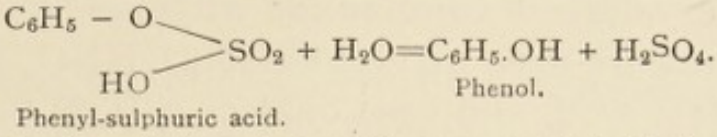
Since the acidity of a sample of urine varies almost directly with the amount of acid phosphates present, as determined by the above method, it is generally held that the acidity of urine is mainly due to the presence of these acid phosphates.

240. Treat 10 c.c. of urine with a few drops of strong hydrochloric acid, and about 3 c.c. of a solution of barium chloride. A precipitate of barium **sulphate** is produced as an opaque milkiness. If the precipitate is thick the sulphates are in excess. (The hydrochloric acid is added to prevent the precipitation of phosphates.)

241. **To demonstrate the presence of ethereal sulphates.** To urine add an equal bulk of baryta mixture (two parts of baryta water to one part of a 10 per cent. solution of barium nitrate). A precipitate is formed consisting of the phosphates and the ordinary *inorganic sulphates*. Filter till quite clear. To the filtrate add a

third of its volume of strong hydrochloric acid, boil in a beaker for five minutes, and allow to stand. A faint white cloud of barium sulphate is formed indicating the presence of *ethereal sulphates* in the urine.

NOTES.—1. The ethereal sulphates form soluble barium salts, but are hydrolysed to sulphuric acid by heating with an acid.



Phenyl-sulphuric acid. Phenol.

The sulphuric acid thus formed is converted into barium sulphate by the excess of barium present.

2. The solution becomes very dark in colour on boiling with the strong acid, owing to the action of the latter on the aromatic chromogenic substances in the urine.

E. Urea.

Urea is the compound in which the greater part of the nitrogen is normally excreted in man. The percentage of the urinary nitrogen in the form of urea varies. Normally it is about 90 per cent., but in starvation, or on a diet deficient in proteins, it is only about 60 per cent. It is also low in cases of diabetes accompanied by acidosis (owing to the relatively high percentage of ammonia), and also in certain cases of hepatic disorder, notably acute yellow atrophy of the liver, owing to the non-formation of urea by the disordered liver, its seat of formation in the body.

The total amount excreted per diem by a normal man on an average diet containing 100 grams. of protein is 30 grams.

Urea is also known as carbamide, since it is the diamide of carbonic acid.

Carbonic acid. Urea.

Urea crystallises in water-free, colourless, long needles, or in four-sided prisms of the rhombic system which melt and decompose at 130 – 132° C.

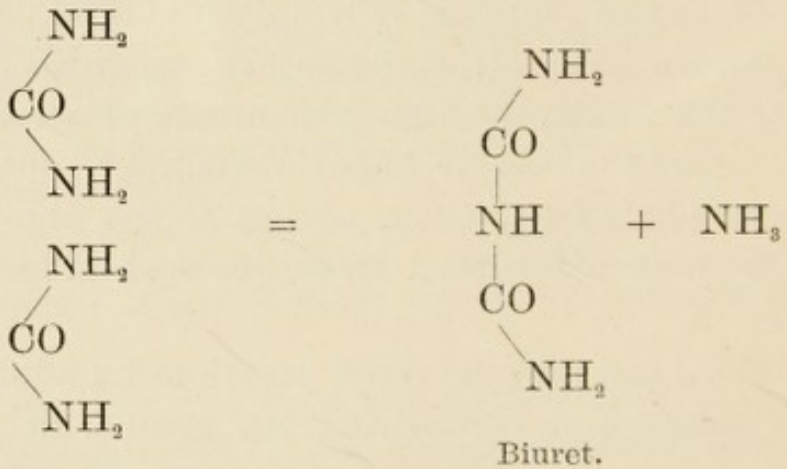
It is soluble in all proportions in hot water, and to the extent 1:1 in cold water. In cold alcohol it is soluble to the extent of 1:5. It is also soluble in acetone. Insoluble in pure ether and chloroform. The solutions are neutral in reaction.

It forms crystalline compounds with acids. The two most important are urea nitrate $CH_4N_2O.HNO_3$, insoluble in strong nitric acid, and urea oxalate $(CH_4N_2O)_2, C_2H_2O_4$, insoluble in oxalic acid.

It forms compounds with the salts of the heavy metals, especially with mercuric nitrate (see below, Ex. 250).

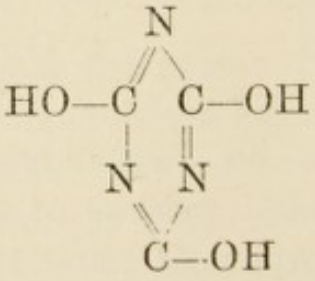
With reducing sugars relatively stable compounds are formed, called ureides. They are of importance in connection with the estimation of urea in diabetic urine.

On heating dry urea to 140° C., ammonia is evolved and biuret formed.



Biuret.

On heating beyond 140° C., cyanuric acid and ammonia are formed. Cyanuric acid is $C_3H_3N_3O_3$.



Solutions of urea are decomposed by boiling alkalies into CO_2 and NH_3. They are also similarly decomposed by heating for several hours at 150° C. with acids. This decomposition is readily effected by the addition of magnesium chloride, zinc sulphate or potassium acetate to the solution for the purpose of raising the boiling point.

Bacteria, as micrococcus ureae, decompose urea into CO_2 and NH_3. This accounts for normal urine rapidly becoming ammoniacal on standing in the air.

Nitrous acid decomposes urea as follows:—

$$CO(NH_2)_2 + 2HNO_2 = 2N_2 + CO_2 + 3H_2O.$$

Hypobromites effect a similar decomposition.

$$CO(NH_2)_2 + 3NaBrO = 3NaBr + CO_2 + N_2 + 2H_2O$$

Sodium hypobromite.

242. To a watch-glass half full of distilled water add as much solid urea as will lie on a sixpenny-piece. Note the solubility of urea in water.

243. Place a drop of the urea solution on a slide, add a single drop of a saturated solution of oxalic acid, mix by stirring with a needle or fine glass rod, cover with a slip and examine the crystals of *oxalate of urea* that separate out. They vary considerably, containing long, thin, flat crystals, often in bundles and rhombic prisms. Draw the crystals.

244. Dilute the urea solution with twice its volume of water. Place a drop on a slide, add a drop of pure nitric acid, cover with a slip, and examine the crystals of *urea nitrate* that separate out. They form octahedral, lozenge-shaped, or hexagonal plates, often striated and imbricated. Draw the crystals.

245. Powder two or three crystals of urea in a watch-glass: rub with a small amount of acetone and warm gently on a water bath. The urea dissolves. Allow most of the acetone to evaporate away, and then place a drop of the remaining solution on a watch-

glass. Urea crystallises out as the acetone passes off. Draw the crystals.

246. Repeat the above exercise, using strong alcohol instead of acetone. Draw the crystals of urea, which are usually very irregular.

247. Dilute the remainder of the aqueous solution left from Ex. 244 with an equal quantity of water, and to a portion of this in a test tube add some yellow nitric acid (or nitric acid to which a little potassium nitrite has been added). An effervescence and evolution of gas take place.

$$CO(NH_2)_2 + 2HNO_2 = CO_2 + 2N_2 + 3H_2O.$$

NOTE.—All compounds containing the amido group (NH_2) react in a similar manner when treated with nitrous acid.

248. To another portion of the solution add sodium hypobromite. A marked effervescence and evolution of gas take place.

$$CO(NH_2)_2 + 3NaBrO + 2NaHO = 3NaBr + Na_2CO_3 + 3H_2O + N_2.$$

249. To a few c.c. of saturated ammonium sulphate add sodium hypobromite. A marked effervescence and evolution of gas take place.

$$(NH_4)_2SO_4 + 3NaBrO + 2NaHO = Na_2SO_4 + 5H_2O + 3NaBr + N_2.$$

NOTES—1. All ammonium salts and all compounds with the amido group give off nitrogen when treated with an alkaline solution of sodium hypobromite.

2. The sodium hypobromite is prepared as follows: dissolve 100 grams. of caustic soda in 250 c.c. of water. Cool, and slowly add 25 c.c. of bromine, cooling under the tap as the bromine is added. The reaction is as follows:

$$2\,NaHO + Br_2 = NaBrO + NaBr + H_2O.$$

It must be freshly prepared before use as it undergoes the following decomposition

$$3\,NaBrO = 2\,NaBr + NaBrO_3.$$

3. As a test for urea the reaction with hyprobromite is only useful in a negative sense; that is to say, if an effervescence is not obtained urea is absent, but if an effervescence is obtained it does not necessarily follow that urea is present.

250. To some of the urea solution add a solution of mercuric nitrate. A white precipitate of mercuric oxide combined with urea

and mercuric nitrate takes place. To the mixture thus obtained add a saturated solution of sodium chloride, drop by drop. The precipitate dissolves, to reappear on a further addition of mercuric nitrate.

NOTES.—1. The precipitate consists of urea and mercuric nitrate and one, two or three molecules of mercuric oxide, depending on the concentration of the two solutions.

2. The solubility in NaCl is due to the formation of mercuric chloride, which is only very feebly ionised in neutral solutions.

3. The reaction is sometimes useful in detecting the presence of urea in solutions. Proteins give a precipitate with mercuric salts, which is soluble or insoluble in NaCl, depending on the nature of the protein. Therefore, to make the test more certain, proteins should be removed by the method given in Ex. 55. Since phosphates give a very similar reaction they must be removed by baryta mixture before testing for urea (See Ex. 255).

251. Treat a solution of urea with Millon's reagent, and heat. A white precipitate is formed, owing to the presence of mercuric nitrate in the reagent. There is also an evolution of gas due to the action of the nitrous acid on the urea.

252. Boil 1 c.c. of a dilute solution of urea with a little strong alkali for fifteen minutes. Cool, neutralise with diluted sulphuric acid and test for urea by the addition of mercuric nitrate. No precipitate is obtained owing to the hydrolysis of the urea by the boiling alkali.

$$CO(NH_2)_2 + H_2O = CO_2 + 2NH_3.$$

253. Place a little urea in a dry test tube. Heat carefully over a flame, keeping the upper part of the tube cool. The urea melts and evolves ammonia, whilst a white sublimate condenses on the cooler parts of the tube. Cool the tube, add a little water and shake. Pour the solution into another tube and treat it with an equal bulk of sodium hydroxide and a drop of copper sulphate. A pink colour is produced, due to the biuret formed from the urea.

254. Repeat the experiment, but heat more strongly till the melt solidifies and becomes opaque. Cool, add two or three c.c. of water, boil and filter whilst still hot. Divide the solution into two portions A and B. To A add a few drops of a solution of

barium chloride and a single drop of diluted ammonia. A white mass of barium cyanurate is formed on cooling.

To B add some ammoniacal copper sulphate solution and boil. On cooling an amethyst precipitate of copper ammonium cyanurate is deposited.

NOTE.—Preparation of ammoniacal copper sulphate. 1 per cent. copper sulphate is treated with *very* dilute ammonia till the precipitate that first forms *just* redissolves.

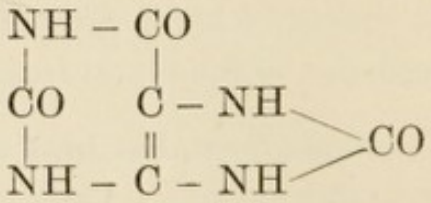
255. **To demonstrate the presence of urea in urine.** Treat 5 c.c. of urine with half its bulk of baryta mixture, and filter off the precipitate of sulphates and phosphates. Neutralise the filtrate with acetic acid and add a little mercuric nitrate. A white precipitate, soluble in sodium chloride, is obtained, indicating the presence of urea. (See Ex. 250.)

NOTE.—Phosphates must be removed, as they give a similar reaction.

256. **Isolation of urea from urine.** Evaporate about 30 c.c. of urine to complete dryness, finishing the evaporation on the water bath (to prevent the destruction of the urea). Turn out the flame and rub the residue with about 10 c.c. of acetone till it is boiling. Allow the acetone to boil, stirring all the time, till about half of it has evaporated away. Pour off the acetone into a dry watch glass and allow it to cool. Crystals of urea separate out as silky needles. Demonstrate that they are urea crystals by evaporating to dryness, taking up in a small amount of water and obtaining characteristic crystals of urea nitrate. (See Ex. 244).

F. Uric Acid.

Uric Acid, $C_5H_4N_4O_3$, is 2-6-8-tri-oxy-purine.



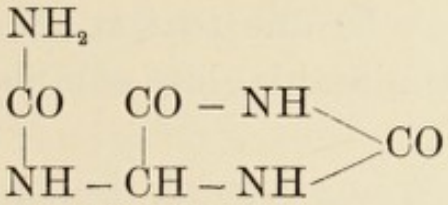
Its relationship to certain of the other purines is indicated on page 20.

When pure it crystallises in microscopic rhombic plates, but when impure it assumes a variety of forms, such as whetstones, dumb-bells, sheaves, rosettes, butchers' trays, etc.

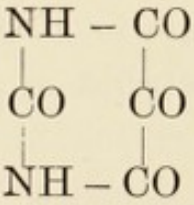
It dissolves to the extent of 1 part in 16,000 parts of cold water and 1600 parts of hot water. It dissolves in alkalies, and the alkali salts of carbonic, phosphoric, boric, lactic and acetic acids, but not in the ammonium salts of these acids. It dissolves in warm concentrated sulphuric acid to form a sulphate, which is decomposed by the addition of water.

It is precipitated by phosphotungstic acid in the presence of hydrochloric acid, slowly by lead acetate, and completely by picric acid, mercuric chloride and ammoniacal silver nitrate.

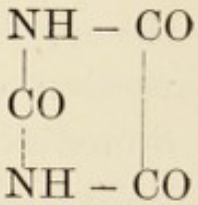
By oxidation, allantoin, alloxan, parabanic acid and urea are formed, depending on the reaction and the reagent employed.



Allantoin.



Alloxan.

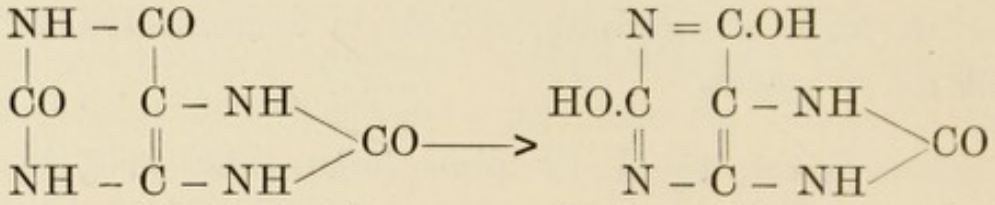


Parabanic acid.

Although the aqueous solutions of uric acid react neutral, it behaves like a disbasic acid $C_5H_2N_4O_3.H_2$ and can form two series of salts, $C_5H_2N_4O_3.Na_2$ (neutral, normal, or di-sodium urate) and $C_5H_2N_4O_3.HNa$ (biurate, acid urate or mono-sodium urate). It is also possible that there is a third form of salt, $C_5H_2N_4O_3.HNa.C_5H_4N_4O_3$ (quadriurate or hemi-sodium urate), though this may be merely a mixture of its two constituents. The di-sodium salts are more soluble than the mono-sodium, but are

only stable in markedly alkaline solutions. In the blood and urine urates exist as mono-sodium salts, which react neutral.

It is interesting to note that there are two modifications of the mono-sodium salt, called the α- and β-form. The α-form is more soluble than the β-form, but is unstable, and slowly passes over into the other form. They are probably the salts of the two tautomeric modifications of uric acid described by Fischer:



Lactam modification forming unstable α-urate.

Lactim modification forming stable β-urate.

It is of great interest to observe that in gout the amount of urate in solution in the blood is in excess of the amount of the β-urate that can be held by normal blood. So that in gout it must be present at least, partly, in the unstable α-form. The deposition of urates in the tissues during an acute attack may be due to the conversion of the unstable α- into the stable, less soluble β-modification.

Urates are completely precipitated as amorphous ammonium urate by saturation with ammonium chloride.

They exert a reducing reaction on Fehling's solution and towards alkaline silver solutions, this being the basis of Schiff's test.

They yield a characteristic colour reaction when evaporated with nitric acid, the so-called murexide test.

Uric acid occurs to the extent of about 0·7 gm. in the 24 hours' urine, but the amount excreted varies with the diet and the individual.

From its close chemical relationship to the purine bases formed by the hydrolysis of the nucleins of the food and tissues (see p. 20), the view is commonly held that uric acid has its origin in the cellular organs of the body from the oxidation of such substances. Thus we can have uric acid arising exogenously from the free or combined purines of the food and also endogenously from those of the tissues. This view is apparently supported by the fact that the administration of foods rich in nucleoproteins, as sweetbreads, or of certain of the pure purine bases, does cause an increased excretion of uric acid.

Plimmer has remarked on the close relationship between the elimination of uric acid and the number of leucocytes in the blood, and makes the suggestion that uric acid is a product of the metabolism of the leucocytes. This is not a revival of the old theory that it is formed by the disintegration of these cells.

It is important to note that a certain proportion of the uric acid formed in the body is destroyed by the liver, so that the amount excreted is a balance between that formed and that destroyed.

In gout, in which there is a deposition of uric acid in the tissues, the excretion is decreased before an acute attack, is increased during the attack, and then falls again. In this condition there is a recognisable amount of uric acid in the blood (see above). For methods of estimation in urine see Exs. 314, 315.

257. Treat a small amount of uric acid with 10 c.c. of 2 per cent. sodium carbonate. Heat nearly to boiling and cool. Note that a considerable portion of the uric acid has dissolved in the form of a urate.

258. Filter the solution and treat a portion with a drop or two of strong hydrochloric acid and shake. A white crystalline precipitate of uric acid separates out, showing that uric acid is very

insoluble in water. Allow the crystals to settle, remove a few by means of a pipette, and examine them microscopically. They usually form rhombic plates. Draw the crystals.

NOTE.—If the solution is very strong, the uric acid may separate out in an amorphous form. Should this be the case, make the solution alkaline and heat to dissolve. Whilst still hot add some HCl and allow the tube to cool slowly.

Uric acid can assume a great variety of crystalline forms, resembling dumb-bells, whetstones, butcher-trays, stars, and sheaves.

259. To another portion of the solution add two drops of ammonia and saturate with ammonium chloride. A white amorphous precipitate of ammonium urate is formed.

NOTE.—This is the basis of Hopkins' original method for the estimation of urates in urine. It is an important reaction for separating urates from physiological fluids, such as urine (see Ex. 268), since no other organic substance, likely to be met with in physiological analysis, is precipitated by saturation with ammonium chloride. The murexide reaction can be applied to the precipitate obtained.

260. Treat a little uric acid with a little strong sulphuric acid: it dissolves. Pour the solution into water: the uric acid may separate out.

261. **Murexide test.** Treat a little uric acid in a porcelain dish with two or three drops of strong nitric acid. Heat on the water-bath till every trace of nitric acid and water has been removed. A reddish deposit remains. Treat this with a dilute solution of ammonia (five drops of ammonia to about a test tube full of water). The residue turns reddish violet in colour. Add a little caustic soda. The colour turns to a blue-violet.

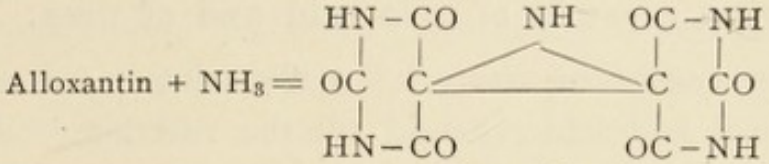
NOTES 1.—This important test needs a certain amount of care. The heating must be performed on the water-bath and should be continued as long as is necessary to ensure the complete removal of every trace of nitric acid.

2. Xanthine and guanine give a yellow substance (nitro-xanthine) when treated with nitric acid. On evaporation the colour goes to a violet shade, which turns yellow with dilute ammonia. Adenine and hypoxanthine give no colour reaction.

3. The chemistry of the reaction is as follows: From uric acid arises by oxidation dialuric acid and alloxan. They condense together to form

alloxantin. By the action of ammonia on alloxantin, purpuric acid is formed. Murexide is ammonium purpurate.

Dialuric acid Alloxan. Alloxantin.



Purpuric acid.

262. **Schiff's test.** Treat a very small amount of uric acid with a few c.c. of sodium carbonate. Pour the solution on to a filter paper moistened with silver nitrate. A black stain of reduced silver immediately results.

NOTE.—This useful test cannot be applied in the presence of chlorides. It is important to note that the uric acid is dissolved in sodium carbonate, not the hydroxide, as the latter gives a precipitate of the brown silver hydroxide, which completely obscures the reduction. An amount of sodium carbonate in excess of that required to dissolve the uric acid must be added, as the reduction only takes place in the alkaline condition.

263. **Folin's test.** To a *very* small pinch of uric acid in a beaker add 20 c.c. of a saturated solution of sodium carbonate. Stir till the uric acid has completely dissolved, add 1 c.c. of Folin's uric acid reagent. A blue colour is obtained.

NOTES.—**Preparation of Folin's solution.** 100 grams. of pure sodium tungstate, 102 c.c. of pure ortho-phosphoric acid (B.P. 66·3%) and 750 c.c. of distilled water in a flask fitted with a reflux condenser are boiled for 2 hours. On cooling the solution is diluted to 1 litre.

2. The solution also gives a blue colour with polyphenols. It is used for the microchemical estimation of uric acid in urine (Ex. 315).

264. Dissolve a little uric acid in sodium carbonate by boiling. Add 5 c.c. of Fehling's solution and boil for a considerable time. Note the peculiar reduction of the copper, and compare it with the reduction obtained with glucose.

265. Similarly try the effect of uric acid on Nylander's (Ex. 70) and Benedict's (Ex. 68) solutions. A reduction is not obtained.

266. Dissolve some uric acid in sodium carbonate, add an excess of ammonia and treat with silver nitrate. A white amorphous precipitate of a silver compound of uric acid is formed.

NOTE.—Xanthine, hypoxanthine and other substances in urine closely related to uric acid are similarly precipitated by ammoniacal silver nitrate.

267. **A solution of sodium urate and urea is provided. To prepare crystals of uric acid and of urea.**

Heat a test tube nearly full of the solution to boiling point and add strong hydrochloric acid till the reaction is distinctly acid. Allow the tube to cool slowly; the uric acid crystals separate out. Cool thoroughly under the tap. Filter off the uric acid. Neutralise the filtrate with sodium carbonate and evaporate to dryness, finishing the process on the water-bath, to prevent the conversion of the urea to biuret. (See Ex. 253.) Extract the residue with strong alcohol or acetone. The alcohol or acetone solution is carefully evaporated to dryness, and the urea crystallises out.

268. **To demonstrate the presence of uric acid in urine.**

Treat 50 c.c. of urine with two drops of ammonia and then stir with powdered ammonium chloride till the solution is saturated. Allow the excess of ammonium chloride to settle for 15 secs., and pour off into another beaker. Note the gelatinous precipitate of ammonium urate. Filter: scrape the precipitate off the paper and transfer it to an evaporating dish. Add three or four drops of strong nitric acid and place the dish on the water bath till a pink, dry residue is obtained. Treat this with a little dilute ammonia: the purple colour produced indicates the presence of urates in urine. (See Exs. 259 and 261.)

269. **Folin's method of demonstrating the presence of uric acid in urine.** To 1 to 2 c.c. (20 drops) of urine in an evaporating dish add one drop of a saturated solution of oxalic acid and evaporate to *complete* dryness on a water bath. Allow to cool, add 10 c.c. of strong alcohol and allow to stand for five minutes to extract the polyphenols. Carefully pour off the alcohol. To the residue add 10 c.c. of water and a drop or two of saturated sodium

carbonate. Stir to secure complete solution of the uric acid and transfer to a beaker. Add 1 c.c. of Folin's uric acid reagent (Ex. 263) and 20 c.c. of saturated sodium carbonate solution. The blue colour that results indicates the presence of uric acid.

270. Urine has been treated with about one-fiftieth its bulk of strong hydrochloric acid, and allowed to stand from twelve to twenty-four hours. Note the brown crystals of uric acid that have formed on the sides of the vessel. Examine them microscopically: they form very irregular crystals, usually arranged in sheaves. Draw the crystals.

NOTE.—The chief pigment that associates itself with uric acid and urates is known as uroerythrin. (See p. 132.)

G. Purine bases, other than uric acid.

The most important of these found in normal urine are hypoxanthine, xanthine and adenine (see p. 20), derived from the metabolism of food and tissue nucleins: heteroxanthine (7-methyl-xanthine) and paraxanthine (1, 7-dimethyl-xanthine) derived from the breakdown of caffeine (1, 3, 7-trimethyl-xanthine) and theobromine (3, 7-dimethyl-xanthine) of the coffee, tea and cocoa ingested.

In man the methylated xanthines constitute the greater part of these purine bases. But it is interesting to note that the non-methylated ones are much increased in fever. Also during severe muscular exercise there is an increase, accompanied by a decrease of uric acid. After the exercise there is an increase of uric acid, and a decrease of the other purines.

The simplest method of estimation is to determine uric acid nitrogen by the method in Exs. 314, 315, and the total purine nitrogen by applying Kjeldahl's method (Ex. 306) to the total purines precipitated by ammoniacal silver nitrate (Ex. 266). The difference is the nitrogen of the purine bases.

H. Creatinine and Creatine.

The chemical relationships of these bodies are described on p. 77. In normal human urine creatinine is always present, but creatine only after a meat diet, being derived from that of the food. Creatine, however, is a normal constituent of the urine of children.

Creatinine seems to be a product of tissue metabolism, and the amount excreted is regarded by Folin as a measure of endogenous metabolism. (See tables B and C, p. 123.) There is an increase in complete starvation and in fevers, due to the increased breakdown of the tissues. Mellanby has drawn attention to the fact that the liver is probably the seat of formation of creatinine. Thus in most diseases of the liver there is a decreased excretion, an important exception being hepatic carcinoma, in which condition the urinary-creatinine is increased and is accompanied by creatine. Creatine is excreted when the muscles of the body are broken down. This explains the presence of creatine in urine during starvation and in fevers.

When creatinine is given by the mouth it is mainly excreted unchanged, but a small portion is broken down into unknown products. When creatine is administered it also is chiefly excreted unchanged, but a certain percentage is destroyed in the body. The amount excreted unchanged is considerably increased with diets rich in proteins.

Properties. Creatinine dissolves in 11 parts of water and 102 parts of alcohol at 16° C. It is insoluble in ether. Its solutions are neutral or very slightly alkaline in reaction.

Creatinine is precipitated by phosphotungstic acid, by picric acid, and by the salts of the heavy metals.

Alkalies convert it slowly into creatine. On boiling with barium hydroxide it is converted into urea and sarcosine (see p. 77).

Creatinine reduces Fehling's solution, but not Benedict's or Nylander's solutions.

For the method of estimation see Ex. 316.

271. **Jaffé's test.** To 5 c.c. of urine add a few drops of a saturated aqueous solution of picric acid and of a 10 per cent. solution of sodium hydroxide. A red colouration is produced owing to the formation of picramic acid.

272. **Weyl's test.** To 5 c.c. of urine add a few drops of a freshly prepared 5 per cent. solution of sodium nitroprusside. Add a 5 per cent. solution of sodium hydroxide, drop by drop. A ruby-red colour appears, which quickly turns yellow.

NOTE.—Acetone gives a similar red colour, but it does not turn yellow.

273. **Salkowski's test.** Heat the yellow solution obtained in the preceding exercise; add an excess of acetic acid and boil. A greenish blue colour results. On standing, a sediment of Prussian blue may separate.

I. Ammonia.

Ammonia is a constituent of normal urine, being present to the extent of about 0·7 gm. per diem. There is an increased excretion following the administration of ammonium salts of inorganic acids, in certain cases of hepatic disease, and as a result of acid poisoning. This last condition ("acidosis") can be produced by the administration of inorganic acids or by the excessive formation of acids in the body, especially if this is not accompanied by an increased intake of alkalies. Thus it is seen in severe diabetes, in starvation, and in delayed chloroform poisoning, the acids formed being aceto-acetic and β-oxy-butyric acids.

For methods of estimation see Exs. 308 to 310.

J. Hippuric Acid.

Hippuric acid is formed in the kidney by the condensation of benzoic acid with glycine.

$$C_6H_5.COOH + H_2N.CH_2.COOH = C_6H_5.CO.NH.CH_2COOH + H_2O$$

Benzoic acid. Glycine. Hippuric acid.

The amount excreted by a normal individual on a mixed diet is about ·7 gm. per diem. It is increased by a vegetable diet, owing to the presence in most plant foods of an aromatic complex that is oxidised to benzoic acid in the body.

Hippuric acid crystallises in 4-sided prisms, somewhat resembling triple phosphate. It melts at 187·5° C.: above this temperature the melt becomes red and is decomposed into benzoic acid, benzonitrile and prussic acid. It is soluble in hot water, alcohol and ethyl acetate: insoluble in benzene and petroleum ether: only slightly soluble in cold water, alcohol, ether and chloroform. It forms an insoluble ferric salt. By hot acids or alkalies it is hydrolysed to benzoic acid and glycine. When evaporated with strong nitric acid, nitrobenzene is formed.

274. **Isolation from urine by Roaf's method.** 500 c.c. of the urine of a horse or cow are treated with 125 grams of ammonium sulphate and 7·5 c.c. of concentrated sulphuric acid. On standing for 24 hours the hippuric acid crystallises out. Filter off the crystals, and wash with a little cold water. Dissolve in a small amount of hot water, boil with a little animal charcoal, filter, concentrate if necessary, and allow to stand for 24 hours.

275. To a little hippuric acid in a small evaporating dish add 1 to 2 c.c. of concentrated nitric acid and evaporate to dryness in a water-bath in the fume chamber. Transfer the residue to a dry test tube, apply heat, and note the odour of nitrobenzene (artificial oil of bitter almonds).

276. Neutralise a solution of hippuric acid with dilute caustic soda. Add a few drops of ferric chloride. A cream-coloured precipitate of the ferric salt of hippuric acid is formed.

K. Certain Constituents of Abnormal Urine.

1. Albumin and Globulin.

"Albuminuria" is the name given to the condition in which a heat-coagulable protein is found in the urine, no matter whether the protein present is albumin or globulin. As a rule both proteins are present, but albumin is generally greatly in excess of the globulin.

Albuminuria can be renal ("true") or accidental ("false"). Renal albuminuria can be brought about by an alteration in the blood pressure in the kidney, by a change in the composition of the blood, or by an alteration in the structure of the kidney. In accidental albuminuria, the protein is not passed by the kidney, but gains access to it lower down in the urinary tract. It is generally accompanied by haemoglobinuria.

For the method of estimating the albumin see Exs. 323, 324.

277. **Boiling test.** Filter the urine till it is clear. If it will not filter clear, as when infected with bacteria, shake with kieselguhr and filter again. If the urine be alkaline to litmus, make it faintly acid by the cautious addition of 1 per cent. acetic acid. Fill a narrow test tube three parts full with the clear urine, incline it at an angle and boil the upper layer by means of a very small flame. A turbidity indicates either albumin or earthy phosphates (see note 2 to Ex. 9). Add one or two drops of strong acetic acic, boiling after the addition of each drop. Any remaining turbidity indicates the presence of albumin.

278. **Heller's test.** Place about 3 c.c. of pure nitric acid in a narrow test tube. Float about 3 c.c. of filtered urine on the surface of this, using a pipette to avoid mixing. A white ring at the junction of the fluids indicates the presence of albumin.

NOTES.—1. The white ring is due to the formation of metaprotein by the action of the acid on the albumin, and the insolubility of the metaprotein in the strong nitric acid. (See Exs. 1, 13 and 42).

2. A coloured ring is usually produced owing to the oxidation of certain urinary chromogens.

3. In very concentrated urine, a white ring of urea nitrate may form. It usually has very sharply defined borders.

4. If the urine is very rich in urates, a precipitate of uric acid may form at the junction of the fluids, or, more commonly, somewhat above the nitric acid. Urea and uric acid are distinguished from albumin by the previous dilution of the urine with two or three volumes of water.

5. The presence of resinous substances in the urine of patients who have been treated with balsams leads to the development of a white ring or cloud that disappears on treatment with alcohol.

6. Urine rich in albumose may give a white cloud that disappears on warming.

7. Urine that has been preserved by the addition of thymol gives a ring of nitrosothymol or nitrothymol. The thymol can be removed by gentle agitation with petroleum ether.

279. **Roberts' test.** Repeat the previous exercise, using Roberts' reagent in place of the nitric acid. A white ring at the junction of the fluids indicates albumin.

NOTES.—1. Roberts' reagent is prepared by adding 1 volume of pure nitric acid to 5 volumes of a saturated solution of magnesium sulphate.

2. Coloured rings are not formed, and so confusion is avoided.

280. **Spiegler's test.** Render the urine faintly acid with acetic acid and repeat the above test, using Spiegler's reagent in place of Roberts'. A white ring indicates the presence of albumin.

NOTES 1. Spiegler's reagent consists of

Mercuric chloride	40 gm.
Tartaric acid	20 gm.
Glycerine	100 gm.
Sodium chloride	50 gm.
Distilled water	1000 c.c.

2. The reaction is also given by albumoses and peptones.

3. The test serves to show 1 part of albumin in 250,000. It is almost too delicate for ordinary clinical work, as a large number of apparently normal urines give a positive reaction.

2. Albumoses.

Albumoses are found in the urine in certain cases of degeneration of the intestinal epithelium ("alimentary albumosuria"). Also in a variety of other conditions such

as in the absorption of pneumonic exudates, in some cases of an increased breakdown of the tissues in certain fevers, in the puerperium, and in urine containing semen.

The albumose present seems to be a secondary albumose.

281. Remove any albumin that may be present by heat coagulation. To the filtrate apply Spiegler's test (Ex. 280). A white ring indicates the presence of albomose.

3. *Bence-Jones' Protein.*

In certain cases of disease of the bone marrow (multiple myeloma), and possibly in osteomalacia, a protein with peculiar properties is found in the urine. It is named after Bence-Jones, who first described the condition. It has the property of coagulating at temperatures under 55° C., of redissolving to a clear solution on boiling and of reappearing on cooling. It is precipitated by half-saturation with ammonium sulphate. It is not precipitated on dialysis.

Hopkins has shewn that the solution of the heat coagulum on boiling depends on the presence of neutral salts, those with divalent cations (as $CaCl_2$) being most potent in neutral or faintly acid solutions, and those with divalent anions (as K_2SO_4) in faintly alkaline solutions.

Hopkins has also shewn that the protein excreted is formed in the body, either in the marrow or as a result of the influence of the growth on general metabolism. The amount in the urine is independent of the nature or amount of the proteins of the food. The nitrogen of the protein excreted may be as high as one-third of the total urinary nitrogen.

282. If necessary make the suspected urine faintly acid with acetic acid. Heat carefully by immersing in a beaker of warm

water. The urine becomes turbid at 40° to 45°C., and shows a flocculent precipitate at 60°C. On raising the temperature to 100°C., the precipitate partially or completely disappears. On cooling it reappears.

4. Blood Pigments.

Blood pigments may occur in pathological urine in intact corpuscles ("haematuria") or free in solution ("haemoglobinuria").

Haematuria can be recognised by determining the presence of red corpuscles by a microscopic examination of the sediment obtained by centrifugalising the urine. It occurs with gross lesions of the kidney or any part of the urinary tract, so that blood passes directly into the urine. If the blood comes from the kidney it is well mixed with the urine. If the blood comes from the bladder or genital organs it often forms a clot. In haematuria the urine often has a characteristic smoky appearance, and it is always associated with albuminuria. Haemoglobinuria is a result of haemolysis. It therefore follows a variety of infectious diseases, transfusion of blood, the absorption of haemolytic substances, such as many aromatic compounds, severe burns and scalds. Methaemoglobin is nearly always present.

283. **Heller's test.** Boil 10 c.c. of urine with a little 40 per cent. sodium hydroxide, and allow the tube to stand for a while. A red deposit indicates the presence of blood-pigment in the urine. Pour off the supernatant fluid and acidify with acetic acid. The precipitate dissolves only partially, leaving a red residue.

NOTES 1.—The alkali converts the pigment into haematin, which is precipitated with the earthy phosphates.

2.—Certain substances, such as cascara sagrada, rhubarb, senna and santonin cause the urine to give a similar red precipitate when boiled with alkali. But in these cases the precipitate dissolves completely in acetic acid.

284. **Schumm's spectroscopic test.** Treat 50 c.c. of the urine with 5 c.c. of glacial acetic acid and 50 c.c. of ether. Shake

thoroughly in a separating funnel. Allow to stand and add a drop or two of alcohol to obtain a separation of the layers. Run off the urinary layer. To the ether add 5 c.c. of water, shake and run off the water. To the washed ether add ammonia and shake for half a minute, cooling under the tap. The reaction must be markedly alkaline after shaking. Run off the lower coloured layer into a tube, add 5 to 10 drops of ammonium sulphide solution and examine spectroscopically for the bands of haemochromogen. (Ex. 219.)

285. **Adler's benzidine test.** To a saturated solution of benzidine in alcohol or glacial acetic acid add an equal bulk of 3 per cent. hydrogen peroxide and 1 c.c. of the urine. If the mixture is not acid, render it so by the addition of acetic acid. The appearance of a green or blue colour indicates the presence of blood pigment.

NOTES.—1. A control test should be performed, substituting water for the urine.

2. The reaction can be applied to the acid ethereal solution prepared in the preceding exercise.

3. Benzidine preparations vary considerably in sensitiveness. The solutions must be kept in the dark.

5. *Bile.*

The constituents of the bile are found in urine when the bile duct is obstructed by a calculus or by catarrh. The bile is absorbed into the lymphatics, passes into the circulation and reaches all parts of the body, the pigments causing a staining of the various tissues. The condition is known as jaundice.

The absence of bile salts from the urine does not exclude the possibility of the presence of bile pigments. With continued obstruction of the bile passages the formation of bile salts seems to decrease. Urine containing bile often has a characteristic appearance.

286. **Cole's test for bile pigments.*** To 25 c.c. of urine add baryta mixture and proceed as directed in Ex. 227.

* See p. 251.

287. **Hay's test for bile salts.** Sprinkle the surface of some urine in a test tube with flowers of sulphur. The particles fall to the bottom of the tube if bile salts are present. (See Ex. 224.)

288. **Oliver's test for bile salts.** Acidify the urine with acetic acid and filter if necessary. To it add a clear 1 per cent. solution of Witte's peptone, also acidified with acetic acid. A white precipitate indicates bile salts. (Ex. 225.)

289. **Jolle's test for bile salts.** Treat 50 c.c. of urine with 15 c.c. of a 3 per cent. solution of casein, add 10 per cent. sulphuric acid, drop by drop, with continued stirring until the casein is completely precipitated (·6 to ·8 c.c. usually required). Filter, and treat the precipitate in a small beaker with 10 c.c. of strong alcohol. Allow to stand for 1 hour at room temperature, stirring frequently. Filter and treat 5 c.c. of the filtrate with one drop of a 5 per cent. solution of rhamnose and 5 c.c. of concentrated hydrochloric acid. Boil over a small flame, and keep gently boiling for about two minutes. Cool, add 2 c.c. of ether and shake. A characteristic green fluorescence indicates the presence of bile salts.

*6. Glucose.**

Glucose seems to be a constituent of normal urine, but the amount present is very small (0·01 to 0·04 per cent.). When present in recognisable quantities the condition is known as glycosuria.

There are two types of glycosuria, alimentary and persistent. Alimentary glycosuria is the condition in which the amount of sugar absorbed exceeds the amount that the individual is capable of assimilating. The limit varies with the individual, and is affected by a variety of pathological conditions. Persistent glycosuria is the condition when large amounts of sugar are excreted for a considerable length of time, and may be quite independent

* The Author's recent method is described on p. 249.

of the administration of carbohydrate food. The condition is known as diabetes mellitus. The urine is generally much increased in amount, of a high specific gravity, and pale in colour.

The classical test for sugar in urine is Fehling's (Ex. 67). It is not a reliable test. Not only is Fehling's solution reduced by certain constituents of normal urine, such as urates and creatinine; but also certain of these bodies, notably creatinine, form soluble compounds with cuprous oxide, and thus markedly interfere with the delicacy of the test. Further, glucose is destroyed by boiling with caustic soda, so that the presence of a small amount of sugar may escape detection.

Benedict's test (Ex. 68) is a great improvement. Owing to the substitution of sodium carbonate for sodium hydroxide it is not reduced by urates or creatinine, and it does not destroy small quantities of sugar.

Nylander's test (Ex. 70) is also valuable. The reagent is not reduced by creatinine or uric acid. But certain substances of unknown composition that are occasionally found in urine cause a slight reduction. If a negative reaction is obtained the urine may be regarded as free from sugar in a clinical sense. But should a positive reaction be obtained this may be due to some other substance.

The osazone test serves to confirm the presence of sugar in doubtful cases, and especially to distinguish between glucose on the one hand and lactose and pentoses on the other. The fermentation test is also valuable, especially in connection with the recognition of lactose and glycuronic acid.

If proteins are present they must be removed by boiling in faintly acid solution before performing the sugar tests.

290. **Benedict's test.** To 5 c.c. of Benedict's reagent (see Ex. 68) in a test tube add eight drops of the urine. Boil vigorously for two minutes and allow to cool spontaneously. If glucose is present the entire body of the solution will be filled with a precipitate which may be red, yellow or green in colour, depending on the amount of sugar.

291. **Nylander's test.** To 2 c.c. of Nylander's reagent (see Ex. 70) in a test tube add 10 c.c. of urine (free from protein). Boil and immerse the tube in a beaker of boiling water for 5 minutes. The presence of sugar is indicated by a black colouration or precipitate of bismuth.

NOTES 1.—Rusting claims that the addition of a couple of drops of chloride of platinum ($PtCl_4$) increases the delicacy of the test.

2.—Bohmansonn suggests removing urochrome and certain other interfering substances by the following method: Treat 10 c.c. with 2 c.c. of 25 per cent. HCl and a small teaspoonful of animal charcoal. Shake at intervals for five minutes and then filter. Neutralise the clear, colourless filtrate with caustic soda.

292. **Fehling's test.** Boil 5 c.c. of Fehling's solution (see Ex. 67) to ascertain whether the Rochelle salt has been decomposed into reducing substances. If no reduction occurs add an equal volume of the urine (previously freed from proteins) and boil for a short time. A red or yellow precipitate indicates the presence of glucose.

NOTES 1.—This test is satisfactory for the demonstration of a considerable quantity of glucose (see small print above).

2.—Chloroform must not be used as a preservative if this test be used. Chloroform is converted into formic acid, a reducing substance, on being boiled with sodium hydroxide.

293. **Phenylhydrazine test.** Treat 10 c.c. of the urine with a few drops of lead acetate and filter. To the filtrate add 5 or 6 drops of strong acetic acid, enough phenylhydrazine hydrochloride to cover a sixpenny piece, and twice this bulk of solid sodium acetate. Dissolve by the aid of heat and filter. Place the filtrate in a tube and immerse this in a boiling water-bath for 30 to 60 minutes. Turn out the flame and allow the tube to cool to room temperature without removing it from the bath. Examine the deposit microscopically for the characteristic crystals of phenylglucosazone. (See Ex. 73.)

NOTES 1.—In doubtful cases 50 c.c. of urine should be used, with a corresponding increase in the amount of the reagents. The precipitate is collected on a small paper and dissolved in a small quantity of hot alcohol. The hot alcoholic solution is treated with boiling water, drop by drop, till a turbidity is produced. The solution is then placed in a boiling water bath to drive off the alcohol. On cooling the osazone is always crystalline. It can be dried and identified by taking its melting point.

294. **Cipollina's test.** Place 5 drops of pure phenylhydrazine (the base) in a test tube. Add ·5 c.c. of glacial acetic acid and 4 c.c. of urine. Heat the mixture over a low flame, shaking continuously to avoid bumping, and keep it boiling for 1 minute. Add 4 to 5 drops of 40 per cent. sodium hydroxide, keeping the reaction acid,

heat for a moment and then cool. Crystals of the osazone usually form at once.

295. **Fermentation test.** Fill a test tube with urine and then transfer the fluid to a mortar. Add a piece of washed yeast about the size of a bean and pound it up with the urine. Transfer the mixture to the test tube and invert, placing the open end under mercury or urine contained in a small dish. Clamp the tube in position, and allow it to stand for at least eighteen hours in a warm place. If glucose is present in the urine there is an accumulation of gas (CO_2) at the top of the tube.

NOTES 1.—Lactose, pentoses and glycuronic acids are not fermented by pure yeast.

2.—A special apparatus called Einhorn's saccharometer has been devised to enable the test to be applied conveniently. Also the volume of CO_2 formed, and the percentage of glucose present can be roughly determined by means of it.

7. Fructose (laevulose).

Fructose occasionally occurs in the urine, sometimes being accompanied by glucose. The significance of fructosuria is not yet clear.

296. **Seliwanoff's test** (Borchardt's modification). To a few c.c. of urine in a test tube add an equal volume of 25 per cent. hydrochloric acid and a speck or two of resorcin. Heat to boiling, cool under the tap, and transfer to an evaporating dish. Make the reaction alkaline by means of solid sodium hydroxide and return it to a test tube. Add 3 c.c. of acetic ether (ethyl acetate) and shake. A yellow colouration in the acetic ether indicates the presence of fructose.

8. Pentoses.

Pentoses, that is carbohydrates with 5 carbon atoms, appear in the urine in three conditions, alimentary, persistent or true pentosuria, and admixed with glucose in cases of glycosuria.

Alimentary pentosuria is sometimes seen after the ingestion of considerable quantities of certain fruits, as

prunes, cherries, grapes and plums. The sugar found varies, but is usually *d*-arabinose. In true pentosuria it is *dl*-arabinose. Its origin and significance have not yet been clearly established.

The presence of pentoses in urine is indicated when Nylander's reaction gives a grey and not a black precipitate: when Fehling's test shows a very slow reduction that often occurs quite suddenly as the mixture cools, and when the fermentation test is negative. The two colour reactions described are also given by glycuronic acid, which can, however, be demonstrated by Ex. 303.

297. **Tollen's test.** To 5 c.c. of urine add an equal volume of strong hydrochloric acid and a little phloroglucin (a piece about the size of a pea) and heat the mixture on a boiling water bath. A cherry-red colour develops and the solution shows an absorption band between D and E. On cooling a dark precipitate separates out. On dissolving this in strong alcohol, the alcoholic solution shows the colour and absorption band of the original mixture.

*298. **Bial's orcin test.** To 2 – 3 c.c. of urine add 4 – 5 c.c. of Bial's reagent and heat till boiling commences. A green colour or the formation of a green precipitate indicates pentoses. The solution shows two absorption bands, one in the red between B and C and the other near the D line.

9. *Lactose.*

Lactose is found in the urine of women during pregnancy, during the nursing period, and soon after weaning. The amount in the urine varies, but rarely exceeds 1 per cent. The excretion usually reaches its maximum 2 to 4 days after parturition.

It is not easy to demonstrate the presence of lactose in urine very satisfactorily. Barfoed's test is not applicable, owing to the fact that the reagent is reduced by the constituents of normal urine.

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* For Bial's Reagent see p. 252.

The osazone cannot be isolated with any certainty, owing to its solubility. Should a marked reduction occur, and if osazone crystals cannot be obtained, the fermentation test should be applied, using pure yeast that has been tested against lactose. If this be negative, then the sugar present is either lactose or a pentose. Should the tests for pentoses yield negative results, lactose is indicated. Its presence can be confirmed by obtaining crystals of mucic acid, which is yielded only by lactose or galactose.

299. **Mucic acid test.** 100 c.c. of the urine and 20 c.c. of pure concentrated nitric acid are evaporated in a wide and rather shallow beaker on a boiling water bath in a fume chamber. The evaporation is continued until the fluid becomes clear, and brown fumes are no longer evolved. The total volume is then about 20 c.c. Remove the beaker from the bath and transfer the contents to a smaller beaker, washing out with a small amount of distilled water. Allow to stand overnight in a cool place. The formation of a white crystalline mass of mucic acid indicates the presence of lactose in the urine. Dilute the fluid, collect the crystals on a small filter and wash with cold water. Microscopically the crystals are seen to be very pointed prisms with oblique angles. The melting point is 213° – 215° C. It can be weighed and titrated with standard alkalies, its equivalent weight being 105.

NOTE.—Mucic acid is $COOH.(CHOH)_4COOH$.

*10. The Acetone bodies.**

The acetone bodies found in urine in the condition known as "acidosis" are

Acetone. $CH_3.CO.CH_3$.

Aceto-acetic acid. $CH_3.CO.CH_2.COOH$.

β-oxy-butyric acid $CH_3.CH(OH).CH_2.COOH$.

β-oxy-butyric acid is readily oxidised to aceto-acetic acid, and this is converted into acetone by the loss of CO_2.

* For methods of Estimation see p. 241.

The two acids are never found in urine unaccompanied by acetone: but acetone may be present without the acids. The excretion of the acetone bodies depends on the inability of the tissues to oxidise completely the fatty acids generally derived from the fats, but sometimes from certain of the amino-acids formed in the metabolism of proteins. The condition that usually gives rise to acetonuria or acidosis is the inability of the tissues to obtain or to utilise an adequate amount of glucose. Thus these acetone bodies are excreted in starvation, on a diet of fats with a limited amount of protein, in certain fevers, severe anaemias, and after phosphorus poisoning, and finally in diabetes mellitus, in which condition the tissues are unable to utilise the glucose provided.

300. **Rothera's test for acetone.** To 10 c.c. of the urine add an excess of solid ammonium sulphate, so that the urine is completely saturated. Then add two or three drops of a freshly prepared 5 per cent. solution of sodium nitroprusside and 2 or 3 c.c. of concentrated ammonia. Mix and allow to stand undisturbed for at least thirty minutes. A characteristic permanganate colouration, that may only develop above the layer of undissolved crystals, indicates the presence of acetone.

301. **Gunning's iodoform for test acetone.*** To 200 c.c. of urine, add a few drops of 25 per cent. hydrochloric acid and distil over about 20 c.c., using an efficient condenser. To 5 c.c. of the distillate add five to ten drops of 10 per cent. ammonia and about five drops of a solution of 1 part of iodine and 2 parts of ammonium iodide in 100 parts of water. A black precipitate of nitrogen iodide is formed, which is converted on standing into iodoform. Examine the sediment microscopically for the characteristic yellow crystals of iodoform (hexagonal plates and rosettes).

NOTES 1.—This test is not given by alcohol or aldehyde.

2.—Aceto-acetic acid is converted into acetone on boiling, and so will also give the test.

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* See also p. 250.

302. **Gerhardt's test for aceto-acetic acid.** A. To 5 c.c. of the urine in a test-tube add ferric chloride solution, drop by drop, till no further precipitate of ferric phosphate is formed. Filter. To the filtrate add some more ferric chloride. A Bordeaux-red colour indicates aceto-acetic acid.

NOTE.—A similar colour is given by a large number of substances, such as salicylic acid, and the bodies excreted after the administration of aspirin, antipyrin, thallin, etc. The majority of these substances are not destroyed by boiling, whereas aceto-acetic acid is converted into acetone.

B. If A is positive shake 50 c.c. of urine and 3 drops of strong sulphuric acid with ether. Pipette off the ether and treat it with very dilute ferric chloride. The lower layer becomes coloured violet. Add more ferric chloride. The colour changes to a Bordeaux-red.

NOTE.—It is advisable to shake the acidified urine first with chloroform or benzene, to extract salicylic acid.

11. Glycuronic Acid.

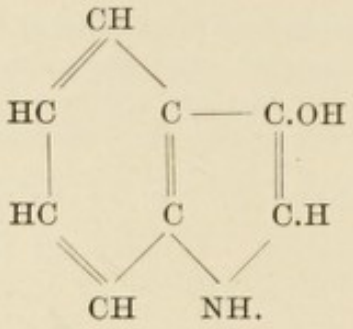
Glycuronic acid, $CHO.(CHOH)_4.COOH$, is not found free in the urine. It is found conjugated with certain drugs, or with substances formed from these in the body. These conjugated glycuronates are excreted after administration of chloral, camphor, naphthol, menthol, phenol, morphine, oil of turpentine, antipyrin, etc. The free and conjugated acids are reducing substances, but are not fermentable. They give the reactions for the pentoses, but can be distinguished by the test given below.

303. **Tollen's test for glycuronates.** To 5 c.c. of the urine in a rather wide test-tube add ·5 to 1 c.c. of a 1 per cent. solution of naphthoresorcin in alcohol and 5 to 6 c.c. of strong hydrochloric acid. Heat slowly to boiling point and keep boiling for 1 minute, shaking the tube the whole time. Set the tube aside for 4 minutes, then cool under the tap and shake with an equal volume of ether. The ether is coloured violet to red, and when examined spectroscopically shows two bands, one on the D line, and one to the right of it.

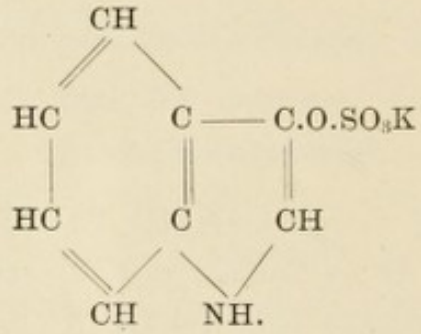
12. Indican.

Indican is the potassium salt of indoxyl sulphuric acid, and is thus one of the ethereal sulphates (see p. 135).

Indoxyl is



Indican is



Indoxyl arises from the bacterial decomposition of tryptophane in the intestine, thus differing from the other ethereal sulphates which are normal tissue metabolites (see p. 135). The excretion of indican is of importance as a measure of the amount of putrefaction occurring, generally in the intestine, but sometimes in a large abscess.

304. **Jaffé's test.** Treat 5 c.c. of urine with a rather larger volume of concentrated hydrochloric acid and about 2 c.c. of chloroform. Add a single drop of 3 per cent. potassium chlorate and shake. Allow the chloroform to settle and examine its colour. If it be blue, indican is present. If not, add another drop of the chlorate and shake again. If no blue colour be found in the chloroform, indican is absent.

305. **Lavelle's test.** Treat 10 c.c. of urine with 2-3 c.c. of Obermayer's reagent. Add 2-3 c.c. of concentrated sulphuric acid slowly, cooling under the tap during the addition. Add 2-3 c.c. of chloroform and shake. A blue colouration in the chloroform indicates the presence of indican.

NOTES.—1. In these reactions the strong acids hydrolyse indoxyl sulphuric acid to free indoxyl and sulphuric acid. The oxidising reagents oxidise the indoxyl to indigo blue, which is soluble in chloroform.

2. Obermayer's reagent is prepared by dissolving 4 gm. ferric chloride in 1 litre of concentrated hydrochloric acid.

L. Urinary Sediments.

For the proper examination of these substances a hand centrifuge is desirable. The sediment obtained should be examined microscopically, and chemically if necessary.

The sediments obtained are either organised or unorganised. Organised sediments consist of casts of the renal tubules, epithelial cells from different parts of the urinary tract, pus, blood cells, spermatozoa, parasites, etc. It is not thought advantageous to describe them in this book.

Unorganised sediments vary with the reaction of the urine. The more common varieties are given below.

In acid urine.

Uric acid: light yellow to dark reddish-brown in colour. Crystalline form very varied: rhombic prisms, wedges, rosettes, dumb-bells, whetstones, butcher's trays, etc. Soluble in sodium hydroxide and reprecipitated by hydrochloric acid.

Urates: pinkish, soluble on warming, sometimes amorphous, sometimes crystalline, as "thorn-apples," fan-shaped clusters of prismatic needles.

Calcium oxalate: octahedra, with an envelope-like appearance (squares crossed by two diagonals); also in dumb-bells. Insoluble in acetic acid, easily soluble in hydrochloric acid.

Calcium hydrogen phosphates (stellar phosphates): in rosettes of prisms and in dumb-bells. Rather rare.

Cystine: colourless hexagonal plates, soluble in ammonia, insoluble in acetic acid. Very rare.

In alkaline urine.

Ammonium magnesium phosphate (triple phosphate): colourless prisms ("coffin-lids" and "knife-rests") or feathery stars. Easily soluble in acetic acid.

Alkaline earthy phosphates of calcium and magnesium: amorphous. Insoluble on warming and in alkalies, soluble in acetic acid.

Calcium hydrogen phosphate: see above.

Calcium carbonate: dumb-bells or spheres with radiating structure

Ammonium urate: yellow, or brownish amorphous masses, or shewing "thorn-apple" crystals. Soluble on warming.

CHAPTER X.

THE QUANTITATIVE ANALYSIS OF URINE.

To determine the nature of the metabolic processes in the body a sample of the measured 24 hours' urine must be analysed. In taking the 24 hours' urine it is best to finish with that voided after the night's rest. The total collected during the 24 hours is mixed and carefully measured. The analyses should be performed as soon as possible, owing to the risk of bacterial decomposition of certain of the constituents. Should it be necessary to postpone the analyses an antiseptic should be added. Toluol or thymol are the best to use (but see Ex. 278, note 7). Chloroform must not be used in any case, since it is decomposed by alkalies and has a marked effect on certain processes.

The analyses performed will vary with the nature of the case that is being investigated, and the time and apparatus at the disposal of the analyst. It is of the utmost importance for the student to acquire skill in the conduction of a complete analysis, and in this connection particular attention is directed to Folin's micro-chemical methods, based on colorimetric comparison, that are described below. They enable a complete analysis of the nitrogenous constituents of a sample of urine to be made in a few hours with a very small amount of special apparatus beyond a good suction pump and a reliable colorimeter, preferably Dubosq's.

Since the fumes arising from the incineration of urine by boiling sulphuric acid are extremely irritating, that operation should be conducted in a fume chamber or under a hood. But these can be dispensed with by use of the special fume-absorber devised by Folin and illustrated in Fig. 6.* A is a bulb (1½ inches in diameter) blown into a piece of $\frac{5}{8}$ths Jena tubing. The lower

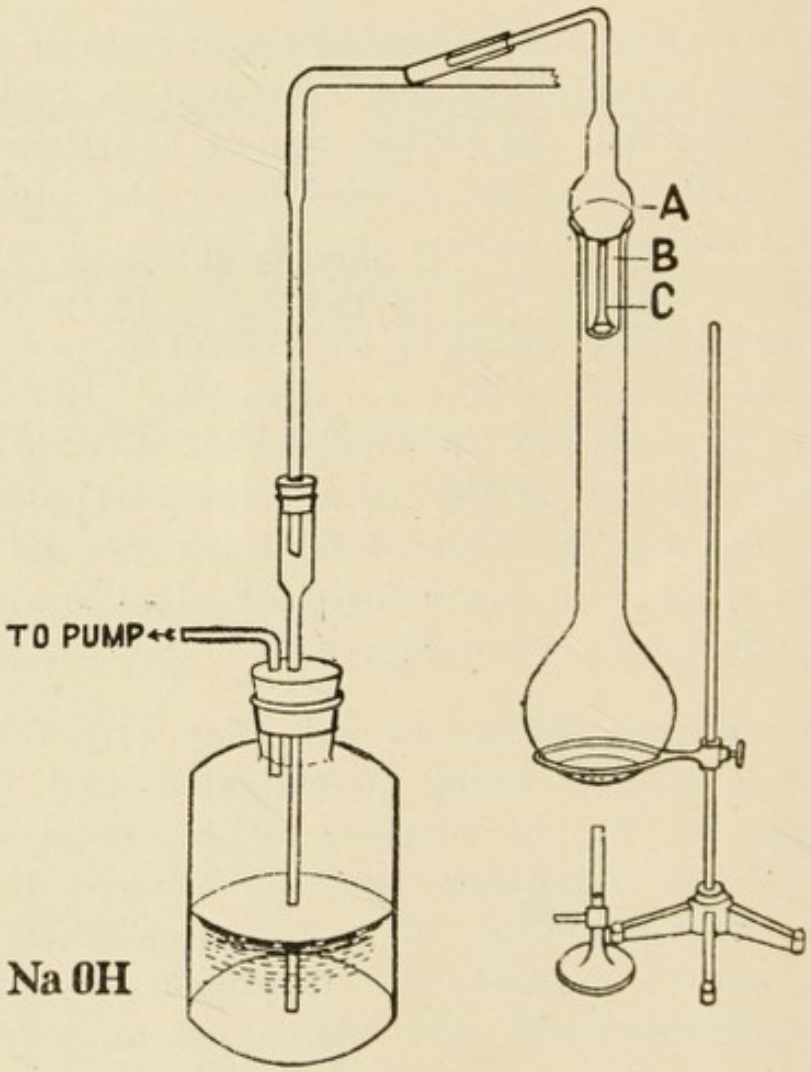


Fig. 6. Folin's fume-absorber.

* The apparatus described in this book can now be obtained from Messrs. J. Griffin and Sons, or from Messrs. Baird and Tatlock, and will be listed in their next catalogues.

end has blown into it a piece of narrow tubing (C) $2\frac{1}{2}$ inches in length. The bulb rests on the neck of the flask or test-tube in which the incineration is conducted.

To the upper end of the tube is fixed a piece of narrow tubing which is bent at a convenient angle, and which slips into a slightly longer tube connected to a good suction pump. The fumes are carried over by the air current into the pump, a wash bottle containing caustic soda being interposed to prevent damage. The condensation water collects in the pocket C and can be removed by inverting the fume-absorber at the end of the experiment. The removal of this condensation water materially hastens the incineration.

One good pump suffices to carry off the fumes from three or four incinerations simultaneously.

By inverting a funnel over an evaporating basin, and arranging the apparatus so that the end of the funnel fits loosely into the neck of the absorber, the fumes from boiling nitric acid can be carried off.

306. **The estimation of total nitrogen by Kjeldahl's method.**

Principle. The nitrogenous compounds in 5 c.c. of urine are converted into ammonium sulphate by boiling with sulphuric acid, copper sulphate being added to aid the oxidation, and potassium sulphate to raise the boiling point. The mixture is diluted with water, made alkaline by the addition of sodium hydroxide and the ammonia distilled into a measured amount of standard acid. The amount of this neutralised by the ammonia is found by subsequent titration with standard alkali. Knowing the amount of ammonia formed from 5 c.c. of urine, the percentage of

Method of Analysis.

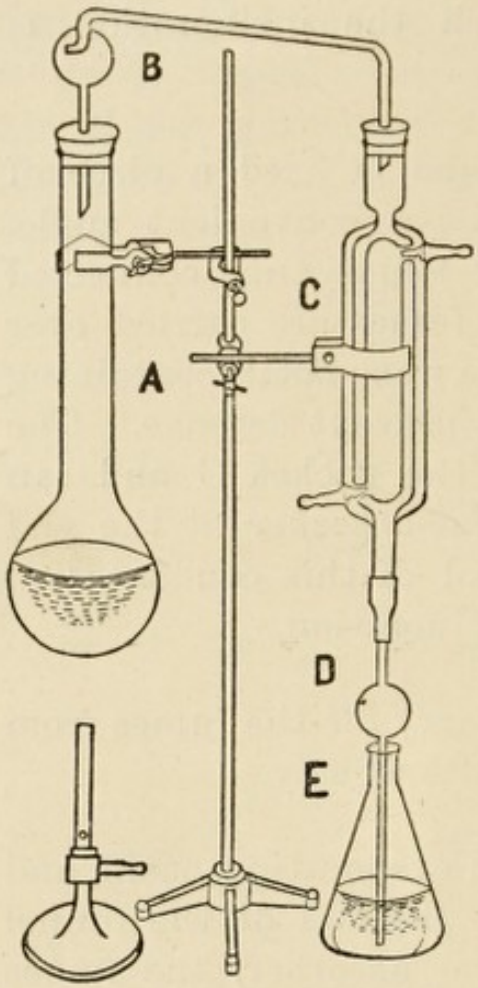


Fig. 7.—Apparatus for Kjeldahl's Method.

nitrogen can be readily calculated. Into a clean, dry, round-bottomed flask of Jena glass A (500 c.c. capacity, with a narrow neck 8 inches in length) place 10 gms. potassium sulphate, 2 c.c. of saturated copper sulphate solution, 5 c.c. of urine (accurately measured) and 10 c.c. of concentrated sulphuric acid, free from nitrogen. Place the flask in the fume-chamber (or use the fume-absorber, described on page 172), and heat by means of a low flame for 10-15 minutes, then boil briskly for 45 minutes. When cool add 250 c.c. of ammonia-free distilled water, about 0·5 gm. of powdered pumice and cool under the tap. Into an Erlenmeyer flask, E, of about 400 c.c. capacity, place 25 c.c. of $\frac{N}{5}$ solution of sulphuric acid.

This flask is then placed on an adjustable stand, so arranged that the lower end of the tube D dips below the surface of the acid in E. The bulb in D is to decrease the risk of the acid in E being sucked back by a sudden cooling of A during the distillation. D is connected to a condenser C. The best pattern is Davies', which is shewn in fig. 7.

To the flask A add 50 c.c. of 40 per cent. sodium hydroxide, pouring it down the neck and wall of the flask so as to form a bottom layer; loss of ammonia is thus prevented.

Fit the glass tube B into the neck of A by means of a well-fitting rubber stopper. The special bulb on B is to prevent any of the alkaline fluid bumping over into the distillate.

Mix the contents of A by shaking and immediately connect up B with C by means of another well-fitting rubber stopper. Heat the mixture in A to boiling by means of a free flame from a Bunsen burner provided with a rose-jet. Allow the fluid to boil till at least half the total volume of fluid has distilled over, lowering E from time to time, so that D does not dip too far under the acid. Finally, lower E so that the tube no longer dips under the surface and continue the boiling for another minute or two to wash down any of the standard acid that may have been sucked up into the tube or bulb. Wash down the exterior of the lower end of D with a jet of distilled water, allowing the washings to run into E.

To the fluid thus obtained add a drop of a dilute solution of neutral red (the best indicator), methyl orange, cochineal or congo red and titrate with $\frac{N}{10}$ sodium hydroxide.

Calculation of results. Example:

16·4 c.c. of $\frac{N}{10}$ = 8·2 c.c. of $\frac{N}{5}$ NaOH found necessary.

$\therefore$ 25 − 8·2 = 16·8 c.c. of $\frac{N}{5}$ acid were neutralised by ammonia from 5 c.c. of urine.

1 c.c. of $\frac{N}{5}$ acid = ·0028 gm. N.

$\therefore$ 16·8 c.c..... = ·04704 gm. N.

So N per cent. = ·04704 × 20 = ·9408 gm.

307. The estimation of total nitrogen by Folin's micro-chemical method.

Principle. A small volume of urine is decomposed by sulphuric acid as in Kjeldahl's method. The ammonia is drawn over into acid and the solution treated with Nessler's reagent. The amount of ammonia is determined colorimetrically by comparison with a standard solution of ammonium sulphate simultaneously Nesslerised.

Incineration. Measure 5 c.c. of urine into a 50 c.c. flask if the specific gravity be over 1018, or into a 25 c.c. flask if the sp. gr. be less than 1018. (The dilution must be such that 1 c.c. of the diluted urine contains between 0·75 and 1·5 mgms. N.) Fill the flask to the mark with distilled water and invert it a few times to secure thorough mixing. Measure 1 c.c. of the diluted urine, by means of an accurate pipette,* into a large test tube of Jena glass (20 to

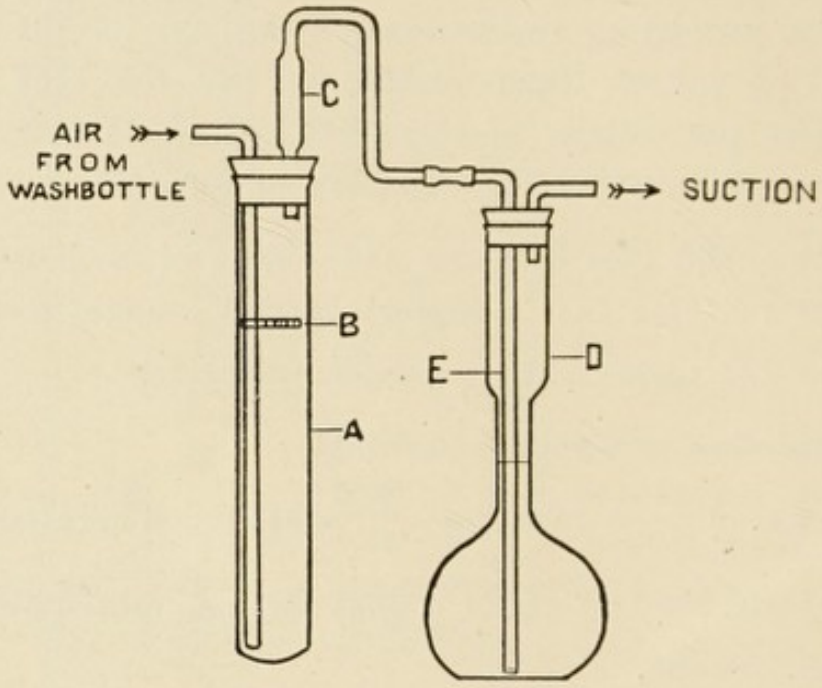


Fig. 8. Apparatus for Folin's micro-chemical methods.†

A. Jena test tube, 20 to 25 mm. by 200 mm.

B. Sheet of rubber, about 2 mm. thick, cut from a 2-holed rubber stopper. It fits loosely into A, and has a small groove cut at the side.

C. Made from a broken 5 c.c. pipette.

D. 100 c.c. measuring flask with wide neck to take a 2-holed rubber stopper.

E. Tube sealed at lower end and holes bored in it by means of a hot platinum wire. It prevents loss of ammonia.

To prevent any ammonia from the room entering the apparatus the air is passed through a wash bottle containing sulphuric acid before it enters A.

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* Ostwald's pipettes are suitable.

† The various parts of the apparatus can be obtained from Messrs. Baird and Tatlock, and from Messrs. J. Griffin and Sons.

25 mm. by 200 mm.) Add 1 c.c. of pure sulphuric acid, 1 gram of potassium sulphate, 1 drop of 5 per cent. copper sulphate and a small, clean quartz pebble, or a small fragment of granulated zinc (to prevent bumping). Boil over a micro-burner for about ten minutes, either in the fume chamber or use the fume-absorber described on page 172. Allow to cool for about three minutes and then add about 6 c.c. of water, at first a few drops at a time, then more rapidly so as to prevent the mixture solidifying.

Distillation of the Ammonia. Use the apparatus shewn in Fig. 8. Transfer 4 c.c. of a saturated solution of sodium hydroxide to the mixture in the test-tube A, and aspirate the ammonia into 2 c.c. of $\frac{N}{10}$ hydrochloric aid and about 20 c.c. of distilled water contained in the special 100 c.c. measuring flask D. The air current may be moderate for the first two minutes but for the next eight minutes it should be as rapid as possible. Disconnect and dilute the contents to about 60 c.c., washing the tube E with some of the water added.

Preparation of the Nesslerised solutions. In another 100 c.c. measuring flask place 5 c.c. of standard ammonium sulphate solution, containing 1 mgm. of nitrogen, and dilute it to 60 c.c. To each flask add 3 c.c. of a cold saturated solution of Rochelle salt (to prevent the formation of a cloud on adding Nessler's solution.* Nesslerise both solutions as nearly as possible at the same time with 5 c.c. of Nessler's reagent diluted *immediately* beforehand with 25 c.c. of water. Fill both flasks to the mark with water and mix.

Determination of the depth of colour. This is done by means of a Dubosq colorimeter. (See Fig. 11, p. 191.) In one of the chambers B place some of the unknown solution, in the other some of the standard ammonia solution. Place the tube D of the standard at a certain depth (10 mm. is usually the best) and adjust the other tube until the colours match. Several readings should be taken, moving the unknown from below and from above.

* The addition of the Rochelle salt is not essential.

Calculation of results. Example:

$$\frac{\text{Height of standard}}{\text{Height of unknown}} = \frac{20 \text{ mm.}}{21{\cdot}3 \text{ mm.}}$$

So the 1 c.c of fluid taken contains $\frac{20}{21{\cdot}3} = 0{\cdot}94$ mgm. nitrogen.

Urine was diluted 1 in 10.

So 100 c.c. of urine contains 0·94 gm. nitrogen.

Preparation of the standard solution of ammonium sulphate. Pure ammonium sulphate is decomposed by means of caustic soda and the ammonia passed into pure sulphuric acid by means of the air current. When all the acid has been neutralised, the solution is partially evaporated and the salt precipitated by alcohol. It is redissolved in water, reprecipitated by alcohol, and dried in a desiccator over sulphuric acid.

9·4285 gm. of the ammonium sulphate are dissolved in water and the volume made up to 1 litre. (Stock solution).

100 c.c. of the stock solution are diluted to form 1 litre (Standard solution).

5 c.c. of the standard solution contain 1 mg. nitrogen.

Preparation of Nessler's reagent.

Dissolve 62·5 gm. of potassium iodide in about 250 c.c. of distilled water, set aside a few c.c. and add gradually to the larger part a cold saturated solution of mercuric chloride (of which about 500 c.c. will be required) until a faint permanent precipitate is produced. Add the reserve portion of the potassium iodide and then mercuric chloride very gradually till a slight permanent precipitate is again formed.

Dissolve 150 gm. of solid potassium hydroxide in 150 c.c. of distilled water, allow the solution to cool and add it gradually to the above solution and make the volume up to 1 litre. Allow to settle, decant the clear liquid into another bottle and keep in the dark. The reagent improves on keeping.

308. The estimation of ammonia by Folin's method.

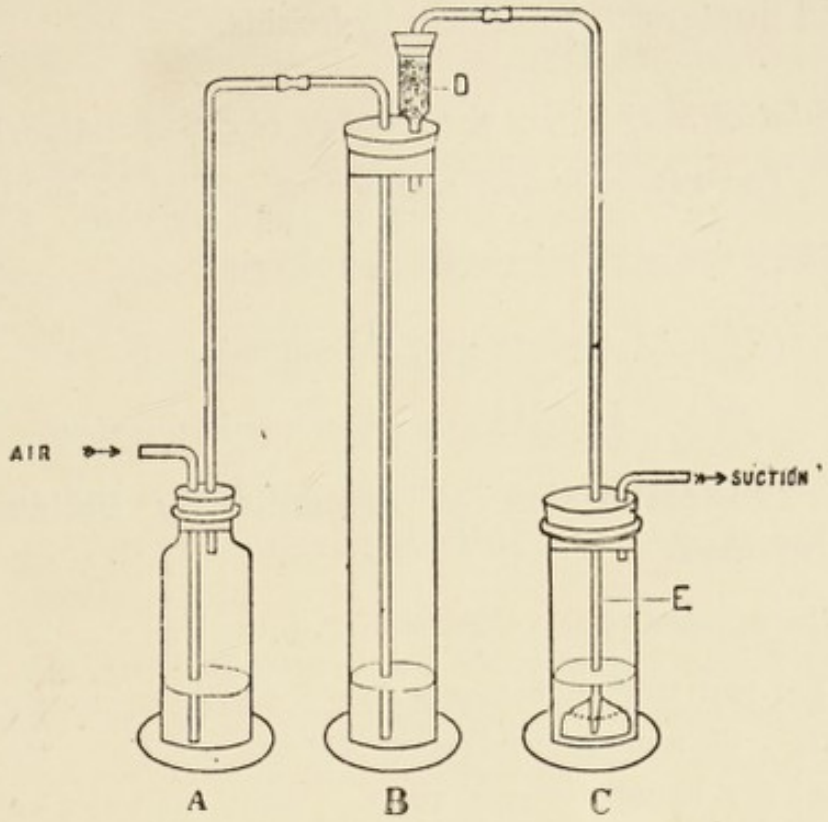


Fig. 9. Folin's apparatus for estimating ammonia.

A. Wash bottle containing acid.
B. Tall aërometer cylinder containing urine.
C. Bottle containing standard acid.
D. Calcium chloride tube, loosely packed with cotton wool, to prevent any sodium carbonate being carried over into C.
E. Folin's absorption tube, to bring the air into intimate contact with the acid.

Use the apparatus shown in Fig. 9*.

Into C measure 20 c.c. of $\frac{N}{10}$ sulphuric acid and two drops of a dilute solution of methyl red, or Alizarin red.

Into B measure 25 c.c. of urine, add 10 c.c. of kerosene oil (to prevent foaming) and one gram of anhydrous sodium carbonate. Connect up the apparatus at once, and draw air through for two hours.

* The parts of the apparatus can be obtained from Messrs. J. Griffin and Sons, or Messrs. Baird and Tatlock.

Disconnect the apparatus, wash the tube E with distilled water into C, and titrate with $\frac{N}{10}$ sodium hydroxide.

Calculation. Subtract the number of c.c. of sodium hydroxide from 20. The result is the number of c.c of $\frac{N}{10}$ sulphuric acid neutralised by the ammonia in 25 c.c. of urine.

1 c.c. of $\frac{N}{10}$ acid = ·0017 gm. of ammonia.

= ·0014 gm. of ammonia nitrogen.

309. **The estimation of ammonia by Folin's micro-chemical method.**

Use the apparatus sketched in Fig. 8, p. 176.

Into the test tube A measure 1 to 5 c.c. of urine, so that 0·75 to 1·5 mgms. of ammonia-nitrogen are dealt with. For normal urine 2 c.c. are usually about right. With diabetic urine, even 1 c.c. may be too much, and the urine must be previously diluted.

Add water, if necessary, to bring the volume to about 5 c.c. Add a few drops of a solution containing 10 per cent. of potassium carbonate and 15 per cent. of potassium oxalate. Also a few drops of kerosene or heavy, crude machine oil (to prevent foaming).

Measure 2 c.c. of $\frac{N}{10}$ hydrochloric acid into the 100 c.c. graduated flask D, add about 20 c.c. of distilled water, connect up the apparatus and pass a strong current of air through for 15 minutes. Nesslerise as described in Ex. 307, and compare with 1 mgm. of nitrogen obtained from the solution of standard ammonium sulphate, similarly and simultaneously Nesslerized.

Calculation. The number of mgms. of ammonia-nitrogen in the volume of urine taken are readily calculated as in Ex. 207, and so the number of grams per 100 c.c. The amount of ammonia is obtained from this by multiplying by $\frac{17}{14} = 1{\cdot}214$.

310. The estimation of ammonia by the formaldehyde method.

Principle. When neutral solutions of ammonium salts are treated with formaldehyde, combination occurs between the ammonia and the formaldehyde, hexamethylene tetramine (urotropin) being formed. A corresponding amount of acid is liberated from the ammonium salt, and this can be titrated with standard alkali.

$$4\,NH_4Cl + 6\,CH_2O = N_4(CH_2)_6 + 6\,H_2O + 4\,HCl.$$

Formaldehyde. Urotropin.

The objection to the method is that amino-acids react in a similar manner, so that the result obtained is ammonia + amino-acids. However, it is of considerable value as a rapid clinical method of determination.

Preparation of formaldehyde solution. 5 c.c. of commercial formalin (40 per cent. formaldehyde) are diluted with 5 c.c. of water, treated with a drop of phenolphthalein and neutralised with $\frac{N}{10}$ sodium hydroxide.

Method of estimation. Neutralise 25 c.c. of urine with $\frac{N}{10}$ sodium hydroxide exactly as described on page 193. To the mixture thus obtained add the whole of the formaldehyde solution. Read the burette and run in more $\frac{N}{10}$ sodium hydroxide until the same point is obtained as before.

Calculation of results. 1 c.c. of $\frac{N}{10}$ sodium hydroxide
= ·0017 gm. NH_3
= ·0014 gm. of ammonia-nitrogen.

311. The estimation of urea by Benedict's method.

Principle. Urine is treated with potassium bisulphate and zinc sulphate and heated to 165° C. for one hour. The urea is thus hydrolysed to ammonium compounds which are retained by the acid mixture. The fluid is diluted, made alkaline with sodium carbonate, and the ammonia distilled into standard acid. The

amount of this neutralised by the ammonia formed is determined by titration with standard alkali. The ammonia nitrogen of the urine must be previously determined.

Method. 5 c.c. of urine are measured into a wide Jena test-tube (200 × 25mm.) and treated with about 3 grams of potassium bisulphate and 1 to 2 grams. of zinc sulphate. A little powdered pumice and a bit of paraffin are introduced to minimise frothing and spattering, and the mixture boiled practically to dryness, either over a small free flame or, more conveniently, by floating the tube in a bath of sulphuric acid kept at about 130° C. A convenient bath is a tall-form Jena glass, or, preferably, porcelain beaker of about 800-1,000 c.c. capacity, two-thirds full of sulphuric acid.

The tube is then immersed for at least three-fourths of its length in the sulphuric bath. This can be done by clamping the tube to the edge of the bath. Raise the temperature of the bath to 162° – 165° C. and maintain it there for one hour. Remove the tube and allow it to cool somewhat. Wash off the acid under the tap. Wash the contents by means of hot water quantitatively into a 500 c.c. Jena flask (A, fig. 7, p. 174). The volume of the fluid in the flask should be about 350 c.c.

Fit up the apparatus as used for Kjeldahl's method, placing 25 c.c. of $\frac{N}{5}$ sulphuric acid in D. To A add about 25 c.c. of a saturated solution of sodium carbonate. Connect up the apparatus and distil for about forty minutes, till about one-half of the fluid has passed over. Boil the fluid in D to remove excess of CO_2, cool and titrate with N/10 sodium hydroxide, using methyl red, cochineal or methyl orange as an indicator.

Calculation of results. Example:

Ammonia-nitrogen of 25 c.c. urine was previously found to correspond to 10 c.c. $\frac{N}{10}H_2SO_4 = 5$ c.c. $\frac{N}{5}H_2SO_4$.

So ammonia-nitrogen of 5 c.c. urine = 1 c.c. $\frac{N}{5}H_2SO_4$.

In this exercise 16 c.c. of $\frac{N}{10}$NaOH, *i.e.* 8 c.c. of $\frac{N}{5}$NaOH neutralised the 25 c.c.$\frac{N}{5}H_2SO_4$.

So amount of $\frac{N}{5}H_2SO_4$ neutalised by urea and ammonia was $25 - 8 = 17$ c.c.

Amount neutralised by urea alone was therefore $17 - 1 = 16$ c.c.
1 c.c. of $\frac{N}{5}H_2SO_4 = \cdot0028$ gm. N = ·006 gm. urea.

So Urea-N in 5 c.c. = $16 \times \cdot0028 = \cdot0045$ gm.
Urea in 5 c.c. = $16 \times \cdot006 = \cdot096$ gm.
Urea per cent. = $\cdot096 \times 20 = 1\cdot92$.

312. **The estimation of urea by Folin's microchemical method.**

Principle. Urine is treated with potassium acetate and acetic acid and boiled. The boiling point of the mixture is about 155° C., and at this temperature the urea is rapidly hydrolysed to CO_2 and ammonia, which is retained as ammonium acetate. Caustic potash is added, and the ammonia aspirated into acid. This solution is Nesslerised and the colour compared with that of a standard solution of ammonium sulphate similarly and simultaneously Nesslerised.

Method. The urine must be diluted so that 1 c.c. contains 0·75 to 1·5 mgms. of urea nitrogen. Usually 1 in 10 is about correct. In a large *dry* Jena test tube (A, fig. 8, p. 176) place 7 grams of dry potassium acetate, 1 c.c. of 50 per cent. acetic acid, a small fragment of granulated zinc (to prevent bumping), and a temperature indicator (see below). To the tube transfer 1 c.c. of the diluted urine by means of an accurate pipette. The test tube is then closed by means of a rubber stopper carrying an empty narrow "calcium chloride tube," without bulb (25 cm. by 1·5 cm.) The test tube is held by a clamp, so that it can be readily raised or lowered. Heat is applied by means of a microburner, which is

shielded from air currents by means of a chimney or a bottomless beaker. The flame should be about 0·5 cm. long.

The acetate dissolves and the mixture begins to boil. The temperature indicator should show that the temperature has reached 153° C. to 160° C. Boiling is continued for ten minutes after this temperature has been attained. (The temperature must not reach 162° C., at which point the acetate cakes and solidifies.) Remove the apparatus from the flame and dilute the contents with 5 c.c. of water, adding it from a pipette through the calcium chloride tube so as to rinse the sides of the tube and the bottom of the rubber stopper from traces of ammonium acetate which may be there, add 2 c.c. of saturated sodium hydroxide solution and aspirate the ammonia into acid exactly as described on p. 177. Estimate the nitrogen colorimetrically against 1 mgm. of nitrogen as described above.

Temperature indicator. This consists of powdered chloride-iodide of mercury (Hg ICl) enclosed in sealed tubes 10 to 15 mm. in length and not over 1 mm. in diameter. The salt is bright red at ordinary temperatures. It turns lemon yellow at 118° C. and melts to a clear dark red liquid at 155° C. The same indicator cannot be used twice within 24 hours.

The salt is prepared by heating in a dry state intimately mixed mercuric chloride (2·7 gm.) and mercuric iodide (4·5 gm.) in molecular proportions for six to eight hours at 150° to 160° C. At the end of the heating the product should be powdered and kept dry till sealed up as indicated.*

Calculation of results Example:

Urine diluted 1 in 10.

$$\frac{\text{Height of standard}}{\text{Height of unknown}} = \frac{20\text{ mm.}}{18\text{ mm.}}$$

So 0·1 c.c. urine contain 1·11 mg. N as urea and ammonia.

* These indicators can be obtained from Messrs. Griffiths, or Messrs. Baird and Tatlock.

So 100 c.c. urine contain 1000 × 1·11 mgm. = 1·11 gm. of urea and ammonia-N.

Ammonia-N was found to be 0·045 gm. per 100 c.c.

So urea-N per 100 c.c. = 1·11 – 0·045 = 1·065 gm.

$$\text{Urea} = 1{\cdot}065 \times \frac{CO(NH_2)_2}{N_2} = 1{\cdot}065 \times \frac{60}{28} = 2{\cdot}28 \text{ per cent.}$$

NOTES.—1. The method has to be modified for diabetic urine, owing to the tendency to the formation with the sugar of stable ureides that resist decomposition.

The urine is diluted 100 times and 1 c.c. of the diluted urine decomposed as above.

The ammonia is driven into another tube containing about 2 c.c. of water and 0·5 c.c. of $\frac{N}{10}$ HCl. To this tube are added first 2 c.c. of water, and then 3 c.c. of 1 in 5 Nessler's solution. The coloured solution is washed into a 10 c.c. measuring flask and the volume made up to 10 c.c. The colour is determined against that of the usual standard containing 1 mgm. of nitrogen per 100 c.c. of solution.

313. The estimation of urea by the hypobromite method.

Remarks. This is the standard method for the clinical estimation of urea. It is of the utmost importance for the student to realise that the method is essentially inaccurate and may lead to very erroneous conclusions. The nitrogen evolved comes from urea, ammonia, and to a small and undetermined extent from creatinine and other nitrogenous constituents. Further, urea does not evolve the whole of its nitrogen in the form of gas, so that allowances have to be made. Since the proportion evolved varies with differences in the composition of the fluid it is obvious that no certain deductions can follow such a determination. It is with the utmost diffidence that the method is given. It is most certainly not to be recommended.

Principle. Urine is treated with an alkaline solution of sodium hypobromite and the amount of urea calculated from the volume of nitrogen evolved.

The reaction that takes place is as follows :—

$$CO(NH_2)_2 + 3NaBrO + 2\ NaOH = N_2 + 3\ NaBr + Na_2CO_3 + 3\ H_2O.$$

Hence 60 grams urea evolve 28 grams N. = 2 × 11·2 litres, and 1 gram urea evolves 373 c.c. N.

Practically it is found that only 357 c.c. are evolved, the other 4·4 per cent. of the nitrogen being converted into nitrates, cyanates, etc.

Apparatus. See fig. 10. A 50 c.c. burette (*a*) is held by a clamp in a tall cylinder of water (*b*). The upper end of the burette is closed by a tightly-fitting rubber stopper, which is pierced by one limb of a glass T-piece. The upper limb of the T-piece is fitted with a short length of pressure-tubing carrying a screw-clamp (*e*). The side limb of the T-piece is connected by about two feet of small rubber tubing to a glass tube piercing the well-fitting rubber stopper of a wide-mouthed bottle (*c*) of about 60 c.c. capacity. This bottle is placed in a jar of water, supported at such a height that the burette can be lifted nearly out of the tall cylinder without stretching the rubber connection. A small glass bottle or short tube of 10 to 15 c.c. capacity is also required (*d*). (For the method of preparing the hypobromite solution see Ex. 249.)

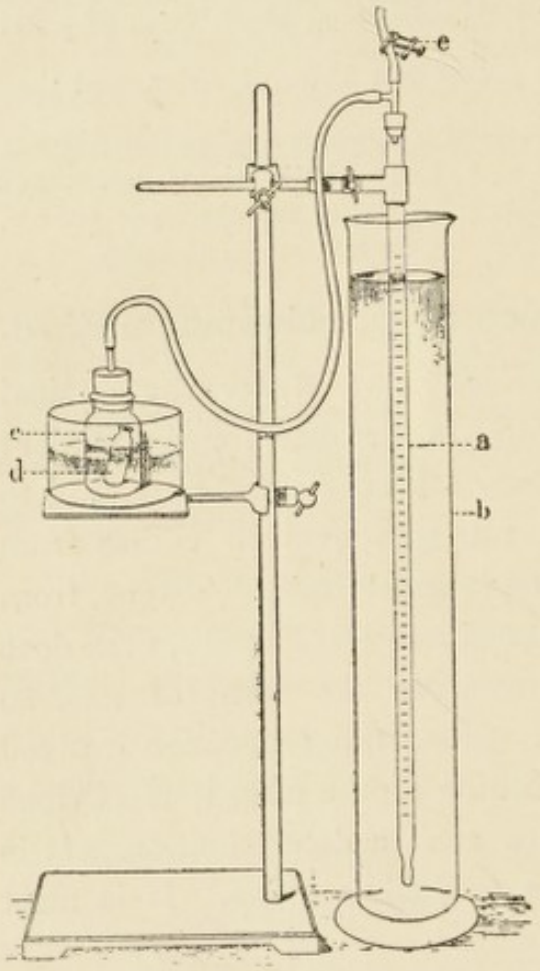


Fig. 10. Apparatus for determination of urea by hypobromite method.

Method of Analysis. Place 25 c.c. of freshly-prepared hypobromite solution in (*c*). Put 5 c.c. of urine, accurately measured, in the small bottle (*d*), and place this inside the other by means of a pair of forceps, taking great care not to upset any urine into the hypobromite. Fit the

rubber cork tightly into the bottle and place this in (*b*) to cool. See that the burette is as low as possible, that the cylinder has sufficient water in it to reach the zero graduation of the burette, and that the screw clamp is open. Leave the apparatus for about a minute to cool to the temperature of the water; clamp the burette in such a position that the water is below the zero mark, and then screw the clamp on the rubber tubing as tight as possible. Note down on paper the level of the water in the tube, keeping the eye level with the meniscus. Take the bottle out of the jar, and gently tilt it so that the urine flows into the hypobromite.

Gently shake the bottle from side to side, keeping the bottle upright to prevent the froth from being forced up into the tube. Tilt the bottle again and repeat the process till the urine and hypobromite are thoroughly mixed. Place the bottle back in the jar of water for about 3 minutes to cool. Raise the burette till the level of water in the tube is the same as that outside, the gas being thus under atmospheric pressure. Read the level of the meniscus as before: the difference in the two readings is the volume of nitrogen evolved. Ascertain the temperature of the water and the barometric pressure.

Calculation of results.

Let the temperature be $t°$ C., the tension of aqueous vapour at this temperature be T mm. (See Appendix), and the barometric pressure be B mm. of mercury. Let v be the volume of nitrogen measured under the conditions: at 0° C. and 760 mm. this will become

$$\frac{v \times 273 \times (B - T)}{(273 + t) \times 760} = v'$$

Now 357 c.c. of N are evolved from 1 grain of urea.

$\therefore$ v' c.c. are evolved from $\frac{v'}{357}$ gram of urea.

$\therefore$ 5 c.c. urine contain $\frac{v'}{357}$ gram urea.

and 100 c.c. urine contain $\frac{20v'}{357}$ gram urea.

NOTE.—Performing these two calculations in one operation we obtain for the percentage of urea

$$\frac{v \times (B - T}{(273 + t)} \times \frac{273 \times 20}{760 \times 357} = \frac{v \times (B - T)}{(273 + t)} \times \cdot 0201$$

314. **The estimation of uric acid by the Folin-Schaffer method.**

Principle. The mucoids and some of the phosphates are precipitated by ammonium sulphate containing uranium acetate and acetic acid. The filtrate is rendered alkaline by ammonia. Ammonium urate separates out. This is washed free from chlorides with ammonium sulphate, suspended in water and titrated with $\frac{N}{20}$ potassium permanganate.

Preparation of Solutions.

1. Uranium acetate solution. Dissolve 500 gm. ammonium sulphate, 5 gm. uranium acetate and 60 c.c. of 10 per cent. acetic acid in 650 c.c. of water. The volume of the solution is almost exactly 1000 c.c.

2. $\frac{N}{20}$ potassium permanganate. Dissolve 1·581 gm. of the pure salt in distilled water and make the volume up to 1000 c.c.

Method. 200 c.c. of urine are treated with 50 c.c. of the Folin-Schaffer reagent, allowed to stand for 20 minutes and filtered through a dry paper into a dry flask.

Measure 125 c.c. of this filtrate into a beaker, previously marked at the 100 c.c. level by means of a label, add 5 c.c. of concentrated ammonia, and allow it to stand for 24 hours. Carefully filter off the supernatant fluid through a hardened filter paper, and wash the precipitate on to the paper with 10 per cent. ammonium sulphate. Wash the precipitate twice more with this reagent to remove the chlorides.

Remove the paper from the funnel, open it, and by a fine jet of hot water, rinse the precipitate back into the beaker. Cool under the tap and make the volume up to 100 c.c. with distilled

water. Add 15 c.c. of concentrated sulphuric acid and titrate at once, without cooling, with $\frac{N}{20}$ potassium permanganate from a burette, which must have a glass tap.

During the titration the fluid in the flask must be kept in vigorous movement. Each drop of the permanganate is at first discoloured almost immediately, before it has had time to diffuse through the liquid and impart to it a pink tinge. The first instantaneous appearance of a diffuse flush through the whole body of the solution marks the end point of the titration. The colour disappears very rapidly, but it will now be found that if another drop of permanganate be added, it has time to diffuse through the liquid, before it, in its turn, is decolourised.

Calculation of the result.

125 c.c. of the fluid taken contains 100 c.c. urine.

1 c.c of $\frac{N}{20}$ permanganate = 0·00375 gm. uric acid.

Add to the result 0·003 gm. for the 100 c.c. to allow for the solubility of ammonium urate in the reagents.

Example.

24 hours urine = 1365 c.c.

12·4 c.c. of permanganate required.

Percentage of uric acid = 12·4 × ·00375 + ·003
= ·0465 + ·003.
= ·0495.

Total in 24 hours = ·0495 × 13·65 = ·675 gm.

Uric acid-nitrogen = ·675 × $\frac{1}{3}$ = ·225 gm.

315. **The estimation of uric acid by Folin's micro-chemical method.***

Principle. Urine is evaporated to dryness and extracted with ether and alcohol to remove polyphenols. The residue is dissolved in dilute alkali and treated with Folin's uric acid reagent. The fluid becomes coloured blue and is compared colorimetrically with a standard solution of uric acid similarly treated.

* An improved method will be found on p. 244.

Preparation of Folin's reagent. See page 149.

Method. 2 to 5 c.c. of urine (depending on the specific gravity) are measured into an evaporating basin, a single drop of a saturated solution of oxalic acid is added and the whole evaporated to complete dryness on the water bath. Allow to cool and add 10 c.c. of a mixture of 2 parts of dry ether (distilled over sodium) and 1 part of pure methyl alcohol.* Allow to stand for five minutes without stirring. Carefully pour off the fluid and extract similarly once more. To the residue add 10 c.c. of water and a drop of saturated sodium carbonate solution and stir till solution is complete. Add 2 c.c. of Folin's reagent and then 20 c.c. of a saturated solution of sodium carbonate. Transfer the blue solution to a 100 c.c. graduated flask, wash the evaporating basin out with water into the flask and make the volume up to 100 c.c. By means of Dubosq's colorimeter (p. 191) compare the colour of this solution with that of a standard solution of uric acid prepared as described below.

Preparation of the standard solution. Weigh out 250 mgms. of Kahlbaum's uric acid. Transfer it to a 250 c.c. measuring flask by means of 25 to 50 c.c. of water. Add 25 c.c. of a 0·4 per cent. solution of lithium carbonate and shake at intervals for an hour before making the volume up to 250 c.c. The solution does not keep for longer than 5 or 6 days.

1 c.c. of this solution is carefully measured by means of a reliable pipette into a 100 c.c. measuring flask, 10 c.c. of water, 2 c.c. of Folin's reagent and 20 c.c. of saturated sodium carbonate solution are added and the volume made up to the mark with water. The reagent and sodium carbonate should be added as nearly simultaneously as practicable both to the unknown and to the standard solution. Five minutes is the maximum allowable interval.

Calculation of results.

Set the standard at a depth of x mm.

* 95 per cent. alcohol can be used as a substitute for the ether-alcohol mixture, but it involves the risk of a slight loss of uric acid.

Let the depth of the unknown be y mm. when an equality of tint is obtained.

Suppose 3 c.c. of urine were taken.

Then 3 c.c. of urine contain $\frac{x}{y}$ mgm. uric acid.

So 100 c.c. contain $\frac{100x}{3y}$ mgm.

316. **The estimation of creatinine by Folin's method.**

Principle. Urine is treated with picric acid and caustic soda. The creatinine yields a colour reaction (see Ex. 271), the intensity of which is compared with that of a standard solution of potassium dichromate by means of a colorimeter.

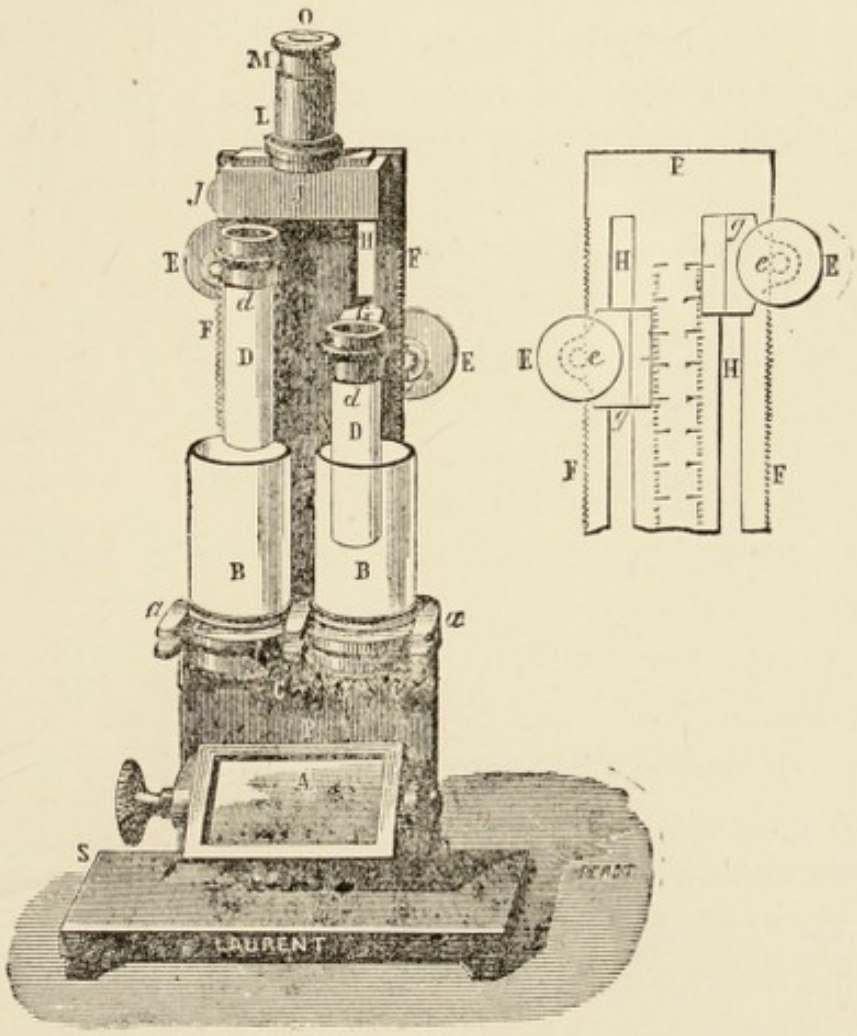


Fig. 11.—Dubosq's Colorimeter.
Inset shows construction of vernier scale.

Apparatus required. A colorimeter. Dubosq's is the most convenient. It is illustrated in Fig. 11. The solutions to be compared are placed in the tubes B. The tubes D are sealed at the bottom by glass plates and are immersed in the solutions, the depths of which are indicated by a scale and vernier at the back of the instrument. Diffused daylight is reflected through the solutions by means of the opal glass A, the path of the rays being shewn in Fig. 12. The heights of the tubes D are so adjusted that an exact equality of tint is obtained.

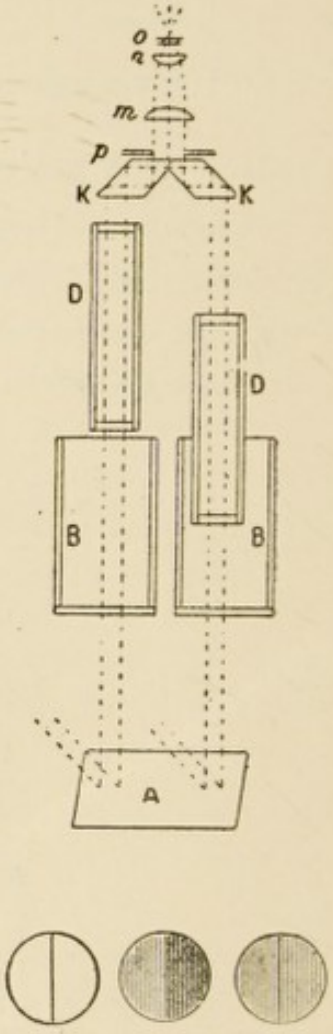


Fig. 12.—Diagram of path of rays in Dubosq's Colorimeter. Below are representations of the appearance of the field under different conditions, that on the left with no fluid in B, and that on the right when the tints are matched.

Before using the colorimeter it is important to test it by placing the bottom of D in contact with the bottom of B. Both indicators should then be at zero. Then fill the tubes B with the standard dichromate solution. Place one of the tubes at a depth of 10 mm. Now look through O and adjust the level of the other tube until the two tints match. The depth of this solution should also be 10 mm. Several determinations should be made, moving the second tube both from above and below. The readings should not differ by more than 0·3 mm.

Preparation of the dichromate solution. Dissolve 24·55 gm. of pure potassium dichromate in water and make the volume up to 1000 c.c.

Method of analysis. Measure 10 c.c. of the urine with a pipette into a 500 c.c. measuring flask. Add 15 c.c. of saturated picric acid in water (about 1·2 per cent.) and 5 c.c. of 10 per cent. caustic soda. Mix and allow to stand for 5 minutes. Fill the

flask up to the mark with distilled water and mix well. Immediately compare with the standard solution. Place the dichromate in one of the cells B and the creatinine in the other. Place the dichromate tube at a depth of 8 mm. and determine the position of the other that gives exactly the same tint. Take several readings working from above and below as before.

Calculation of results.

When 10 mg. creatine are treated in this way the colour of a layer 8·1 mm. in depth is the same as that of a layer of 8 mm. of the standard dichromate solution.

If the depth of the layer be x mm., then the creatinine in 10 c.c. of urine $= \frac{10 \times 8{\cdot}1}{x}$ mg.

NOTE.—If the reading be less than 5 mm., the urine must be carefully diluted and another determination made using less urine. Should it be greater than 13 mm., 20 c.c. must be taken instead of 10.

317. **The estimation of the titration acidity by Folin's method.** Place 25 c.c. of urine in a 200 c.c. Erlenmeyer flask, add 15 gm. of finely powdered neutral potassium oxalate, 2 drops of 1 per cent. phenolphthalein and shake the mixture vigorously for 2 to 3 minutes. Titrate with $\frac{N}{10}$ sodium hydroxide, until a permanent faint pink colour is produced.

Calculation. Express the result in terms of $\frac{N}{10}$ soda. Thus if 7 c.c. of soda are required for 25 c.c. of urine, the acidity of the same is equivalent to 28 c.c. of $\frac{N}{10}$ sodium hydroxide per 100 c.c.

NOTES.—1. The method only gives approximate results owing to the difficulty in determining the end-point. Since the point varies with the amount of indicator used, this must be kept constant. Each individual worker should always choose the end-point that he can most readily determine, and always proceed to this point. In this way reliable information oan be obtained concerning the relative titration acidity of various samples of urine.

2. For the estimation of the true acidity (the concentration of the hydrogen ions) see page 129.

3. The potassium oxalate is added to precipitate calcium salts, which interfere with the sharpness of the end-point of the titration.

4. The urine thus neutralised can be used for the determination of the ammonia of the urine by the formaldehyde method. (See p. 181.)

318. *The estimation of chlorides by Volhard's method.

Principle. The chlorides are precipitated from urine by a known excess of standard solution of silver nitrate in the presence of nitric acid. The excess of silver is estimated in an aliquot portion of the filtrate by titration with a solution of potassium sulphocyanide, that has been previously standardised against the silver solution, a ferric salt being used as an indicator in both titrations.

Reagents required.

(i) Standard silver nitrate solution prepared by dissolving 29·063 grams of pure fused silver nitrate in distilled water and filling up accurately to one litre. The solution should be kept in the dark.

1 c.c. corresponds to ·01 gram NaCl (·00606 gram Cl).

(ii) Solution of potassium sulphocyanide made by dissolving 8 grams of the salt in a litre of distilled water.

(iii) Pure nitric acid, quite free from chlorine.

(iv) A concentrated solution of iron alum.

Standardisation of the Sulphocyanide. In a beaker place 10 c.c. of the silver nitrate, accurately measured: add 5 c.c. of pure nitric acid, 5 c.c. of iron alum and 80 c.c of distilled water. Titrate the whole with the sulphocyanide from a burette until a faint permanent red tinge is obtained. Note the amount required for the 10 c.c. of silver nitrate.

Method of Analysis. In a 100 c.c. cylinder or measuring flask place 10 c.c. of urine, accurately measured by a pipette, 20 c.c of the standard silver solution, also accurately measured, about 4 c.c. of pure nitric acid, and 5 c.c. of the iron alum. Add distilled water till the 100 c.c. mark is just reached, and mix thoroughly by

* For a simple alternative method see p. 240.

pouring into a beaker and stirring well. Filter off the precipitated silver chloride through a dry paper into a dry vessel. Of the filtrate take 50 c.c., accurately measured, and titrate it with the potassium sulphocyanide solution till a faint permanent red tinge is obtained.

NOTE.—It is very important to remember to add the nitric acid. It renders the silver chloride insoluble and prevents the precipitation of the silver compounds of the purine bases in those cases in which the urine is alkaline.

Calculation of results.

Standardisation of sulphocyanide shows that

1 c.c. KCNS = xc.c. standard silver.

Now 50 c.c. of urinary filtrate = S c.c. KCNS.

∴ 100 c.c. „ „ „ = 2S c.c. KCNS

2 S c.c. KCNS = 2 S × x c.c. of standard silver.

We added 20 c.c. standard silver to 10 c.c. urine.

So (20 − 2 S × x) c.c. have been precipitated by chlorides in 10 c.c. urine.

1 c.c. standard silver = ·01 gram NaCl.

So 10 c.c. urine contain (20 − 2S × x) × ·01 gram NaCl.

Thus percentage is obtained.

Example.

19·6 c.c. KCNS = 10 c.c. $AgNO_3$.

So 1 c.c. KCNS = $\frac{10}{19{\cdot}6}$ c.c. $AgNO_3$ = x.

50 c.c. urinary filtrate required 11·6 c.c. KCNS = S.

100 c.c. „ „ „ 23·2 c.c. = 2 S.

23·2 c.c. KCNS = $\frac{23{\cdot}2 \times 10}{19{\cdot}6}$ = 11·8 c.c. $AgNO_3$.

So 20 − 11·8 = 8·2 c.c. $AgNO_3$ = NaCl in 10 c.c. urine.

NaCl in 10 c.c. is 8·2 × ·01 = ·082.

NaCl in 100 c.c. is 0·82 gram.

319. **The estimation of phosphates.**

Principle. Urine is heated to boiling point, and titrated whilst hot with a standard solution of uranium acetate, which gives a precipitate of $(UO_2)HPO_4$ with phosphates in acetic acid solution.

Cochineal tincture is used to indicate by a change in colour when the uranium is in excess.

Reagents required.

(i) A solution containing 100 grams of sodium acetate and 100 c.c. of strong acetic acid to a litre of distilled water.

(ii) Cochineal tincture, prepared by extracting the insects with 30 per cent. alcohol and filtering after two days.

(iii) A standard solution of sodium phosphate. Dissolve twelve grams of pure sodium phosphate in a litre of distilled water. Take 50 c.c. of the filtered solution and evaporate it to dryness in a weighed dish or crucible on a water bath. When dry, raise the temperature to about 130° C., and leave for some hours. Allow the dish to cool in a desiccator and weigh. Let x be the weight of the pyrophosphate obtained. Then to every 100 c.c. of the remaining solution add from a burette $\frac{(2x - \cdot 3746)}{\cdot 003746}$ c.c. of distilled water. A solution is thus obtained of such a strength that 50 c.c. = ·1 gram P_2O_5.

It is more convenient, but not quite so accurate, to prepare the standard solution by dissolving 3·85 grams of KH_2PO_4 in water and making the volume up to 1 litre.

(iv) Standard solution of uranium acetate. Dissolve by the aid of heat 36 grams of uranium acetate in a litre of water. Allow the solution to cool and then filter. Standardise the solution as follows: In a beaker place 50 c.c. of the phosphate solution, add 5 c.c. of the sodium acetate solution and a few drops of the cochineal tincture. Bring the solution to the boiling point, remove the flame and titrate with the uranium acetate solution from a burette till the red tinge just changes to a green, heating the mixture to boiling before the last few drops are added. Suppose x c.c. of the uranium are necessary: then to every 100 c.c. of the uranium solution add $\frac{(20 - x) \times 100}{x}$ c.c. of distilled water. A standard solution of uranium acetate is thus obtained, of which 1 c.c. = ·005 gram P_2O_5.

Method of Analysis. In a beaker of about 100 c.c. capacity place 50 c.c. urine, add 5 c.c. of the sodium acetate solution and a few drops of the cochineal tincture. Have a burette ready containing the standardised uranium acetate solution. Heat the urine to boiling point, remove the flame and run in the uranium acetate as long as a precipitate is formed. Heat the mixture again just to boiling point, and cautiously add uranium acetate, drop by drop, till the red colour is converted to a green.

Calculation of results.

1 c.c. of the uranium acetate = ·005 gram P_2O_5.

Thus if 50 c.c. of urine require 31 c.c. uranium, the percentage of P_2O_5 is $2 \times 31 \times \cdot005 = \cdot31$ gram.

320. **The estimation of total sulphates by Folin's method.**

Place 25 c.c. of urine in a 250 c.c. Erlenmeyer flask, add 20 c.c. of hydrochloric acid (1 volume of concentrated HCl to 4 volumes of water) and boil gently for 30 minutes, covering the mouth of the flask with a small watch glass. Cool the flask under the tap and dilute to about 150 c.c. with water. Add 10 c.c. of 5 per cent. barium chloride solution slowly, drop by drop, to the cold solution, which must not be stirred or shaken during the addition, nor for at least one hour after. Then shake well, filter through a weighed Gooch crucible, wash with 250 c.c. of cold water, dry in an air bath, or over a very low flame. Ignite, cool and weigh.

Calculation. Weight of $BaSO_4 \times 1{\cdot}373 = SO_3$ per cent.

NOTES.—Instead of using a Gooch crucible a washed filter paper (Shleicher and Schüll, No. 589, blue ribbon) may be used.

After washing and drying the ignition may be carried out in a platinum or porcelain crucible, previously weighed. After ignition, the ash should be treated with a drop of 25 per cent. sulphuric acid, cautiously dried and heated again.

A correction must be made for the weight of the ash of the paper.

321. **The estimation of inorganic sulphates by Folin's method.**

Place 25 c.c. of urine and 100 c.c. of water in a 250 c.c. Erlenmeyer flask. Acidify with 10 c.c. of hydrochloric acid (1 volume of concentrated HCl to 4 volumes of water). Add 10 c.c.

of 5 per cent. barium chloride, drop by drop, as in the previous exercise, and proceed as there directed.

Calculation. The same as for total sulphates.

Ethereal Sulphates.

This can be found by the difference total sulphates less inorganic sulphates.

322. **The estimation of total sulphur by Benedict's method.**

Place 10 c.c. of urine in a small (7-8 c.c.) porcelain evaporating dish and add 5 c.c. of Benedict's sulphur reagent.* Evaporate over a free flame, keeping the solution just below the boiling point, to prevent loss by spattering. When dry, raise the flame slightly until the entire residue has blackened. Raise the flame still more and heat to redness for ten minutes after the black residue (which first fuses) has become dry. Allow the dish to cool. Add 10 to 20 c.c. of 1 in 4 hydrochloric acid, and heat again till the residue has completely dissolved to a clear solution. Wash the contents quantitatively into an Erlenmeyer flask, and dilute with cold water to 100 to 150 c.c. Add 10 c.c. of 10 per cent. barium chloride, drop by drop, and allow to stand for about an hour. Shake thoroughly and proceed as in Ex. 320.

323. **The estimation of albumin by Esbach's method.**

Fill the albuminometer to the mark U with urine. Add Esbach's reagent (Ex. 16) to the mark R. Stopper the tube, and invert it slowly several times to mix the fluids. Allow the tube to stand upright for 24 hours.

Calculation. The graduations on the albuminometer indicate grams of albumin per litre.

Fig. 13. Esbach's albuminometer.

* For preparation of Reagent see p. 252.

324. The estimation of albumin by Scherer's method.

Measure 50 c.c. of urine into a beaker. Place it on a water bath and raise the temperature to 50° C. Add 1 per cent. acetic acid, drop by drop, to obtain a complete separation of the protein (care must be taken to avoid an excess). Raise the temperature to boiling, and keep it so for a few minutes. Filter the urine through a small paper that has previously been washed, dried and weighed. Wash the precipitate in turn with hot water, 95 per cent. alcohol and ether. Dry the paper and precipitate in an air bath at 110° C. till the weight is constant. The weight of protein in 50 c.c. is obtained by subtracting the weight of the paper.

CHAPTER XI.

DETECTION OF SUBSTANCES OF PHYSIOLOGICAL INTEREST.

A. Fluids.

1. Neutralise a considerable portion, and evaporate it to dryness, completing the process on a water bath to prevent charring. This evaporation to dryness is only necessary in tests for such substances as urea and the sugars. If these be known to be absent it can be omitted. It is as well to start this evaporation as soon as possible, as it takes a considerable time. Neutralisation is necessary to obviate any chemical changes produced by boiling acids or alkalies.

2. Note any characteristic smell of urine, bile, etc.

3. Note the colour and appearance of the fluid: opalescence suggests starch, glycogen, or certain protein solutions; coloured fluids suggest bile, blood or urine.

4. Note the reaction to litmus. An acid reaction excludes the presence of mucin, nucleoproteins, caseinogen, and usually, earthy phosphates.

5. If acid test for free HCl by Gunsberg's test. (Ex. 187A.)

6. Sprinkle some flowers of sulphur on the surface of a portion of the fluid in a test-tube. If the particles fall through the surface, bile salts are present. (Ex. 224.) Confirm by Pettenkofer's test. (Ex. 223.)

7. If the fluid be brown or green, apply Cole's test (Ex. 227) for bile pigments.

8. If the fluid be red or brown, examine for blood-pigments or derivatives by Table F.

9. If there are any reasons for suspecting the presence of ferments, examine by Table G. If none of the colour reactions for proteins are obtained, ferments are probably absent.

10. Examine for proteins by Millon's and the biuret reactions. (Ex. 2 and 4.) If they be present, proceed as directed in Table A, B or C, according to the reaction of the fluid.

11. If proteins are absent, proceed to Table E.

12. Test for uric acid if the fluid be alkaline, neutral or only faintly acid. Acidify with a drop or two of strong hydrochloric acid; uric acid may separate out as a crystalline powder. Make another portion of the solution alkaline with ammonia, saturate with NH_4Cl and apply the murexide reaction to the precipitate thus obtained. (Ex. 261.)

13. If the fluid be alkaline, treat a little with a solution of calcium chloride. A white curdy precipitate indicates the presence of soaps. (Their presence should be confirmed by the methods given in Ex. 128.)

Table A.

Analysis of an **acid** solution containing proteins.

<table>
<tr><td colspan="4">Add sodium carbonate solution till neutral to litmus or till the maximum precipitate has been obtained and filter.</td></tr>
<tr><td colspan="2">Precipitate. Treat with 5 c.c. of dilute NaOH on the filter paper, passing the filtrate through two or three times.</td><td colspan="2">Filtrate A. Boil. Whilst boiling add 1 % acetic acid, drop by drop, to keep the reaction faintly acid. Filter.</td></tr>
<tr><td>Residue. Dissolve in nitric acid. Add ammonium molybdate & boil. Yellow ppt. indicates

Earthy phosphates.</td><td>Filtrate. Neutralise with dilute acetic acid. Precipitate, soluble in excess of acid indicates

Metaprotein.</td><td>Coagulum indicates albumin or globulin. If obtained, treat a portion of Filtrate A with an equal volume of saturated ammonium sulphate solution and filter.

Precipitate. Scrape off the paper. Dissolve in a small amount of cold water and boil. Coagulum indicates

Globulin.</td><td>Filtrate. Boil. Coagulum indicates

Albumin.</td><td>Filtrate B. Proceed as in Table D.</td></tr>
</table>

Table B.

Analysis of a **neutral** solution containing proteins.

Acidify a portion with dilute acetic acid.	
Precipitate. (i.) **Bile salts with any protein.** Test original solution for bile salts. (Ex. 224.) (ii.) **Mucin.** Insoluble in strong acetic acid. (iii.) **Casein, caseinogen or nucleoprotein.** Soluble in strong acetic acid. Contain phosphorus. (Ex. 39.)	*Filtrate.* Neutralise and proceed as for filtrate A, Table A.

Table C.

Analysis of an **alkaline** solution containing proteins.

Add a drop of litmus and then acetic acid till distinctly acid. Filter.		
Precipitate. (i.) **Bile salts with any protein.** Test original solution for bile salts by Hay's test. (Ex. 117.) (ii.) **Fatty acids.** Test original solution for soaps by Ex. 128. (iii.) **Mucin.** Insoluble in strong acetic acid. (iv.) **Casein, caseinogen or nucleoprotein.** Soluble in strong acetic acid. Contain phosphorus. (See Ex. 21.)	*Filtrate.* Neutralise with sodium carbonate.	
	Ppt. Soluble in excess of alkali. **Metaprotein.**	*Filtrate.* Proceed as for filtrate A, Table A.

Table D.

Examination of Filtrate B for **albumoses, peptones** and **gelatin.**

<table>
<tr><td colspan="4">Treat a portion with caustic soda and a drop of copper sulphate solution.</td></tr>
<tr><td rowspan="3">No biuret reaction.</td><td colspan="3">Positive biuret reaction. To portions of filtrate B apply Millon's and glyoxylic tests.</td></tr>
<tr><td rowspan="2">Negative reactions.</td><td colspan="2">Positive reactions. Saturate filtrate B with ammonium sulphate by heating with excess of solid. Cool under tap and filter.</td></tr>
<tr><td>Precipitate. Mostly sticking to tube. Wash with cold saturated ammonium sulphate. Dissolve in a little boiling water and cool under tap. To portions apply biuret test (using 40 per cent. NaOH) and the glyoxylic test. If both are positive—</td><td>Filtrate. Treat 3 c.c. with 6 c.c. of 40 per cent. NaOH and a drop of copper sulphate. Pink colour indicates</td></tr>
<tr><td>Proteins absent.</td><td>Gelatin present.</td><td>Albumoses.</td><td>Peptones.</td></tr>
</table>

Table E.

Examination of a solution for **carbohydrates** and **urea.**

If proteins be present they must be removed, as far as possible, by neutralising, boiling and filtering.

In any case the solution tested must be neutral.

(*a*) To a small portion add diluted iodine drop by drop, until an excess has been added. If a pure blue colour be obtained at any stage of the addition of iodine, starch is present. If a purple or brown colour be produced and the fluid be quite clear, erythro-dextrin

is present and glycogen absent. If a blue colour be produced, or if the fluid be opalescent, proceed as follows:

To a portion of the fluid, prepared as directed above, add an equal bulk of saturated $(NH_4)_2SO_4$, shake vigorously, and filter through a dry paper after about ten minutes.

Precipitate.	*Filtrate.* To a small portion add a drop or two of iodine. If a reddish or purple colour be produced, glycogen or dextrin is present. If the fluid be opalescent after warming, **glycogen** is present. Saturate the remainder with $(NH_4)_2SO_4$ and filter.
Scrape off the paper, dissolve in a little hot water, cool and add a drop of iodine. A blue colour shows the presence of **starch.**	*Precipitate.* Neglect. / *Filtrate.* Add a drop of diluted iodine, a red-brown colour shows the presence of **erythro-dextrin.**

(*b*) Apply Benedict's (Ex. 68) or Fehling's test (Ex. 67) for **reducing sugars.** Note that the tests do not succeed in the presence of any considerable amount of ammonium salts. Also that if albumoses, peptones or gelatin are present they should be removed by alcoholic extraction as described in Ex. 55.

(*c*) If a reduction be obtained, apply Barfoed's test (Ex. 69) to distinguish between mono- and di-saccharides. The osazone test (Ex. 73) also can be applied if necessary.

(*d*) Test for **cane-sugar** by Exs. 74-76.

(*e*) Examine for **urea.**

Apply hypobromite test. (Ex. 248.) Effervescence indicates urea or ammonium salts. If obtained boil with a little strong sodium hydroxide: if a marked smell of ammonia be not obtained, ammonium salts are absent and urea is probably present. In any case attempt to obtain crystals of urea nitrate by the following method:

Evaporate the fluid to dryness on a water bath. Add alcohol, stir till boiling, rub up thoroughly and filter. Evaporate the alcohol to dryness on a water bath. Add a few drops of water to the residue, transfer a drop of the solution to a slide, add a drop of strong nitric acid and examine for crystals of urea nitrate. (Ex. 244.)

Table F.

Examine the solution spectroscopically: gradually dilute the solution, noting the spectrum at all stages of dilution.

Take the reaction of the undiluted fluid to litmus paper, washing the surplus off the paper with a stream of distilled water, if you are unable to note the reaction directly.

If the fluid be neutral or alkaline, treat it with Stokes' fluid or warm it with ammonium sulphide, and note whether the spectrum is altered by reduction. This should be done after various dilutions of the original solution.

- **Fluid red**
  - Acid—*Acid haematoporphyrin*, two bands. (Ex. 220.)
  - Neutral — Dilute till two bands are well seen and then reduce.
    - *Oxyhaemoglobin*, the two bands merge into one faint band. (Ex. 207.)
    - *CO-haemoglobin*, the two bands are unaltered. (Ex. 209.)
  - Alkaline
    - *Alkaline haematoporphyrin*, four bands, converted into acid haematoporphyrin by strong acids. (Exs. 220 and 221.)
    - *Haemochromogen*, two bands in green, one much more distinct than the other, unaffected by reducing reagents. (Ex. 219.)

- **Fluid brown**
  - Acid—*Acid haematin*, band in red. (Ex. 215.)
  - Neutral. *Methaemoglobin*, band in red: gives spectrum of oxyhaemoglobin and then of reduced haemoglobin if reduced. (Ex. 213.)
  - Alkaline
    - *Alkaline haematoporphyrin*—four bands. (Ex. 221.)
    - *Alkaline haematin*, faint band in red, converted to haemochromogen by reducing reagents. (Exs. 217, 219.)

Table G.

Examination of a solution for **ferments.**

Take the reaction of the fluid to litmus.

I. Markedly acid.
   Examine for pepsin. (Ex. 185.)
   Neutralise very carefully and examine for rennin. (Ex. 143.)

II. Faintly acid or neutral.
   Examine for ptyalin. (Ex. 177.)
   Examine for pepsin. (Ex. 185.)
   Examine for rennin. (Ex. 143.)
   Examine for trypsin. (Ex. 190.)
   Examine for lipase. (Ex. 116.)

III. Distinctly alkaline.
   Examine for ptyalin. (Ex. 177.)
   Examine for trypsin. (Ex. 190.)
   Examine for lipase. (Ex. 116.)

Perform control experiments in all cases. (See Ex. 118.)

A few special hints on the examination of physiological fluids.

1. It is impossible to obtain a heat coagulum of albumin or globulin in an acid or alkaline fluid. The reaction must be *neutral* or only very faintly acid.

2. A little litmus solution in the fluid does no harm, and often reminds one that the reaction changes after boiling (owing to the evolution of CO_2).

3. In testing for peptones, after removing the albumoses by saturation with ammonium sulphate, the biuret test succeeds only if at least two volumes of 40 per cent. soda are used. The test will not be obtained with the ordinary 5 per cent. soda.

4. Gelatin reacts very much like the albumoses, except that it does not yield the glyoxylic reaction.

5. It is impossible to obtain Fehling's or Benedict's test for the reducing sugars in the presence of ammonia or ammonium salts.

6. The sugars reduce only in an alkaline medium. If the fluid under examination be acid, it must be neutralised before boiling with the Fehling's or Benedict's solution.

7. In testing for cane sugar do not forget that starch and the dextrins are hydrolysed to dextrose by boiling acids. But whereas cane sugar is hydrolysed very easily, starch, etc., are only slowly acted on.

8. Starch, glycogen and the erythro-dextrins do not give any colour with iodine solutions, if the reaction of the fluid be alkaline. If this be the case, make the reaction acid with acetic acid.

9. The proteins interfere with the iodine tests for these substances, and should therefore as far as possible be removed before testing for the polysaccharides.

10. Fat is insoluble in water, so do not waste time in testing an ordinary solution for fats.

11. The only reliable test for urea is to obtain crystals of the nitrate or oxalate. In this connection it must be remembered that urea is soluble in alcohol, and can thus be separated from the proteins and other substances likely to interfere with crystal formation.

12. Ammonium chloride is a very valuable reagent in testing for uric acid or urates. The only other physiological substance precipitated by it is soap.

13. Never omit "control" tests when investigating the ferment action of a solution.

14. Use "carmine fibrin" in testing for pepsin; never when testing for trypsin.

15. In testing solutions for pigments, examine spectroscopically in various dilutions. Note the reaction of the fluid; it is no good looking for haemochromogen in a markedly acid solution.

B. Solids.

1. Examine a little microscopically, both dry and with the addition of a drop of water. Look for starch grains, crystals of urea, uric acid, urates, leucine, tyrosine, cholesterin, and haemin scales.

2. Heat a small amount of the solid in a dry tube, at first gently and then more strongly.

(*a*) If sublimation take place and an odour of amylamine be given off, leucine is present.

(*b*) If sublimation take place and a strong smell of ammonia be evolved, urea is indicated.

(*c*) A smell of phenol and nitro-benzol indicates tyrosine.

(*d*) A smell of burning feathers indicates proteins, gelatin, etc.

(*e*) A smell of acrolein indicates fats.

3. Boil some of the solid with a small amount of water in a tube, cool under the tap and leave the test-tube in a beaker of cold water for 10 minutes. If gelatin be present, the solution will set to a jelly. (Starch, if present, may form a thick paste, which may be confused with the clean jelly given by gelatin. If the tube be subsequently placed in boiling water, gelatin becomes quite limpid, whilst starch remains thick.)

4. If the solid be of a dark brown or red colour, boil a portion with dilute alkali, filter, heat the filtrate with Stokes' fluid or $(NH_4)_2S$, and examine for the spectrum of haemochromogen. (Ex. 219.) If this be obtained, the solid contains dried blood or haematin. Confirm by obtaining haemin crystals. (Ex. 222.)

5. Examine by the method indicated in the Table on the next page.

Analysis of a Solid for substances of Physiological Interest.

Heat a considerable amount of the solid with strong alcohol on a water bath, stirring well for some time. Remove the dish from the bath, and filter the alcohol into a dry vessel. Extract the residue once more with alcohol, filter and mix the alcohols.

<table>
<tr><td colspan="2">Alcoholic Solution. Evaporate to dryness on the water bath. Add 5 c.c. of water, heat and stir.</td><td colspan="3">Residue insoluble in alcohol. Treat with water, warm to about 40° C., and add acetic acid till the reaction is just acid. Cool and filter.</td></tr>
<tr><td>Residue. May contain fats, fatty acids or cholesterin.</td><td>Aqueous Solution. May contain urea, reducing sugars, cane sugar, bile-salts, soaps.</td><td>Solution. Test for albumoses and peptones (Table D). Glycogen and dextrin. Earthy phosphates.</td><td colspan="2">Residue. Treat with 2 per cent. Na_2CO_3, shake well and filter.</td></tr>
<tr><td colspan="3"></td><td>Solution. Test for urates (by Schiff's test) (Ex. 262), nucleo-proteins and caseinogen</td><td>Residue. Boil with water and filter.

Solution. Test for starch and urates.</td><td>Residue. Test for coagulated proteins.</td></tr>
</table>

APPENDIX.

1 grain = ·0648 gram.

1 ounce = 437·5 grains = 28·3595 grams.

1 lb. = 16 oz. = 7000 grains = 453·5925 grams.

1 gram = 15·432 grains.

1 kilogram = 1000 grams = 2 lb. $3\frac{1}{4}$ oz. (approximately).

1 minim = ·059 c.c.

1 fluid drachm = 60 minims = 3·55 c.c.

1 fluid ounce = 8 fluid drachms = 28·4 c.c.

1 pint = 20 fluid oz. = 567·9 c.c.

1 c.c. = 16·9 minims.

1 litre = 1000 c.c. = 35·2 fluid oz.

1 gallon = 8 pints = 4·548 litres.

1 inch = 2·54 cm.

1 foot = 30·48 cm.

1 yard = 91·44 cm.

1 cm. = ·39 in.

1 metre = 39·37 in.

Conversions.

To convert grams per 100 c.c. into grains per fluid ounce, multiply by 4·375.

To convert grams into ounces, multiply by 10 and divide by 284.

To convert litres into pints, multiply by 88 and divide by 50.

To convert kilos into pounds, multiply by 1000 and divide by 454.

To convert degrees Fahrenheit into degrees Centigrade, subtract 32, multiply the remainder by 5, and divide the result by 9.

To convert Centigrade into Fahrenheit, multiply by 9, divide by 5, and add 32.

TENSION OF AQUEOUS VAPOUR

in millimetres of mercury from 8° to 25° C.

°C.	mm.	°C.	mm.	°C.	mm.
8	8·0	14	11·9	20	17·4
8·5	8·3	14·5	12·3	20·5	17·9
9	8·6	15	12·7	21	18·5
9·5	8·9	15·5	13·1	21·5	19·1
10	9·2	16	13·5	22	19·6
10·5	9·5	16·5	14	22·5	20·2
11	9·8	17	14·4	23	20·9
11·5	10·1	17·5	14·9	23·5	21·5
12	10·5	18	15·3	24	22·2
12·5	10·8	18·5	15·8	24·5	22·9
13	11·2	19	16·3	25	23·5
13·5	11·5	19·5	16·8		

INTERNATIONAL ATOMIC WEIGHTS.

		Revised **0 = 16.**	Approx.			Revised **0 = 16.**	Approx.
Barium	Ba.	137·37	137	Manganese	Mn.	54·93	55
Bromine	Br.	79·92	80	Nitrogen	N.	14·01	14
Calcium	Ca.	40·07	40	Oxygen	O.	16	16
Carbon	C.	12	12	Phosphorous	P.	31·04	31
Chlorine	Cl.	35·46	35·5	Potassium	K.	38·6	39
Copper	Cu.	63·57	63	Silver	Ag.	107·88	108
Hydrogen	H.	1·008	1	Sodium	Na.	23	23
Iodine	I.	126·92	127	Sulphur	S.	32·07	32
Iron	Fe.	55·84	56	Uranium	U.	238·5	239
Magnesium	Mg.	24·32	24	Zinc	Zu.	65·37	65
Mercury	Hg.	200·3	200				

Preparation of Normal Solutions of Acids and Alkalies.

A normal solution of a substance contains in one litre that weight in grams which corresponds to 1 equivalent in grams (1·008) of available hydrogen or its equivalent.

Thus normal hydrochloric acid contains 35·46 + 1·008 = 36·468 gm. of HCl per litre.

Normal sulphuric acid contains $\frac{2 \cdot 016 + 32 \cdot 07 + 64}{2} = 49 \cdot 043$ gm. H_2SO_4 per litre.

Other normal solutions are :—

Crystallised oxalic acid $(COOH)_2\ 2H_2O$	= 63·03 gm. per litre.
Anhydrous sodium carbonate	= 53 ,, ,, ,,
Sodium hydroxide	= 40·01 ,, ,, ,,
Ammonia	= 17·034 ,, ,, ,,

The most reliable starting point for the preparation of the standard solutions is anhydrous sodium carbonate, obtained by igniting sodium bicarbonate.

Powdered sodium bicarbonate in a platinum dish is placed in an air bath previously heated to 200° C. The temperature is raised to 270°—280°, but not more than 300° C. It is kept at this temperature for an hour, and cooled by placing the dish in a desiccator. Before it is quite cold it is transferred to warm, dry, stoppered weighing tubes, which are allowed to cool in the desiccator.

Have ready an approximately normal solution of sulphuric acid. This is obtained as follows :—Weigh 104 grams of pure sulphuric acid into a counter-balanced beaker, pour the acid into a litre flask containing about 500 c.c. of distilled water, rinse the beaker out several times with water, adding the rinsings to the flask, make up to 1000 c.c., mix and allow to stand several hours to acquire room temperature. Transfer to a dry Winchester quart bottle and add 1 litre of water.

Weigh one of the tubes of sodium carbonate, transfer 2 or 3 grams to a 200 c.c. beaker of Jena glass, and weigh the tube again to determine the exact amount of sodium carbonate taken. Dissolve this in 80 to 100 c.c. of distilled water, add a single drop of methyl orange (0·1 per cent.) and titrate with the sulphuric acid from a burette till the mixture just turns pink.

Suppose that 2·55 gm. of sodium carbonate are neutralised by 42·4 c.c. of the sulphuric acid.

Then $2 \cdot 55 : 5 \cdot 3 = 42 \cdot 4 : x$ = 88·1 c.c.
That is 88·1 c.c. of the acid = 100 c.c. Normal H_2SO_4
And 1000 c.c. ,, ,, $= \frac{1000 \times 100}{88 \cdot 1}$ c.c.
= 1135 c.c.

To one litre of the sulphuric acid add 135 c.c. of distilled water and mix. The solution thus obtained should be normal sulphuric acid. It should be tested against sodium carbonate as above directed.

Normal sodium hydroxide is prepared by dissolving 85 gm. of pure NaOH in water, and making the volume up to 2 litres. When quite cold, fill a burette with the solution and titrate 20 c.c. of normal sulphuric acid, using methyl orange as an indicator. Suppose 19·6 c.c. of the NaOH are required,

Then 19·6 c.c. has to be diluted to make 20 c.c.

So 1 ,, ,, ,, ,, $\frac{20}{19{\cdot}6}$ c.c.

And 1000 c.c. ,, ,, ,, $\frac{20 \times 1000}{19{\cdot}6}$ c.c.

$= 1020$ c.c.

To 1 litre of the sodium hydroxide solution, add 20 c.c. of distilled water and mix. Check the mixture against the normal sulphuric acid.

Thus having normal solutions of an acid and an alkali, normal solutions of other acids and alkalies can readily be prepared.

Strengths of Acids and Alkalies.

ACIDS.

	100 c.c. contain
Acetic acid, glacial, sp. gr. 1·06	111·1 gm.
Acetic acid, "strong"	33 gm.
Acetic acid, 1 per cent.	1 gm.
(9 c.c. of glacial acetic acid made up to 1 litre)	
Hydrochloric acid, concentrated, sp. gr. 1·16	36·6 gm.
Hydrochloric acid, 0·4 per cent.	0·4 gm.
(11 c.c. conc. HCl added to 1 litre)	
Nitric acid, fuming, sp. gr. 1·40	about 65 gm.
Nitric acid, concentrated, sp. gr. 1·42	99 gm.
Sulphuric acid, concentrated, sp. gr. 1·84	175·9 gm.

ALKALIES.

Ammonia, concentrated, sp. gr. ·880	31 gm.
Sodium hydroxide, sp. gr. 1·34	40 gm.
(410 gm. of 98% NaOH or	
426 gm. of 94% NaOH made up to 1 litre)	

Phenyl-glucosazone (Ex. 73).

Fine yellow needles in fan-shaped aggregates, sheaves or crosses.

Phenyl-lactosazone (Ex. 84).

Ovoid or spherical clusters of fine yellow needles.

Phenyl-maltosazone (Ex. 81).

Broad yellow plates, either singly or arranged in spherical clusters.

Potato Starch (Ex. 85).

Ovoid or elliptical grains, with concentric markings and an eccentric hilum.

Wheat Starch (Ex. 151).

Small circular grains with a central hilum.

Tyrosine (Ex. 191).

Feathery masses and sheaves of fine white needles.

Leucine (Ex. 191).

Rounded cone or balls with a radiating striation.

Oxyhaemoglobin (dog) (Ex. 203).

Thin rhombic prisms.

Haemin (Teichmann's Crystals, Ex. 222).

Brown rhombic prisms.

Cholesterin (Ex. 229).

Rhombic plates, often incomplete.

Urea crystallised from acetone (Ex. 245).
Long four-sided prisms or fine needles.

Urea, crystallised from alcohol (Ex. 246).
Irregular branching masses.

Urea Oxalate (Ex. 243).

Long thin flat crystals, often in bundles. Rhombic prisms.

Urea Nitrate (Ex. 244).

Octahedral, lozenge-shaped, or hexagonal plates, striated or imbricated.

Uric Acid (Exs. 258, 270).

(*a*) Rhombic plates

(*b*) Irregular forms, such as dumb bells, whet-stones, butcher-trays, stars and crosses

Urinary Sediments (page 169).

(*a*) Urates (spheres with projecting spines)

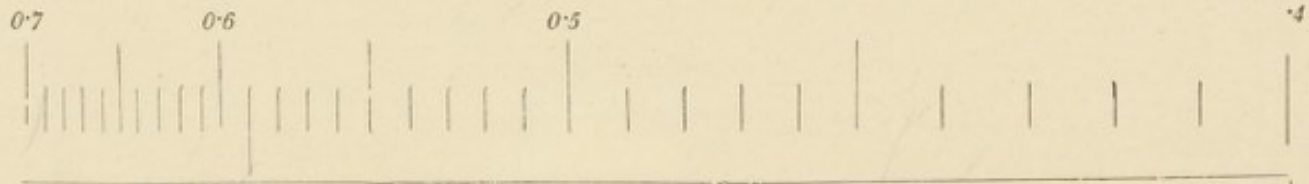
(*b*) Calcium oxalate (envelopes or dumb-bells)

(*c*) Calcium hydrogen phosphate (stellar phosphate)

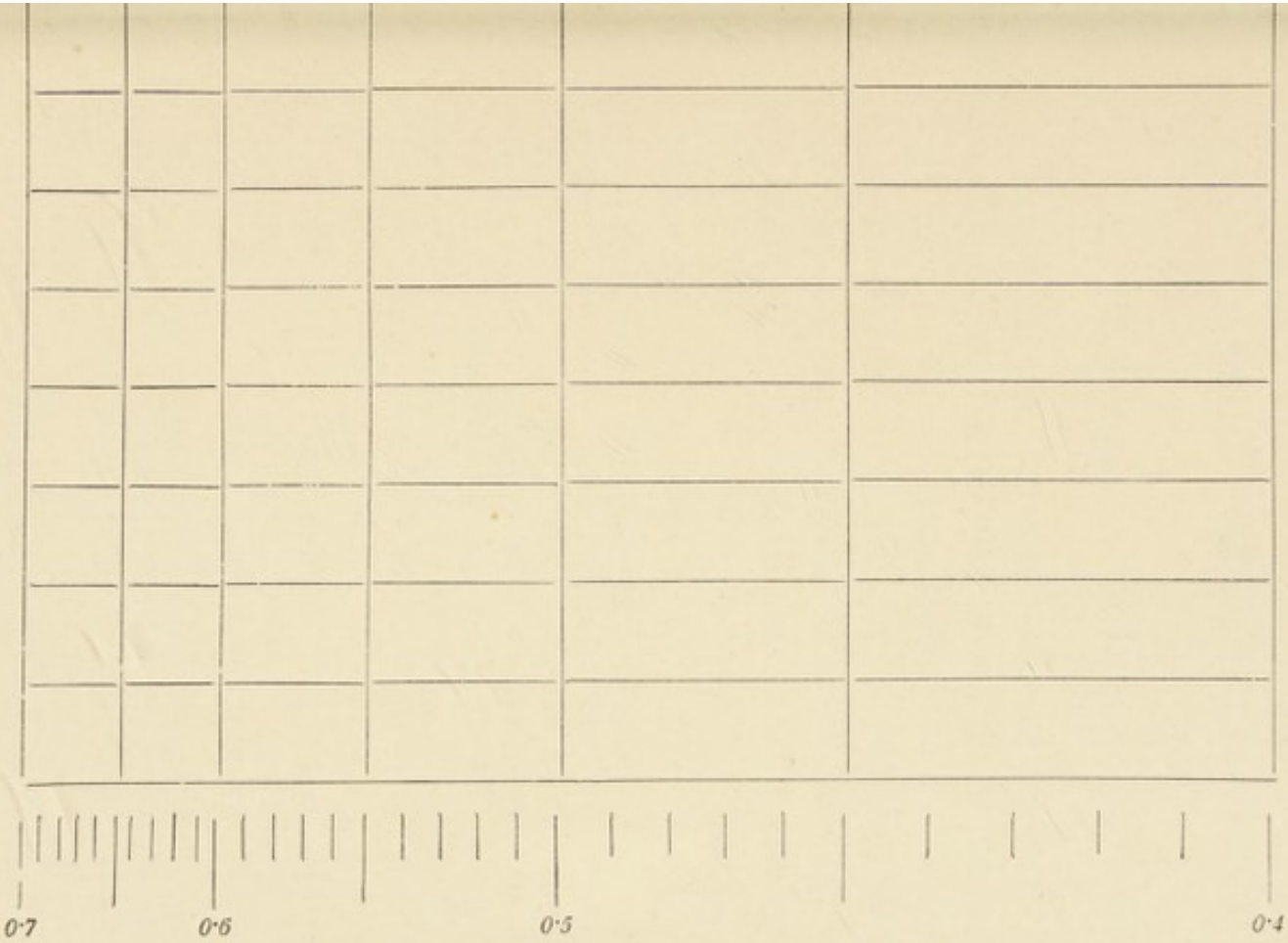
(*d*) Ammonium-magnesium phosphate (triple phosphate), prisms (coffin lids) or feathery stars

(*e*) Calcium carbonate, dumb-bells or spheres with radiating structure

CHART FOR RECORDING THE ABSORPTION-SPECTRA OF PIGMENT SOLUTIONS.



	C	D	E	b	F	G	
							1.
							2.
							3.
							4.
							5.
							6.
							7.
							8.



1. Fraunhofer's lines
2. Oxy-haemoglobin (dilute)
3. Oxy-haemoglobin (medium)
4. Oxy-haemoglobin (strong)
5. Reduced haemoglobin
6. CO-haemoglobin
7. Methaemoglobin (strong)
8. Methaemoglobin (dilute)
9. Acid haematin
10. Acid haematin in ether
11. Alkaline haematin
12. Haemochromogen
13. Haematoporphyrin (Acid)
14. Haematoporphyrin (Alkaline)
15. Urobilin
16. Pettenkofer's reaction

MORE RECENT METHODS.

325. The Estimation of Glucose by Bang's Method II.

Principle. A known volume of the sugar solution is boiled with an alkaline solution of cupric chloride. Cuprous chloride is thus formed. The amount of cuprous chloride is determined by titration with a standard solution of iodine. From the volume of iodine required the amount of glucose in the solution can be calculated.

Preparation of Solutions.

1. Stock copper solution. In a litre flask place 700 c.c. of boiled out, cold distilled water. Warm to about 30°C. Add 160 gm. of pure powdered potassium bicarbonate. When dissolved, add 66 gm. of pure potassium chloride. Cool and add 100 gm. of potassium carbonate. Then 100 c.c. of a 4·4 per cent. solution of pure crystalline copper sulphate. Allow to stand for a while and fill up to the mark with boiled out distilled water. Vigorous shaking of the fluid must be avoided. The solution should not be used for 24 hours.

2. Diluted copper solution. 300 c.c. of the stock solution are diluted to 1 litre with a cold saturated solution of potassium chloride. Avoid excessive shaking and allow to stand for some hours before use.

3. N/10 iodine solution. 12·685 gm. of pure resublimed iodine are weighed out from a stoppered tube into 25 gm. of potassium iodide in 100 c.c. of distilled water. The volume is made up to 1 litre with distilled water.

4. N/100 iodine solution. 10 c.c. of N/10 iodine are diluted to 100 c.c. with boiled out distilled water. The solution is only stable for 24 hours.

5. Soluble Starch. 1 gm. of Kahlbaum's soluble starch is dissolved in 100 c.c. of a boiling solution of potassium chloride, saturated at room temperature. After cooling the volume is made up to 100 c.c. with distilled water.

Method. The flange of the neck of a 100 c.c. Jena flask is cut off by means of a file. The neck is fitted with a piece of rubber tubing 4 to 5 cm. in length and about 3 mm. thick. This rubber collar should extend about 2 cm. above the neck. (See Fig. 14.)

In the flask place 0·1 to 2 c.c. of the sugar solution, containing not more than 10 mg. of glucose. Add 55 c.c. of the diluted copper solution. Heat to boiling on a piece of asbestos gauze over a burner. Allow the boiling to continue for exactly 3 minutes. Seal the rubber tubing completely by means of a screw clip, remove the flask from the gauze and cool it by immersion in cold water for about 1 minute. Loosen the screw to admit air, remove the rubber tubing, add 4 or 5 drops of the soluble starch and titrate at once with the N/100 iodine. Vigorous shaking must be carefully avoided. The addition of iodine to the mixture gives rise to a deep blue which is at first rapidly discharged. The titration is completed when the "starch-blue" tinge is permanent for 20 secs. It is essential that the titration be conducted rapidly to minimise the re-oxidation of cuprous to cupric chloride by the air. It is convenient, but not absolutely essential, to titrate in an atmosphere of CO_2, as described in the method for the analysis of sugar in blood (p. 235).

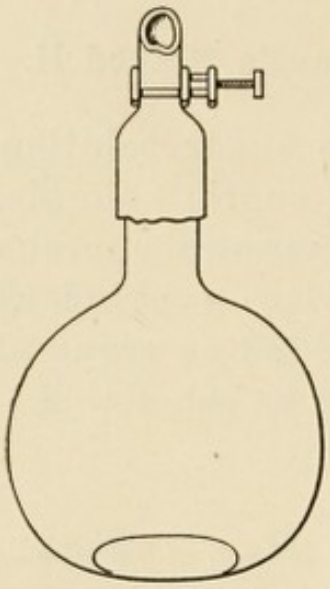


Fig. 14. Flask fitted for sugar estimation.

Calculation. c.c. of iodine divided by 2·7 (or multiplied by 0·3704) = mg. of glucose in the volume of solution taken.

NOTES.—1. The solution must not contain proteins.

2. The method is well adapted to the estimation of glucose in urine.

3. N/25 or N/10 iodine can be used for the titration, but a microburette is necessary. If N/25 is used, divide by 0·7 ; if N/10, divide by 0·285.

4. It is advisable to use the standard heating apparatus devised by the author. It is illustrated and described in fig. 15 (p. 229). The heating should be such that the mixture begins to boil in $1\frac{1}{2}$ mins.

326. **The estimation of glucose by the Method of Amos Peters.**

Principle. A known amount of the sugar solution is boiled with a measured amount of an alkaline solution of copper sulphate. The cuprous oxide is filtered off and the copper in the filtrate determined by treatment with potassium iodide and titration of the iodine liberated by means of a solution of sodium thiosulphate.

Solutions required.

1. Copper sulphate. 69·278 gm. of the purest crystalline salt $CuSO_4$, $5H_20$, is dissolved in water and the volume made up to 1 litre. It is advisable to use Kahlbaum's copper sulphate "with certificate of analysis," and to allow for the very small amount of impurity stated on the certificate.

2. Alkaline tartrate. 346 gm. of Rochelle salt and 250 gm. of pure potassium hydroxide are dissolved in water and the volume made up to 1 litre.

3. N/5 Sodium thiosulphate. 99·2 gm. of the purest thiosulphate are dissolved in boiled out distilled water and the volume made up to 1 litre with boiled out distilled water. It should be prepared at least a week before it is standardised.

4. Potassium iodide. Saturated solution. 100 gm. of the solid are treated with 70 c.c. of hot distilled water and the solution allowed to cool.

5. Soluble starch. Shake 1 gm. of Kahlbaum's soluble starch with about 10 c.c. of distilled water and pour the suspension into 90 c.c. of boiling water.

Standardisation of the thiosulphate. Measure 20 c.c. of the copper sulphate into a 200 c.c. Erlenmeyer flask. Add 40 c.c. of distilled water and 20 c.c. of strong (33 per cent.) acetic acid. Insert a thermometer and cool or warm to 20° C. Run in about 6·5 c.c. of the saturated potassium iodide, the thermometer being withdrawn and its stem washed with this solution. The iodine liberated is titrated at once with the thiosulphate. When approaching the end point add about 1 c.c. of the soluble starch. The colour changes to a chocolate brown when very near the end point. This is best determined by the "spot test" method. Allow a drop of the thiosulphate to fall on the quiet surface of the liquid.

If the end point has not been reached, a very perceptible white area is seen around the drop. This is very readily distinguished from the diminution of the slightly yellowish colour of the suspended cuprous iodide. The volume of the drop delivered by the burette must be deducted from the total volume added.

The copper value of the thiosulphate is calculated as shewn in the following example :—

20 c.c. of the copper sulphate = 352·93 mg. Cu.

This required 27·6 c.c. of thiosulphate.

So 1 cc. of thiosulphate $= \frac{352{\cdot}93}{27{\cdot}6} = 12{\cdot}78$ mg. Cu.

The heating apparatus. Use the apparatus shewn in fig. 15. In a 200 c.c. Erlenmeyer flask of Jena glass, and of about 6 cm. basal diameter, place 60 c.c. of distilled water. The flask is fitted with a 2-hole rubber stopper carrying a thermometer so graduated that the stem above 34° C. is visible above the upper edge of the stopper. The lower end of the thermometer should be about 2 mm. from the bottom of the flask.

Turn on the tap B to its full extent and light the flame of a Bunsen or Meker burner which is placed under a piece of asbestos gauze carried by an adjustable ring stand. The gauze should be from 4 to 6 cm. above the top of the burner. Tighten the screw A till the pressure is reduced about one-third. Allow the gauze to get thoroughly heated and then place the flask in the centre of the heated gauze. By means of a stop-watch note the time for the temperature to rise from 35° to 95°. If the time is greater or less than 120 secs. loosen or tighten the screw A and repeat the experiment with another 60 c.c. of distilled water until the temperature of the water rises from 35° to 95° in 120 ± 2 secs. The height of the ring and the thickness of the asbestos should be such that the pressure is well under the minimum supplied to the

laboratory and yet sufficient to prevent any risk of the flame striking back. Note the manometer reading. The standard heating power can be rapidly obtained for further experiments by adjusting the screw A so that the manometer shews the requisite pressure.

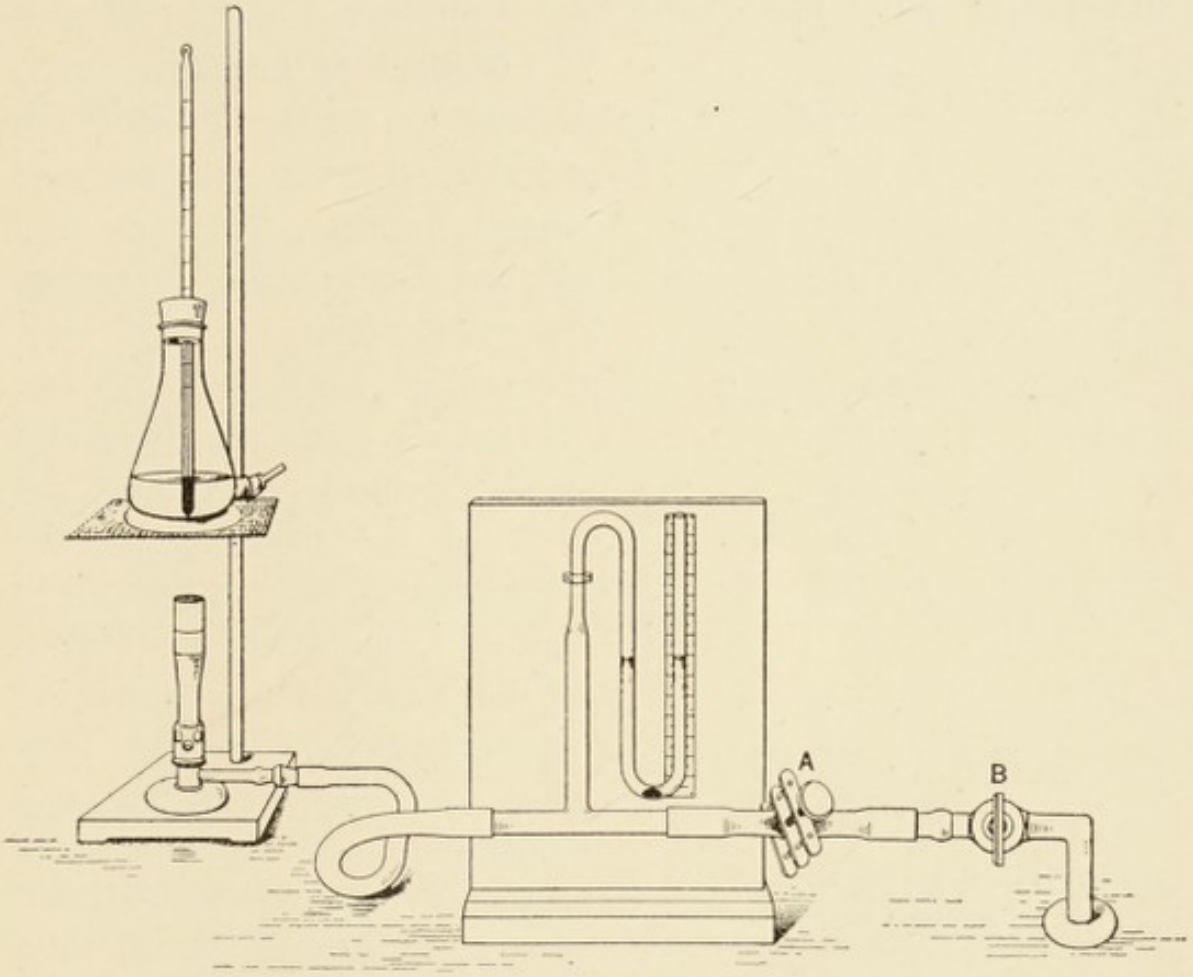


Fig. 15. Apparatus for maintaining a standard heating power. The manometer tube contains a dilute solution of eosin or other dye. It also contains a globule of mercury which nearly fills the bottom of the tube. This prevents the rapid oscillations of pressure due apparently to the explosions of local gas engines.

Filtering Apparatus. It is convenient to use the apparatus shown in Fig. 16. *A* is a Jena flask of 200 c.c. capacity. Tube *B* is an ordinary calcium chloride tube. The lower end should reach at least 3 cm. below the lower edge of the stopper to prevent loss by splashing during filtration. The filtering mat is made of glass wool, asbestos fibre, powdered pumice and asbestos fibre added in

that order. The mat should be washed with nitric acid and then thoroughly washed with water. After a test the cuprous oxide on the mat is dissolved in nitric acid diluted with an equal volume of water and then thoroughly washed.

An ordinary Gooch crucible can be used with a mat prepared in the same way. The arrangement is shewn in Fig. 20, p. 239.

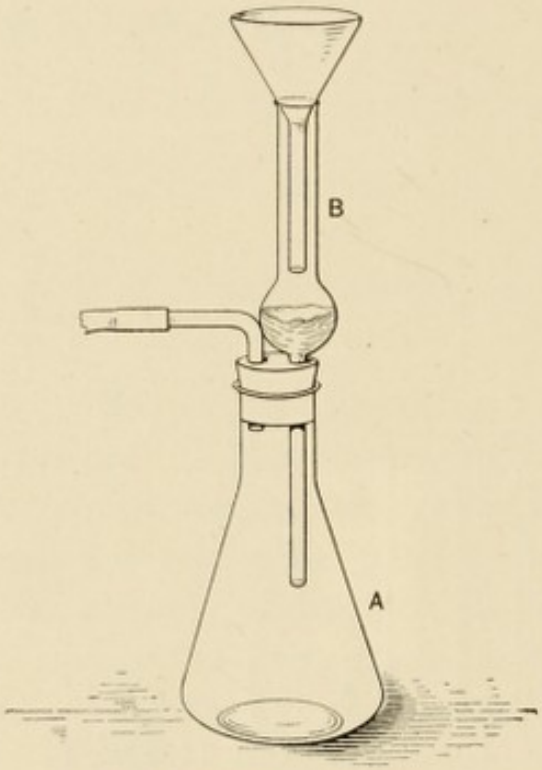


Fig. 16. Filtering apparatus for reduced copper.

Method of Analysis. Into a 200 c.c. Erlenmeyer flask measure 20 c.c. of the standard copper sulphate, 20 c.c. of the aklaline tartrate and 20 c.c. of the sugar solution (which must not contain more than 180 mg. of glucose). Fit the two-holed rubber stopper firmly into the neck of the flask, adjust the thermometer so that its lower end is 2 mm. from the bottom of the flask and place on the heated gauze. Note the time when the mercury indicates a temperature of 95° C. Allow the heating to continue for exactly 20 secs. beyond this. Remove the flask by gripping the rubber stopper and swill it for a second or two under the tap or in a bowl of water. The reduction of the temperature practically stops the reduction. Filter the hot fluid at once, using the stem of the thermometer as a stirring rod. Wash the flask twice with about 7 c.c. of distilled water. Cool the filtrate by holding the flask under the tap. Add exactly 4 c.c. of strong sulphuric acid, insert a thermometer and cool to 20°. Add 6·5 to 7 c.c. of the saturated solution of potassium iodide, washing the stem of the thermometer with this solution. Titrate at once with the standardised solution of sodium thiosulphate as described above, using soluble starch as an indicator when near the end point.

Calculation of results. From the amount of thiosulphate required the amount of copper in the filtrate is determined. Knowing the amount taken (352·9 mg.), the amount reduced by the sugar can be calculated. The amount of glucose corresponding to this copper can be determined by a reference to the curve in Fig. 17.

Example. The copper in the filtrate required 14·62 c.c. of thiosulphate.

1 c.c. of thiosulphate = 12·86 mg. Cu.
So copper in filtrate = 14·62 × 12·86 = 188·1 mg. Cu.
So copper reduced by glucose in 20 c.c. = 352·9 − 188·2 = 164·7 mg.
From the curve this is seen to correspond to 86·3 mg. glucose.
So 20 c.c. contain 86·3 mg. glucose.
So 100 c.c. contain 431·5 mg. glucose. = 0·431 per cent.

NOTE.—If the amount of reduced copper is between 60 and 200 mg., the amount of glucose corresponding to this can be obtained by multiplying by 0·522.

327. **The estimation of lactose by the copper-iodide method.**

The method is exactly similar to that described in the previous exercise. The author is responsible for the copper values for lactose. They are represented graphically in Fig. 18.

It must be noted that the results are given as anhydrous lactose and not as the crystalline hydrate.

In the case of lactose as much as 250 mg. may be present in the 20 c.c. taken.

The copper values above 25 mg. Cu. can be converted to anyhrous lactose by the use of the following formula:

mg. anhydrous lactose = 1·25 + mg. Cu. × 0·778.

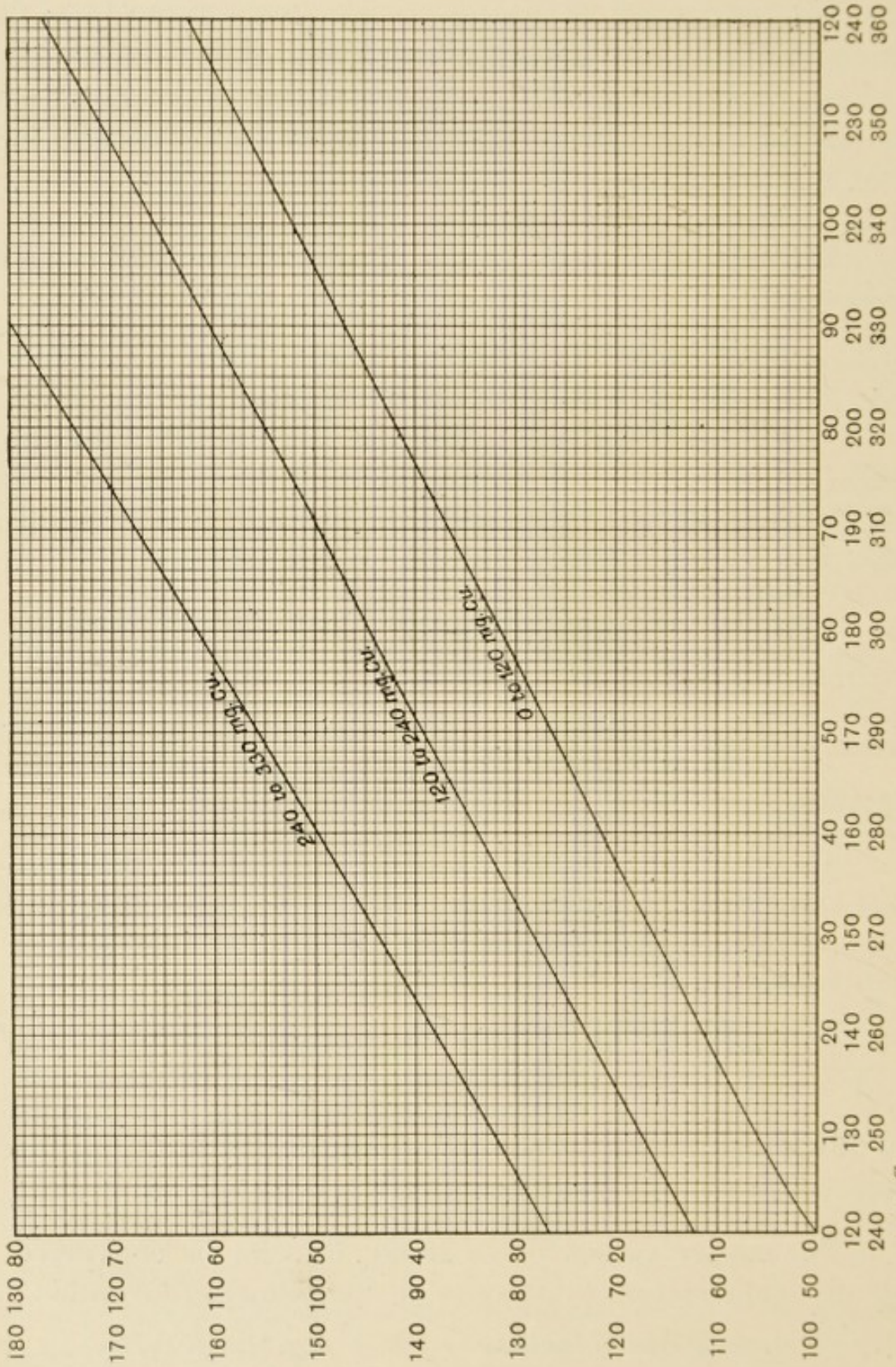


Fig. 17. Curve showing amount of copper reduced by glucose.

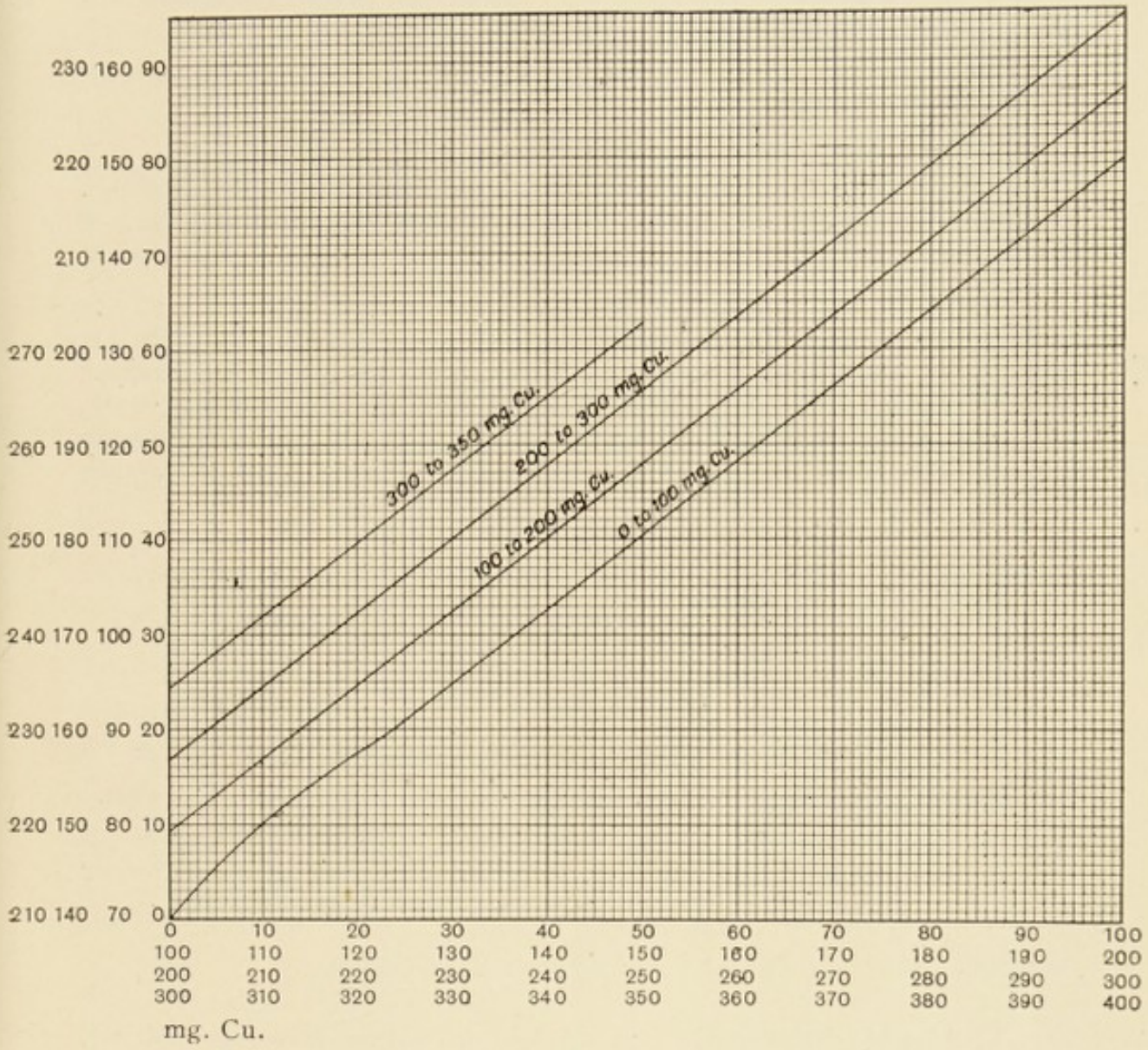


Fig. 18. Curve showing amount of copper reduced by lactose anhydride.

328. **The micro-analysis of sugar in blood by Bang's Method.**

Principle. A few drops of blood are drawn up on a weighed piece of special absorbing paper. The paper is again rapidly weighed on a microbalance or torsion balance. The proteins are coagulated by the addition of a boiling acidified solution of potassium chloride.

The sugar diffuses out into the solution and is estimated by a modification of Bang's II. method.

Solutions required.

1. Stock copper solution (see p. 225).

2. Acid solution of potassium chloride obtained by mixing 1360 c.c. of a cold saturated solution of pure potassium chloride, 640 c.c. of distilled water and 1·5 c.c. of 25 per cent. hydrochloric acid.

3. Soluble starch (see p. 225).

4. N/200 iodine. Obtained by diluting 5 c.c. of N/10 iodine to 100 c.c. with boiled out distilled water. It may also be prepared by treating 2 gm. of potassium iodide with 1 to 2 c.c. of 2 per cent. potassium iodate, adding 5 c.c. of N/10 hydrochloric acid, and making the volume up to 100 c.c. with boiled out distilled water. The solution must be freshly prepared each day.

Method of Analysis.

1. *To obtain a measured amount of blood.*

Special absorbing papers can be obtained from Grave of Stockholm, or of Messrs. J. J. Griffin & Sons, Kingsway, London.

A paper is held in a small spring clip and the clip and paper are weighed together on a torsion balance or other form of micro-balance.

The hand of the subject is washed in warm water and dried. The subject is instructed to swing the arm backwards and forwards, keeping the hand as low as possible. The finger is pricked with a sterile bayonet-pointed probe on the back of the finger about $\frac{1}{8}$ inch above the nail. A piece of rubber tubing is wound tightly round the middle joint of the finger. On firmly flexing the finger the blood usually wells up in sufficient amount. The blood is taken up on the paper, until the paper is fairly covered. It is undesirable to have the paper fully saturated with blood. The paper and clip are immediately weighed on the torsion balance, and the weight of blood taken is thus known. A convenient amount is about 120 mg.

2. *Coagulation of the proteins.* Place the paper in a clean, dry, rather wide test-tube. Measure $6\frac{1}{2}$ c.c. of the acid solution of potassium chloride into another test-tube. Boil and rapidly pour the vigorously boiling solution on to the paper in the other tube. Allow the tube to stand for at least 30 minutes. Transfer the fluid to a 50 c.c. Jena flask with a straight neck, *i.e.* with the flange cut off. Wash the paper with another $6\frac{1}{2}$ c.c. of the acid potassium chloride, and add this to the fluid in the flask.

3. *Method of heating the solution.* To the flask add 1 c.c. of the copper solution, and fit on to the neck of the flask a piece of rubber tubing about 3 mm. thick and about 5 cm. in length (see Fig. 14, p. 226). For heating it is advisable to use the apparatus designed by the author and shewn in Fig. 15, p. 229. The pressure of gas or the height of the asbestos gauze must be so adjusted that the solution begins to boil in $1\frac{1}{2}$ minutes $\pm$ 5 secs. Allow the solution to boil for exactly 2 minutes. Just before the two minutes is completed, commence to tighten the screw clip C. When the time has expired tighten the clip very firmly, remove the flask at once and plunge it into cold water. Allow it to cool in a stream of water for about 1 minute.

NOTE.—A special pair of forceps has been devised for holding the flask and sealing the rubber tube.

4. *Titration of the fluid.* Owing to the rapidity with which cuprous chloride is oxidised it is necessary to exclude air by means of an atmosphere of CO_2. This is best accomplished by means of the simple apparatus shewn in Fig. 19. The CO_2 can be obtained from a cylinder or by the action of HCl on marble in a Kipp's apparatus. Loosen the clip, remove the rubber, and immediately fit in the tube from the CO_2 apparatus, the gas having previously been turned on. Add 3 or 4 drops of the starch solution and titrate with N/200 iodine from a microburette. The titration is completed when the "starch-blue" tint persists for about 20 seconds.

There is usually no difficulty in determining the difference between the greenish blue of the copper and the bright blue of the starch-iodine compound. The titration should be done against a white ground and preferably by daylight.

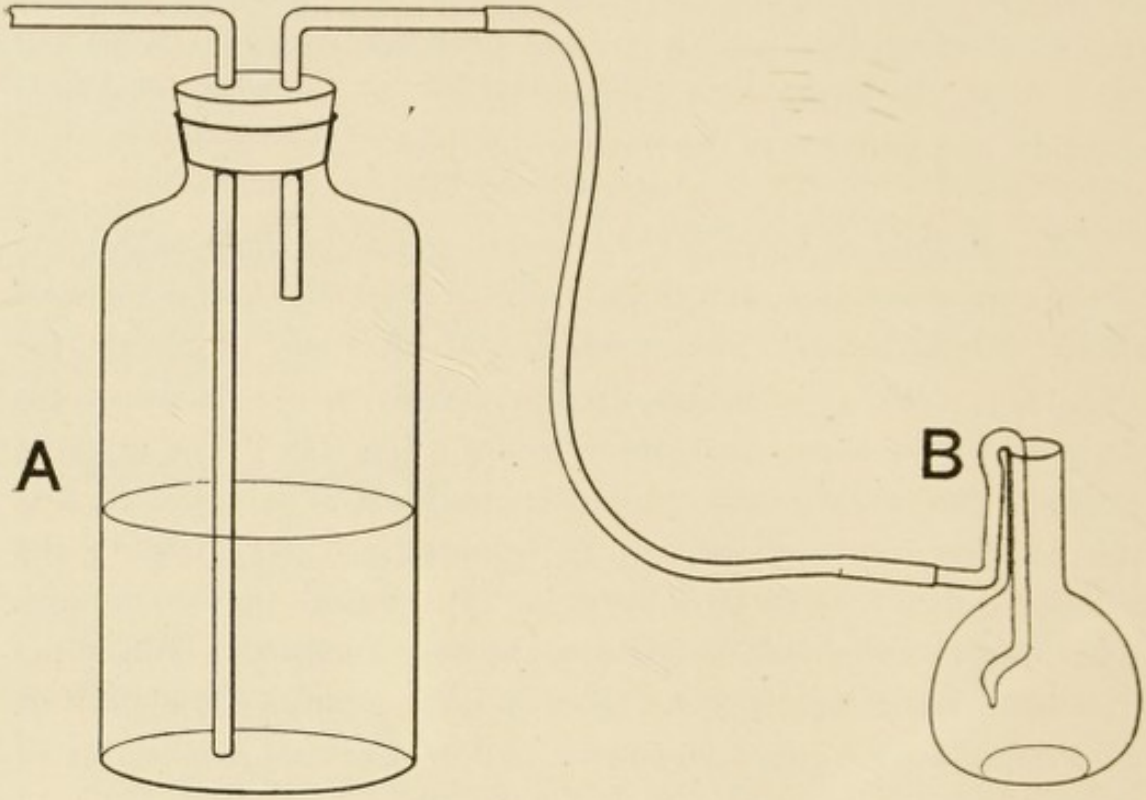


Fig. 19. Apparatus for titration in an atmosphere of CO_2.

A. Wash bottle containing water.

B. Tube fitted into flask so that the iodine can fall from burette directly into the fluid.

5. *Calculation.*

$$\frac{\text{c.c. of iodine} - 0{\cdot}16 \text{ c.c.}}{4} = \text{mg. of glucose in the blood taken.}$$

6. *Example.*

Weight of blood = 118 mg.

Iodine required = 0·68 c.c.

$$\frac{0{\cdot}68 - 0{\cdot}16}{4} = \frac{0{\cdot}52}{4} = 0{\cdot}13 \text{ mg. glucose in 118 mg. blood.}$$

$$= 0{\cdot}11 \text{ per cent.}$$

NOTES.—1. The special papers, micro-balance, flasks, rubber-tubing, etc., can be obtained from Grave, of Stockholm, or Messrs. J. J. Griffin and Sons, Kingsway, London. The latter firm will also supply the apparatus shewn in Figs. 14, 15, 16 and 19.

2. It is advisable to allow the blood to thoroughly soak into the paper before coagulating the proteins. But a delay of more than 20 minutes entails a possibility of loss of sugar by glycolysis.

3. It is of the utmost importance to use pure reagents, clean tubes, flasks, etc. Impurities usually result in the figure being too high.

329. The micro-analysis of chlorides in blood by Bang's Method.

Principle. A few drops of blood are taken up from a finger prick on to a piece of weighed absorbing paper. The paper is again weighed on a micro-balance and the weight of blood thus found. The proteins are coagulated by pouring on a boiling acid solution of magnesium sulphate. After cooling, 2 c.c. of standard silver nitrate are added and the silver chloride filtered off, a little kieselgur being added to aid filtration. The silver nitrate in the filtrate is treated with 2 c.c. of a standard solution of potassium iodide and potassium iodate and a few drops of starch solution. The iodate yields free iodine owing to the presence of the acid in the coagulating fluid. The mixture is then titrated with standard silver till the blue colour disappears. From the amount of silver required to effect this, the amount of silver in the filtrate can be determined. Thus the amount of silver that has disappeared in the formation of silver chloride can be calculated, and so the amount of chloride in the blood taken.

Preparation of reagents.

1. N/100 silver nitrate. 1·7 gm. of pure silver nitrate are dissolved in distilled water and the volume made up to 1 litre. It should be stored in a dark bottle and kept in the dark.

2. Iodide and iodate solution. 0·015 gm. of potassium iodate and 1·7 gm. of potassium iodide are dissolved in water and the volume made up to 1 litre. 2 c.c. of the solution are measured and treated with 10 c.c.

of solution 3 and a few drops of the soluble starch. This mixture is titrated with N/100 silver nitrate from a microburette until the blue colour disappears. The strength of the solution must be adjusted by the addition of water or of a dilute solution of potassium iodide until 2 c.c. require exactly 2 c.c. of the silver nitrate.

3. Acid magnesium sulphate. 2 litres of a 30 per cent. solution of magnesium sulphate, 120 c.c. of strong pure nitric acid (sp. gr. 1·42) and 280 c.c. of distilled water are mixed.

4. Starch solution. 1 gm. of Kahlbaum's soluble starch are suspended in a little cold water and poured into about 80 c.c. of boiling distilled water. 20 gm. of pure potassium nitrate are added to the mixture. The solution is poured into a number of small sterile bottles and stoppered whilst still hot. In this way the solution can be preserved for a considerable time.

5. Kieselgur. This should be purified by heating to a dull red, washing with 10 per cent. acetic acid and then with distilled water and heating again to redness.

Measure 0·2 c.c. of distilled water into a tube about 5 cm. in length and about 5 mm. bore which has one end sealed. Make a mark with a file or a label to show the height of the fluid. Dry the tube thoroughly. The amount of kieselgur for each experiment is measured by filling the tube to the mark after gently tapping.

Since kieselgur adsorbs silver nitrate it is essential to determine the amount of this for every specimen by a blank experiment conducted as follows: To 10 c.c. of solution 3 add 2 c.c. of the silver nitrate and the measured amount of kieselgur. Shake and filter through a Gooch crucible as described below. Wash the tube and the kieselgur twice with 5 c.c. of distilled water. To the filtrate add 2 c.c. of the solution 2 and a few drops of the starch solution. Titrate cautiously with N/100 silver nitrate from a microburette till the blue colour is discharged. The amount of silver required corresponds to the amount adsorbed by the kieselgur.

Method of Analysis.

1. *To obtain a measured amount of blood.* The procedure is exactly the same as described in the section on the micro-analysis of sugar (p. 234).

2. *Coagulation of the proteins.* Place the paper in a clean, dry, rather wide test-tube. Into another clean tube measure 10 c.c. of the acid magnesium sulphate and boil. Whilst vigorously boiling pour the solution on to the paper and allow it to stand for 30 minutes.

3. *Removal of the silver chloride.* To the tube, still containing the paper, add 2 c.c. of N/100 silver nitrate and the measured amount of kieselgur. Grease the rim of the tube with a smear of vaseline and shake vigorously. Filter through a 6 c.c. Gooch crucible into a 125 c.c. filtering flask connected to a water pump (see Fig. 20). The bottom of the crucible is covered with a piece of filter paper cut a trifle larger than the bottom. The paper is then washed with a little water which is sucked through. Care must be taken to see that all the perforations of the crucible are covered. Empty the flask and before turning on the pressure fill the crucible with the mixture in the tube, being careful to get as much kieselgur as possible into the crucible. Allow a few drops to filter through before turning on the pressure. Filtration is rapid and the filtrate is usually quite clear. If it is cloudy it must be refiltered.

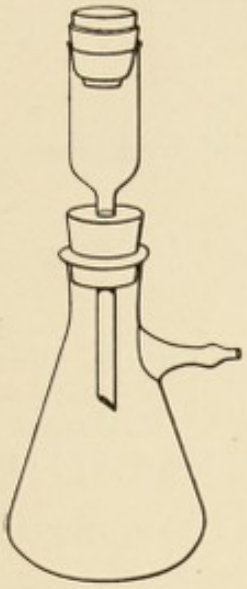


Fig. 20. Gooch crucible and filtering apparatus for micro-analysis of chlorides.

4. *Washing the paper.* When the whole of the fluid has been filtered add 5 c.c. of distilled water to the tube, shake vigorously, pour it into the crucible and filter. Repeat this operation once more.

5. *Titration of the silver.* To the fluid in the flask add 2 c.c. of the iodide-iodate solution and a few drops of the starch solution. Titrate against a white ground with N/100 silver nitrate from a microburette. The blue colour gradually disappears as the silver iodide is formed and then sharply disappears, leaving a yellowish green solution.

6. *Calculation of results.* 2 c.c. of N/100 silver = 2 c.c. of the iodide-iodate solution. The volume of N/100 silver required to effect the disappearance of the blue colour is the volume of silver that has disappeared from the tube. Of this volume a certain

amount has been adsorbed by the kieselgur. The remainder has formed silver chloride with the chlorides of the blood. Since 1 c.c. of N/100 silver = 0·585 mg. NaCl, the amount of NaCl in the blood taken can be calculated.

7. *Example.* Weight of blood taken = 116 mg.
Volume of N/100 si ver adsorbed by kieselgur = 0·12 c.c.
Volume of N/100 silver required for titration = 1·21 c.c.
Volume of N/100 silver precipitated as silver chloride = 1·21 − 0·12 = 1·09 c.c.
NaCl in 116 mg. blood = 1·09 × 0·585 = ·638 mg.
NaCl per cent. = 0·55.

330. **The estimation of chlorides in urine in Larrson's Method.**

Principle. The pigments, urates and other interfering substances are removed from the urine by adsorption with blood charcoal. The chlorides are estimated in a measured amount of the filtrate by direct titration with silver nitrate, using potassium chromate as an indicator.

Reagents required.

1. Standard silver nitrate (see p. 194).
2. Merck's pure blood charcoal (Carbo sanguinis puriss). Ordinary animal charcoal is quite useless.
3. A 5 per cent. solution of potassium chromate.

Method of analysis. To 1 gm. of the blood charcoal in a dry 50 c.c. flask add 20 c.c. of the urine. Shake vigorously and repeat the shaking at intervals for 10 minutes. Filter through a small dry paper into a dry tube. Measure 10 c.c. of the filtrate by means of a pipette and transfer it to a small beaker. Add 5 or 6 drops of the chromate and titrate with the standard silver nitrate

from a burette until the end point is reached, as indicated by the appearance of a reddish-brown colour.

Calculation. 1 c.c. of silver = 0·01 gm. NaCl.

Example. 10 c.c. of the filtered urine required 10·6 c.c. of silver.
So 10 c.c. contain 10·6 × 0·01 gm. NaCl.
So 100 c.c. contain 1·06 gm. NaCl.

331. The estimation of acetone and aceto-acetic acid in urine by the method of Scott-Wilson.

Principle. The urine is distilled into an alkaline solution of silver mercuric cyanide. The aceto-acetic acid is decomposed into acetone, which passes over with any preformed acetone into the cyanide. An insoluble keto-mercuric-cyanide compound is formed. This is filtered off, dissolved in acid, and the amount of mercury determined by titration with standard sulphocyanide. From the amount of mercury present the total amount of acetone in the urine taken can be calculated.

Solutions required.

1. Silver mercury cyanide. Dissolve 9 gm. of pure caustic soda and 0·5 gm. of mercuric cyanide in 60 c.c. of distilled water. Add 20 c.c. of 0·7268 % silver nitrate slowly with constant stirring. If necessary filter through a layer of washed asbestos in a Gooch crucible. The silver nitrate solution is prepared by diluting 5 c.c. of the standard silver nitrate used for Volhard's method (p. 194) with 15 c.c. of distilled water.

2. Acid mixture.

Strong nitric acid	40 c.c.
Strong sulphuric acid	5 c.c.
Distilled water	55 c.c.

3. N/5 potassium permanganate. 6·324 gm. of permanganate dissolved in water and the volume made up to 1 litre.

4. Standardised solution of potassium sulpho-cyanide. Dilute 125 c.c. of the stock solution described on p. 194 to make 1 litre with distilled water. Standardise the stock solution against standard silver nitrate as described on p. 194. Divide 21·4 by the number of c.c. of KCNS required for 10 c.c. of silver nitrate.

The result = mg. of Hg per c.c. of the diluted KCNS.

5. A saturated aqueous solution of iron alum.

Method.

1. *The distillation of the acetone.* Use the apparatus shewn in Fig. 21. Into flask *A* measure an amount of urine that yields between 0·4 and 2 mg. acetone. Add water to make the volume up to about 100 c.c. and then 1 c.c. of strong sulphuric acid. Into flask *C* place 10 c.c. of 40 per cent. caustic soda and a few glass beads. Into *E* place 10 c.c. or more of the silver mercury cyanide reagent (there must be at least 25 c.c. to each mg. of acetone). Close the tube with the glass rod *B* and then light the burners. The soda in *C* must boil before the fluid in *A*. The soda is kept just boiling whilst *A* is allowed to boil briskly. The first appearance of turbidity in *E* is noted and the distillation allowed to proceed for another six minutes. Remove plug *B* and turn out the flames. Detach tube *D* from the condenser and wash it with a jet of distilled water into *E*. Allow the fluid to stand for 10 minutes.

2. *Filtration of the mercury compound.* Use an apparatus similar to that shewn in Fig. 20, p. 239. The Gooch crucible should be of 10 c.c. and the filtering flask of 250 c.c. capacity. First prepare the crucible. Cut a filter paper slightly larger than the bottom of the crucible, place it in position and moisten it. Then pour in a suspension of washed asbestos fibre and form a mat of this by applying suction. A small amount of a suspension of washed powdered pumice should next be filtered to partly close the pores of the filter.

Filter the fluid in flask *E* through this. If the first portions of the filtrate are cloudy they must be refiltered. Wash the precipitate with cold water till free from silver.

3. *Solution of the mercury compound.* By means of a glass rod with a pointed hook transfer the filter mat to an Erlenmeyer flask. Place the crucible in the neck of the flask and wash it through into the flask with 10 c.c. of the acid mixture. The point of the rod should also be washed with a little of this solution.

Add 1 c.c. of the potassium permanganate and boil till colourless. Add more of the permanganate, a few drops at a time, till the brown tinge persists in the boiling mixture for about two minutes. Discharge the colour by the addition of a few drops of yellow nitric acid.

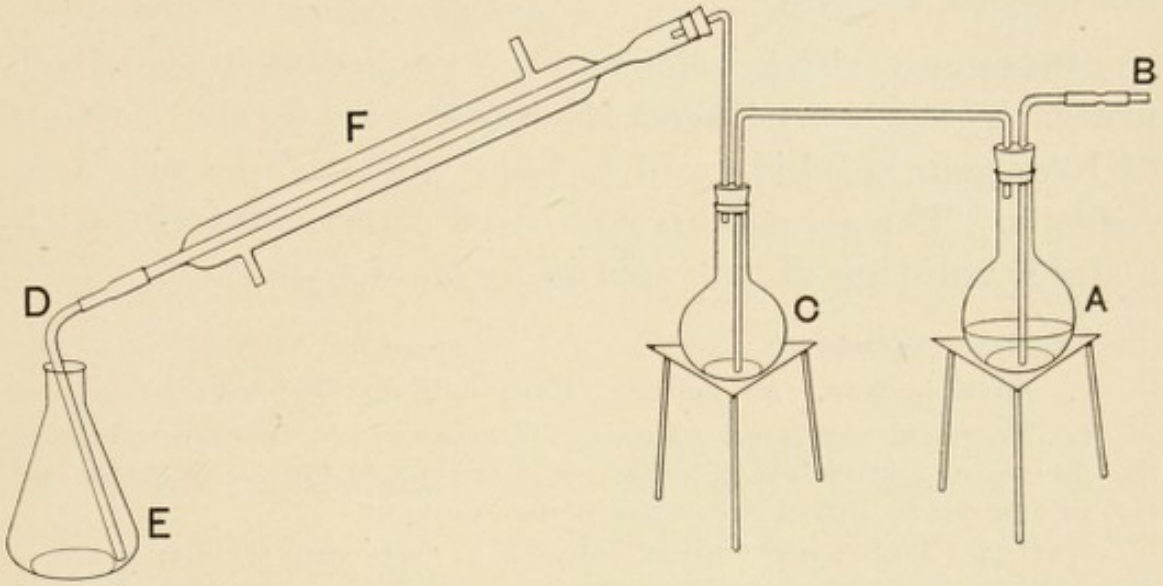


Fig. 21. Apparatus for the estimation of acetone.

A. Jena flask. B. Solid glass rod for sealing tube. C. Jena flask. D. Glass tube connected by rubber to condenser tube. E. Erlenmeyer flask. F. Liebig condenser.

4. *Titration of the mercury.* Cool thoroughly under the tap and add 2 c.c. of the iron alum. Titrate with the diluted sulpho-cyanide against a white ground until a very faint pinkish brown tinge is permanent. The end point is quite sharp, but it must be noted that after it is reached a considerable amount of sulpho-cyanide can be added without appreciably darkening the tint.

Calculation. From the amount of sulpho-cyanide required the amount of mercury can be found.

1 mg. Hg = ·0606 mg. acetone.

Example. 10 c.c. standard silver = 20·2 c.c. of stock KCNS.

$$\text{So 1 c.c. dilute KCNS} = \frac{21{\cdot}4}{20{\cdot}2} = 1{\cdot}06 \text{ mg. Hg.}$$

2 c.c. of diabetic urine taken. Mercury in ppt. required 24·2 c.c. KCNS.

So mercury = 24·2 × 1·06 = 25·65 mg.

So total acetone in 2 c.c. = 25·65 × ·0606 = 1·55 mg.

So total acetone in 100 c.c. = 1·55 × 50 = 77·5 mg.

332. The microchemical estimation of uric acid by the Folin-Denis Method.

Principle. The uric acid is precipitated as silver urate. This is centrifuged off, decomposed by the addition of hydrogen sulphide and treated with Folin's uric acid reagent. The solution is compared colorimetrically with a stable solution of uric acid in formaldehyde.

Solutions required.

1. Silver lactate. A 3 per cent. solution in distilled water.

2. Magnesia mixture. 55 gm. of crystallised magnesium chloride and 70 gm. of ammonium chloride are dissolved in 650 c.c. of water and the volume made up to 1 litre with strong ammonia.

3. Uric acid-formaldehyde solution. One gm. of uric acid is dissolved in a graduated litre flask by means of 200 c.c. of a 0·4 per cent. solution of lithium carbonate (solution is effected rather slowly). Add 40 c.c. of 40 per cent. formaldehyde solution, shake and allow to stand for some minutes. Acidify by the addition of 20 c.c. of normal acetic acid and make up to 1 litre with distilled water. The solution should be perfectly clear and must stand at least a day before it is standardised.

4. Folin's uric acid reagent. See page 149.

5. Sodium carbonate. A saturated solution.

6. A freshly prepared saturated solution of hydrogen sulphide in water.

Standardisation of the uric acid—formaldehyde solution. Prepare a fresh standard solution of uric acid as described on pape 190. Measure 1 c.c. of this by means of a standard pipette into a 50 c.c. measuring flask. Add 2 c.c. of the uric acid reagent, 10 c.c. of a saturated solution of sodium carbonate, and make up to the mark with distilled water. As nearly simultaneously as possible treat 5 c.c. of the uric acid formaldehyde solution in another 50 c.c. flask with 2 c.c. of the reagent, 10 c.c. of saturated sodium carbonate and water up to the mark. Each solution is well mixed and immediately compared in a Dubosq's Colorimeter (p. 192). Place

the tube containing the standard uric acid at either 10 or 20 mm. and determine the depth of the other tube that gives exact equality of tint. The amount of reacting uric acid in the uric acid—formaldehyde solution can then be calulated as shewn in the following example :

Height of standard solution of 1 mg. of uric acid = 10 mm.
Height of 5 c.c. of uric acid-formaldehyde solution = 10·10 mm.
So 5 c.c. of uric acid-formaldehyde corresponds to

$$\frac{10}{10{\cdot}10} = 0{\cdot}99 \text{ mg. uric acid.}$$

Or if, in subsequent estimations, the tube containing the uric acid-formaldehyde solution be kept at 10·1 mm., this corresponds to 1 mg. of uric acid.

Method of Analysis. Into the tube of a hand centrifuge accurately measure 1 to 2 c.c. of urine and add water to make about 5 c.c. Add 6 drops of the silver lactate, 2 drops of the magnesia mixture and a sufficient amount (10 to 20 drops) of strong ammonia to dissolve the silver chloride. Mix and centrifuge for one to two minutes. Pour off the supernatant fluid and to the residue in the tube add six drops of the hydrogen sulphide solution and one drop of concentrated hydrochloric acid. Place the tube in a beaker of boiling water for seven minutes or until all excess of hydrogen sulphide has been removed. Add a single drop of a 0·5 per cent. solution of lead acetate. If a brown precipitate is produced the hydrogen sulphide has not been removed, and the tube must be replaced in the boiling water bath.

Cool the tube, add 2 c.c. of the uric acid reagent, 10 c.c. of the saturated solution of sodium carbonate, transfer to a 50 c.c. graduated flask and make up to the mark with distilled water. As nearly simultaneously as possible measure 5 c.c. of the standardised uric acid-formaldehyde solution into another 50 c.c. flask, add 2 c.c. of Folin's reagent, 10 c.c. of saturated sodium carbonate and make up to the mark with distilled water. Immediately compare the

colour of the two solutions by means of a Dubosq's colorimeter. It is convenient to set the tube containing the uric acid-formaldehyde solution at the height determined when it was standardised (10·10 mm. in the example given above).

Calculation and Example.

2 c.c. of urine taken.

Height of urine tube = 9·8 mm.

Height of standard tube = 10·1 mm. (corresponds to 10 mm. of a solution of 1 mg. uric acid).

$$\text{So uric acid in } 2 \text{ c.c.} = \frac{10}{9{\cdot}8} \text{ mg.} = 1{\cdot}02 \text{ mg.}$$

$$\text{So uric acid in } 100 \text{ c.c.} = 1{\cdot}02 \times 50 = 51 \text{ mg.}$$

$$= 0{\cdot}051 \text{ per cent.}$$

333. The Estimation of the diastatic power of urine by Wohlgemuth's Method.

Principle. Varying amounts of urine are treated with a given amount of soluble starch for 30 minutes at 38° C. After cooling, a drop of dilute iodine is added to each tube. The tubes that contain considerable amounts of urine have all the starch digested so that no colour is obtained on adding the iodine. The tube with the smallest amount of urine that completely digests the starch is found and so the diastatic value calculated.

Reagents required.

1. Stock solution of soluble starch. Accurately weigh out 2 gms. of Kahlbaum's soluble starch and transfer it to a dry test tube. Add about 10 c.c. of distilled water and shake. Pour the suspension into about 70 c.c. of boiling distilled water and stir well. Wash the tube three successive times with 5 c.c. of distilled water, transferring the washings to the boiling solution. Now add 10 gms. of pure sodium chloride. Allow to cool and make the volume up to 100 c.c. with distilled water. The solution is stable for months.

2. One per mille soluble starch in 0·5 per cent. sodium chloride. 5 c.c. of the stock are diluted with distilled water to make 100 c.c. This solution must be freshly prepared each day.

3. N/50 iodine, prepared from N/10 iodine (see page 225) by diluting 2 c.c. with 8 c.c. of distilled water. The diluted iodine must be freshly prepared each day.

Method. Label a series of clean dry test-tubes 1 to 10.

Into the tubes measure the volume of urine and of distilled water stated in the table.

Tube.	c.c. of Urine.	c.c. of Water.	$d\frac{38°}{30'}$	Tube.	c.c of Urine diluted 1 in 10 with water.	c.c. of Water.	$d\frac{38°}{30'}$
1	0·5	0·5	4	6	0·9	0·1	22·2
2	0·4	0·6	5	7	0·8	0·2	25
3	0·3	0·7	6·6	8	0·7	0·3	28·6
4	0·2	0·8	10	9	0·6	0·4	33·3
5	0·1	0·9	20	10	0·5	0·5	40

To each tube add 2 c.c. of the one per mille starch, commencing with tube 10. Mix the contents by agitation and place in a thermostat or a water bath at 38° C. for exactly 30 minutes.

Remove the tubes and place them in a beaker of cold water for 3 minutes to cool.

Arrange the tubes in order in a stand.

Commencing with tube 1 add 1 drop of the N/50 iodine to each tube and carefully note the colour produced.

Should a colour be produced and it rapidly fades, add 1 more drop of iodine to each tube.

Note the tube with the lowest number that shows a blue tinge. The next lower tube contains an amount of urine that completely digests 2 c.c. of 0·1 per cent. starch in 30 minutes at 38° C.

From this the number of c.c. of 0·1 % starch that would be digested by 1 c.c. of urine can be calculated. This index is written as $d\frac{38°}{30'}$.

NOTES.—1. It is customary to use a freshly prepared 0·1 per cent. solution of starch in water and to make the volume of the urine up to 1 c.c. with 1 per cent. sodium chloride. The author has determined that 2 per cent. starch in 10 per cent. sodium chloride is quite stable and that the results obtained agree with these found by the original method. It is suggested that the present more convenient method be adopted as a standard.

2. The $d\frac{38^{\circ}}{30'}$ of normal urine varies between 5 and 20, with an average of 10. In acute pancreatitis the value is high and may be over 200. In such cases the urine is still further diluted to 1 in 100 and the d calculated.

Thus if 0·006 c.c. of urine is required $d = \frac{2}{\cdot 006} = 333$.

3. It is important that the same amount of iodine be added to each tube. Very uneven results are obtained if varying amounts of iodine be employed.

4. Samples of the mixed 24 hours' specimen should be used.

5. The diastase in the urine is quite stable if the urine be preserved by the addition of toluol. 3 c.c. are ample for an estimation.

6. The pipettes for measuring the solutions must be accurate 1 c.c. pipettes graduated to 1/100 c.c.

334. The estimation of pepsin by Fuld's Method.

Principle. An acid solution of edestin (the protein of hemp seeds) is precipitated by sodium chloride: the peptic digestion products are not precipitated.

Solutions required.

1. Hydrochloric acid. Dilute 30 c.c. of N/10 HCl to 100 c.c. with distilled water.

2. 0·1 per cent. solution of edestin. Dissolve 0·1 gm. of Merck's edestin in 100 c.c. of the hydrochloric acid at boiling point. Cool and make up to 100 c.c. with the hydrochloric acid. If the solution is not clean it must be filtered.

3. Saturated (33 per cent.) solution of sodium chloride.

Method. Number a series of clean tubes from 1 to 10. Into tubes 2 to 10 measure 1 c.c. of the hydrochloric acid. Into tubes 1 and 2 measure 1 c.c. of the gastric juice. Mix the fluid in tube 2 and transfer 1 c.c. to tube 3. Mix this and transfer 1 c.c. of the mixture to tube 4. Proceed in this way till each tube contains 1 c.c. of fluid and each tube contains one half of the amount of enzyme present in the tube with the next lower number. To each tube add 1 c.c. of the edestin solution. Mix and allow the tubes to stand at room temperature (15 to 17° C.) for 30 minutes. To each tube add 10 drops of the sodium chloride. The tubes with low numbers are probably clear, whilst the tubes with high

numbers are cloudy. Note the tube with the lowest number that shews a cloud. The tube with the number next below it has an amount of gastric juice that just digests 2 c.c. of the edestin in 30 minutes. Thus the number of c.c. of edestin digested by 1 c.c. of gastric juice can be calculated.

This is best denoted by $pe\frac{16^\circ}{30'}$

Thus if tube 6 shews a cloud, then in tube 5 (containing 0·0625 c.c. gastric juice) digestion is complete. Supposing the temperature is 16° C. then $pe\frac{16^\circ}{30'} = \frac{2}{\cdot 0625} = 32.$

335. **Cole's test for small amounts of glucose in urine.**

In a dry boiling tube or large test-tube place about 1 gm. of Merck's pure blood-charcoal. Add 10 c.c. of the urine, shake, heat to boiling and then cool under the tap. Shake at intervals for 5 minutes. Filter through a small paper into a dry test-tube. To the filtrate add 6 drops of pure glycerine and 0·5 gm. of anhydrous sodium carbonate. Shake and heat to boiling. Maintain the boiling for exactly 50 secs. Immediately add 4 drops of a 5 per cent. solution of crystalline copper sulphate, shake to mix and allow the tube to stand without further heating for one minute. With normal urine the fluid remains blue. If glucose is present to the extent of 0·03 per cent. above the normal amount in urine the blue colour is discharged and a yellowish precipitate of cuprous hydroxide forms.

NOTES.—1. The treatment with blood charcoal removes practically the whole of the urates, creatinine and pigments that interfere with Fehling's test (see small print on p. 161). It also adsorbs so much of the normal amount of glucose present that the filtrate from normal urine fails to give a reduction.

2. Ordinary animal charcoal is quite useless. Merck's "blood charcoal, purified by acid" must be employed. 1 gm. is roughly measured by piling up about half the large blade of an ordinary pen-knife twice with the charcoal.

3. 0·5 gm. of anhydrous sodium carbonate is carried by about $\frac{3}{4}$ the length of a large blade well piled up once.

4. Should the specific gravity of the urine exceed 1025 it is advisable to use 5 c.c. of the urine + 5 c.c. of water.

5. The test is not given by chloroform nor by glycuronates: it is given by pentoses.

6. Should there be any reason to suspect lactose the procedure should be modified as follows: treat 20 c.c. of the urine with 1 gm. of charcoal as described above. Treat the whole of the filtrate with another gm. of charcoal and repeat the process. To 5 c.c. of this filtrate add the glycerine and sodium carbonate and proceed as above directed. A reduction indicates the presence of glucose, the whole of any lactose up to even 1 per cent. being removed by this double adsorption, whilst 0·04 per cent. of glucose in the original urine still shows in the filtrate.

336. **On the detection of acetone and aceto-acetic acid.**

It is now recognised that Rothera's test (Ex. 300) is a test for aceto-acetic acid as well as for acetone. It is also clear that fresh pathological urine never contains acetone unless aceto-acetic acid is also present.

Rothera's test is the simplest one to apply to urine for the recognition of the presence of the acetone bodies. The author has determined that it is not necessary to have a freshly prepared solution of nitroprusside. A mixture of 1000 parts of pure solid ammonium sulphate and one part of solid sodium nitroprusside is prepared by grinding and intimate mixing. The test can then be performed as follows: in a test-tube place the solid for a depth of 2 inches. In another similar tube place a like depth of the urine. Pour the urine on to the solid and mix by repeatedly pouring from tube to tube. Add 2 c.c. of strong ammonia, mix and allow to stand. The development of a permanganate colour indicates the presence of acetone bodies in the urine.

Hurtley has recently described the following test which is specific for aceto-acetic acid.

To 10 c.c. of urine add 2·5 c.c. of concentrated hydrochloric acid and 1 c.c. of a freshly prepared 1 per cent. solution of sodium nitrite. Shake and allow to stand for

two minutes. Now add 15 c.c. of strong ammonia, then 5 c.c. of a 10 per cent. solution of ferrous sulphate. Shake, pour into a large boiling tube and allow to stand undisturbed. A violet or purple colour slowly develops if aceto-acetic acid be present. The speed at which the colour develops depends on the concentration of aceto-acetic acid. With small amounts the colour may not develop for about 5 hours. The test shows in a dilution of 1 in 50,000.

337. **Cole's test for bile pigments.**

The following method has recently been elaborated as an improvement on that described on page 119.

Boil about 15 c.c. of the fluid or suspected urine in a test tube. Add two drops of a saturated solution of magnesium sulphate, then add a 10 per cent. solution of barium chloride, drop by drop, boiling between each addition. Continue to add the barium chloride until no further precipate is obtained. Allow the tube to stand for a minute. Pour off the supernatant fluid as cleanly as possible or use a centrifuge. To the precipitate add 3 to 5 c.c. of 97 per cent. alcohol, two drops of strong sulphuric acid, and two drops of a 5 per cent. aqueous solution of potassium chlorate. Boil for half a minute and allow the barium sulphate to settle. The presence of bile pigments is indicated by the alcoholic solution being coloured a greenish blue.

To render the test more delicate, pour off the alcoholic solution from the barium sulphate into a dry tube. Add about one-third its volume of chloroform and mix. To the solution add about an equal volume of water, place the thumb on the tube, invert once or twice and allow the chloroform to separate. It contains the bluish pigment in solution.

338. **Preparation of haemin crystals (Nippe's Method).**

A small drop of blood is spread to form a film on a glass slide and *slowly* evaporated till it is quite dry. To the film add two drops of a solution of 0·1 gm. of potassium chloride in glacial acetic acid. Cover with a slip and heat over a *very small* flame till bubbles appear and the solution is boiling. Allow a drop or two more of the reagent to run under the cover slip and examine under a microscope.

NOTE.—The advantage of the method is that it is rapid and that crystals of the inorganic chloride do not separate. It is *very* important not to burn the blood whilst drying, and also to be sure that the solution is heated to boiling with the acid mixture.

Bial's reagent consists of 1 to 1·5 gm. orcine, 500 c.c. of concentrated hydrochloric acid, and 30 drops of a 1 per cent. solution of ferric chloride.

Benedict's sulphur reagent is:

Crystallised copper nitrate, 200 gm.
Potassium chlorate, 50 gm.
Distilled water to 1 litre.

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LOGARITHMS.

	0	1	2	3	4	5	6	7	8	9	Differences. 1	2	3	4	5	6	7	8	9
10	0000	0043	0086	0128	0170	0212	0253	0294	0334	0374	4	8	12	17	21	25	29	33	37
11	0414	0453	0492	0531	0569	0607	0645	0682	0719	0755	4	8	11	15	19	23	26	30	34
12	0792	0828	0864	0899	0934	0969	1004	1038	1072	1106	3	7	10	14	17	21	24	28	31
13	1139	1173	1206	1239	1271	1303	1335	1367	1399	1430	3	6	10	13	16	19	23	26	29
14	1461	1492	1523	1553	1584	1614	1644	1673	1703	1732	3	6	9	12	15	18	21	24	27
15	1761	1790	1818	1847	1875	1903	1931	1959	1987	2014	3	6	8	11	14	17	20	22	25
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17	2304	2330	2355	2380	2405	2430	2455	2480	2504	2529	2	5	7	10	12	15	17	20	22
18	2553	2577	2601	2625	2648	2672	2695	2718	2742	2765	2	5	7	9	12	14	16	19	21
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20	3010	3032	3054	3075	3096	3118	3139	3160	3181	3201	2	4	6	8	11	13	15	17	19
21	3222	3243	3263	3284	3304	3324	3345	3365	3385	3404	2	4	6	8	10	12	14	16	18
22	3424	3444	3464	3483	3502	3522	3541	3560	3579	3598	2	4	6	8	10	12	14	15	17
23	3617	3636	3655	3674	3692	3711	3729	3747	3766	3784	2	4	6	7	9	11	13	15	17
24	3802	3820	3838	3856	3874	3892	3909	3927	3945	3962	2	4	5	7	9	11	12	14	16
25	3979	3997	4014	4031	4048	4065	4082	4099	4116	4133	2	3	5	7	9	10	12	14	15
25	4150	4166	4183	4200	4216	4232	4249	4265	4281	4298	2	3	5	7	8	10	11	13	15
27	4314	4330	4346	4362	4378	4393	4409	4425	4440	4456	2	3	5	6	8	9	11	13	14
28	4472	4487	4502	4518	4533	4548	4564	4579	4594	4609	2	3	5	6	8	9	11	12	14
29	4624	4639	4654	4669	4683	4698	4713	4728	4742	4757	1	3	4	6	7	9	10	12	13
30	4771	4786	4800	4814	4829	4843	4857	4871	4886	4900	1	3	4	6	7	9	10	11	13
31	4914	4928	4942	4955	4969	4983	4997	5011	5024	5038	1	3	4	6	7	8	10	11	12
32	5051	5065	5079	5092	5105	5119	5132	5145	5159	5172	1	3	4	5	7	8	9	11	12
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34	5315	5328	5340	5353	5366	5378	5391	5403	5416	5428	1	3	4	5	6	8	9	10	11
35	5441	5453	5465	5478	5490	5502	5514	5527	5539	5551	1	2	4	5	6	7	9	10	11
36	5563	5575	5587	5599	5611	5623	5635	5647	5658	5670	1	2	4	5	6	7	8	10	11
37	5682	5694	5705	5717	5729	5740	5752	5763	5775	5786	1	2	3	5	6	7	8	9	10
38	5798	5809	5821	5832	5843	5855	5866	5877	5888	5899	1	2	3	5	6	7	8	9	10
39	5911	5922	5933	5944	5955	5966	5977	5988	5999	6010	1	2	3	4	5	7	8	9	10
40	6021	6031	6042	6053	6064	6075	6085	6096	6107	6117	1	2	3	4	5	6	8	9	10
41	6128	6138	6149	6160	6170	6180	6191	6201	6212	6222	1	2	3	4	5	6	7	8	9
42	6232	6243	6253	6263	6274	6284	6294	6304	6314	6325	1	2	3	4	5	6	7	8	9
43	6335	6345	6355	6365	6375	6385	6395	6405	6415	6425	1	2	3	4	5	6	7	8	9
44	6435	6444	6454	6464	6474	6484	6493	6503	6513	6522	1	2	3	4	5	6	7	8	9
45	6532	6542	6551	6561	6571	6580	6590	6599	6609	6618	1	2	3	4	5	6	7	8	9
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52	7160	7168	7177	7185	7193	7202	7210	7218	7226	7235	1	2	2	3	4	5	6	7	7
53	7243	7251	7259	7267	7275	7284	7292	7300	7308	7316	1	2	2	3	4	5	6	6	7
54	7324	7332	7340	7348	7356	7364	7372	7380	7388	7396	1	2	2	3	4	5	6	6	7
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55	7404	7412	7419	7427	7435	7443	7451	7459	7466	7474	1	2	2	3	4	5	5	6	7
56	7482	7490	7497	7505	7513	7520	7528	7536	7543	7551	1	2	2	3	4	5	5	6	7
57	7559	7566	7574	7582	7589	7597	7604	7612	7619	7627	1	2	2	3	4	5	5	6	7
58	7634	7642	7649	7657	7664	7672	7679	7686	7694	7701	1	1	2	3	4	4	5	6	7
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60	7782	7789	7796	7803	7810	7818	7825	7832	7839	7846	1	1	2	3	4	4	5	6	6
61	7853	7860	7868	7875	7882	7889	7896	7903	7910	7917	1	1	2	3	4	4	5	6	6
62	7924	7931	7938	7945	7952	7959	7966	7973	7980	7987	1	1	2	3	3	4	5	6	6
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68	8325	8331	8338	8344	8351	8357	8363	8370	8376	8382	1	1	2	3	3	4	4	5	6
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73	8633	8639	8645	8651	8657	8663	8669	8675	8681	8686	1	1	2	2	3	4	4	5	5
74	8692	8698	8704	8710	8716	8722	8727	8733	8739	8745	1	1	2	2	3	4	4	5	5
75	8751	8756	8762	8768	8774	8779	8785	8791	8797	8802	1	1	2	2	3	3	4	5	5
76	8808	8814	8820	8825	8831	8837	8842	8848	8854	8859	1	1	2	2	3	3	4	5	5
77	8865	8871	8876	8882	8887	8893	8899	8904	8910	8915	1	1	2	2	3	3	4	4	5
78	8921	8927	8932	8938	8943	8949	8954	8960	8965	8971	1	1	2	2	3	3	4	4	5
79	8976	8982	8987	8993	8998	9004	9009	9015	9020	9025	1	1	2	2	3	3	4	4	5
80	9031	9036	9042	9047	9053	9058	9063	9069	9074	9079	1	1	2	2	3	3	4	4	5
81	9085	9090	9096	9101	9106	9112	9117	9122	9128	9133	1	1	2	2	3	3	4	4	5
82	9138	9143	9149	9154	9159	9165	9170	9175	9180	9186	1	1	2	2	3	3	4	4	5
83	9191	9196	9201	9206	9212	9217	9222	9227	9232	9238	1	1	2	2	3	3	4	4	5
84	9243	9248	9253	9258	9263	9269	9274	9279	9284	9289	1	1	2	2	3	3	4	4	5
85	9294	9299	9304	9309	9315	9320	9325	9330	9335	9340	1	1	2	2	3	3	4	4	5
86	9345	9350	9355	9360	9365	9370	9375	9380	9385	9390	1	1	1	2	3	3	4	4	5
87	9395	9400	9405	9410	9415	9420	9425	9430	9435	9440	0	1	1	2	2	3	3	4	4
88	9445	9450	9455	9460	9465	9469	9474	9479	9484	9489	0	1	1	2	2	3	3	4	4
89	9494	9499	9504	9509	9513	9518	9523	9528	9533	9538	0	1	1	2	2	3	3	4	4
90	9542	9547	9552	9557	9562	9566	9571	9576	9581	9586	0	1	1	2	2	3	3	4	4
91	9590	9595	9600	9605	9609	9614	9619	9624	9628	9633	0	1	1	2	2	3	3	4	4
92	9638	9643	9647	9652	9657	9661	9666	9671	9675	9680	0	1	1	2	2	3	3	4	4
93	9685	9689	9694	9699	9703	9708	9713	9717	9722	9727	0	1	1	2	2	3	3	4	4
94	9731	9736	9741	9745	9750	9754	9759	9763	9768	9773	0	1	1	2	2	3	3	4	4
95	9777	9782	9786	9791	9795	9800	9805	9809	9814	9818	0	1	1	2	2	3	3	4	4
96	9823	9827	9832	9836	9841	9845	9850	9854	9859	9863	0	1	1	2	2	3	3	4	4
97	9868	9872	9877	9881	9886	9890	9894	9899	9903	9908	0	1	1	2	2	3	3	4	4
98	9912	9917	9921	9926	9930	9934	9939	9943	9948	9952	0	1	1	2	2	3	3	4	4
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	0	1	2	3	4	5	6	7	8	9	1	2	3	4	5	6	7	8	9