UNIVERSAL
LIBRARY

PRACTICAL BIOCHEMISTRY

Churchill's Empire Series

A COURSE IN PRACTICAL BIOCHEMISTRY FOR STUDENTS OF MEDICINE

BY

A. T. CAMERON
M.A., D.Sc.(Edin.), F.I.C., F.R.S.C.
Professor of Biochemistry, Faculty of Medicine, University of Manitoba; Biochemist, Winnipeg General Hospital

AND

FRANK D. WHITE
A.R.T.C., Ph.D.(Edin.), F.I.C.
Assistant Professor of Biochemistry, Faculty of Medicine, University of Manitoba

FOURTH EDITION

With 4 Plates and 23 Text Figures

LONDON
J. & A. CHURCHILL LTD.
104 GLOUCESTER PLACE
PORTMAN SQUARE
1940

First Edition . 1930
Second Edition . 1932
Third Edition . 1935
Fourth Edition . 1940

Printed in Great Britain

PREFACE TO THE FOURTH EDITION

It has seemed to us that a practical text designed to be used in training students should err if anything on the conservative side, preferring old tested methods rather than newer less tested ones, and thus enabling teachers to use the same ordered arrangement from year to year until marked advances render this undesirable. We have therefore made few alterations in this edition.

The sequence of certain exercises has been altered to make the order of treatment conform more closely to the fifth edition of the senior author's theoretical text. Some of the less illustrative qualitative work has been omitted, while a number of tests have been slightly modified, to render their performance by the student less open to any error.

It has seemed wise to introduce an account of the micro-Kjeldahl technique, while the simple estimation of ascorbic acid has been introduced as helping to strengthen the student's conception of the reality of vitamins.

No attempt has been made to incorporate an account of any of the electrical instruments for measurement of *p*H, or of the photo-electric colorimeters, since it is felt that while these expensive types of apparatus can be demonstrated to students, yet there can be but few laboratories in which individual medical students are permitted to carry out experiments with them.

A. T. Cameron.
F. D. White.

Winnipeg.

PREFACE TO THE FIRST EDITION

THIS book consists of a series of exercises designed to train medical students in the practical tests and procedures used in biochemical work. It is in no sense a reference book, though it should afford a useful introduction to the larger texts such as Hawk and Bergeim's "Practical Physiological Chemistry." To this we have been especially indebted as a reference book both for practical teaching and for clinical procedures for many years.

The purposes of a course in Practical Biochemistry such as that given in this volume should be in the first place to demonstrate the accuracy of a reasonably large number of statements taught in lecture courses, in the second place to afford some training in the technique of the quantitative procedures used in the science, and in the third place to continue to inculcate those methods of scientific accuracy of observation, deduction, and measurement in which presumably previous training has been given in the more basic sciences of chemistry and physics.

Such a course designed for medical students is circumscribed (and justifiably circumscribed) by the more limited time which their curriculum affords for the study of biochemistry than can be allowed in a pure science course, and by the desirability of utilising such portions of the subject and such procedures as have a bearing upon their subsequent medical course and the clinical applications of the subject. Hence certain more complex procedures, such as the electrometric determination of hydrogen-ion concentration, have been deliberately excluded

as unnecessary and relatively undesirable in a course for which only a limited amount of time should be provided.

This book is designed to be used with any of the smaller theoretical text-books of biochemistry, although it more particularly follows the order of the " Text-book of Biochemistry " by the senior author. The work covered is that which has been given for the past several years to the students of medicine in the University of Manitoba, with a few additions to facilitate that selection of work which every teacher prefers. All methods given have been tested repeatedly.

In the quantitative section only one approved method has been usually detailed for each estimation. References to others have been given, with comments on them in so far as our experience seemed to warrant such comments. The order of the quantitative exercises has been selected mainly from the standpoint of gradually increasing difficulty; thus exercises on urine constituents precede those on blood. The exercises illustrate as many different types of procedure as possible, with the exception of gravimetric procedures.

Matter extraneous to the purposes outlined has been excluded as far as possible, since large and costly handbooks seem both undesirable and unnecessary for the student at this stage; he merely becomes confused with a multiplicity of detail. He is presumed to be well acquainted with the ordinary types of chemical apparatus, and the illustrations have therefore been limited to a minimum.

Demonstrations have been introduced throughout the course. These we have found to serve two useful purposes; many new procedures are most easily taught to students in groups before these students themselves attempt them; in tests involving use of costly chemicals the wastage is very materially decreased.

A note at the head of each exercise indicates the time that we have found by experience to be adequate.

In the quantitative exercises calculation formulæ have been rigidly excluded, since we have long found that the

majority of students use them without comprehension, and often through lack of comprehension use them wrongly.

Colorimeters, and certain other pieces of apparatus, are too costly to be purchased in large numbers for students' courses by most universities. A simple system of alternating groups of students renders such large expenditures unnecessary.

No attempt has been made to obtain unusually fine crystal preparations for the micro-photographs, but rather we have sought to reproduce such pictures as students themselves, following the directions in the text carefully, may reasonably expect to obtain.

Our thanks are due to Professor William Boyd, of the Department of Pathology of this University, and his technician, Miss L. Nason, who have made these micro-photographs from our preparations, and to Messrs. J. & A. Churchill, for permitting us to reproduce the first two plates from Cameron's "Biochemistry."

We desire to express our appreciation of the co-operation of Messrs. J. & A. Churchill, whereby the rapid publication of this volume has been greatly facilitated.

A. T. C.
F. D. W.

TABLE OF CONTENTS

PART I. QUALITATIVE PROCEDURES

PART II. QUANTITATIVE AND CLINICAL PROCEDURES

PRACTICAL BIOCHEMISTRY

PART I

QUALITATIVE PROCEDURES

GENERAL INSTRUCTIONS

STUDENTS should not be satisfied with any result which is not in accordance with that stated in the text-book. If repetition does not bring agreement, assistance should be sought to explain the discrepancy. One of the most frequent sources of error is the use of **dirty test-tubes.**

Students should learn as soon as possible to use only small quantities of materials and of reagents. A test, especially a test requiring the application of heat, can always be carried out more satisfactorily in a test-tube one-third filled with liquid than in one half filled. Students should familiarise themselves with the appearance of 1, 2 and 5 c.c. of liquid in a test-tube.

Dilute reagents should always be used unless concentrated reagents are specifically called for in the text.

Reagents must never be poured back into reagent bottles. Since many reagents are expensive, only the small amounts actually required should be used.

All procedures in which noxious or unpleasant odours may be produced should be carried out in a fume-chamber.

All procedures in which ether is being evaporated must be carried out away from the vicinity of flames.

When concentrated acids or alkalies have to be pipetted, connect a 6 or 8-in. length of rubber tubing to the pipette and apply suction to the tubing (and not directly to the pipette).

Since some students only learn to appreciate inhibitory instructions by the unpleasant results following failure to observe them a few simple first aid procedures are given in Appendix III.

EXERCISE I

DETECTION OF THE ELEMENTS IN ANIMAL AND PLANT MATERIAL

(One or two Three-hour Periods)

THE delicacy of the tests employed determines to a great extent what elements can be detected in animal and plant material; the more delicate the tests, the greater the list of elements which can be shown to be present.

In this Exercise a few of the easier tests are detailed; these apply to the elements present in largest amount, with the exception of oxygen; the presence or absence of this element can only be determined by quantitative procedures.

Experiment 1. Place a little stearic acid (an organic compound) in a porcelain crucible and heat it over a Bunsen flame *in the fume-chamber*. It melts, chars, burns with a smoky flame (both charring and the smoky flame indicating the presence of **Carbon**), and on continued heating completely oxidises, leaving no **Ash**. Repeat with a little cane sugar. Similar results are obtained.

Experiment 2. Repeat (1), using a little powdered sodium chloride (an inorganic salt). There is no charring. The sodium chloride may partly fuse, but remains otherwise unaltered.

Now take a little of a salt of an organic acid, such as sodium citrate, and treat it in the same way. Charring takes place. The carbon gradually disappears, and a colourless or slightly grey **Ash** is left.

Plant and animal materials are usually mixtures of organic and inorganic constituents. If subjected to similar treatment part will burn away, and ash will remain.

Experiment 3. Repeat (1), using a tiny piece of lean meat. The charring, as before, signifies the presence of **Carbon.** An unpleasant odour— the odour of "burning flesh"—becomes noticeable, and this is characteristic of *proteins* containing **Nitrogen.** After ten or fifteen minutes a slightly grey ash remains. (The greyness is due to traces of unburnt carbon.)

Repeat, using instead of the meat a fragment of cabbage leaf. Similar results will be obtained.

Comparison of the results of Experiments 1 and 3 illustrates the fact that cane sugar contains no nitrogen and no mineral constituents. The following are more specific tests for certain of the elements :—

Carbon and Hydrogen

Experiment 4. Warm some cupric oxide in a dry test-tube to drive off any water that may have condensed with it. Then mix with it in a dry mortar about one-fifth the amount of powdered lactose (milk sugar). Transfer sufficient of the mixture to fill one-third of a dry test-tube. Close this with a perforated cork through which passes a bent glass tube. Allow the other end of the tube to dip into either lime water or baryta water in a second test-tube. Gently heat the test-tube containing the mixture over a Bunsen flame ; gas will bubble through the lime (or baryta) water, and gradually a cloudiness will appear. This is due to the formation of insoluble carbonate, and that indicates the presence of carbon dioxide in the gas evolved, and therefore of carbon in the original material.

Note that drops of *water* condense on the upper (cool) end of the heated tube. These obviously indicate the presence of **Hydrogen** in the original material (provided that material was properly dried).

Nitrogen

Experiment 5. Whenever a preliminary heating of plant or animal material gives the characteristic odour of burning flesh (horn, hair, feathers, etc.), this in itself is reasonable evidence that the element nitrogen is present. Its presence can be confirmed in either of the two following ways :—

(i.) Take a little dried (commercial) egg or blood albumin, and mix it with ten to fifteen times its volume of soda lime

in a mortar. Transfer enough to a test-tube to one-third fill it, and heat over a Bunsen flame in the fume-chamber. The smell of ammonia should be noticeable. Hold at the mouth of the tube a piece of moistened red litmus paper. It will be turned blue. (The ammonia gas dissolves to give an alkaline solution.) Then hold at the mouth of the tube a glass rod that has been dipped in a **test-tube** containing $\frac{1}{2}$ c.c. of concentrated hydrochloric acid. (*Do not dip the rod in the bench reagent bottle.*) Dense fumes result. The ammonia and hydrochloric acid vapours have combined to give **solid** ammonium chloride.

The formation of ammonia in this test indicates the presence of nitrogen in the original substance. Most plant and animal materials when heated with strong alkali or acid liberate their nitrogen as ammonia.

(ii.) Mix thoroughly some dried albumin with about ten times its weight of a mixture of equal parts of magnesium powder and anhydrous sodium carbonate. One-third fill a dry test-tube with this mixture and heat in the fume-chamber. Gradually increase the heat until finally the mixture is kept at red heat for half a minute. Then immediately dip the red-hot tube into a beaker containing about 3 to 4 c.c. of distilled water. The bottom of the tube breaks up to small fragments, and its contents partly dissolve in the water. Filter. Add to the filtrate 2 drops of a cold saturated solution of ferrous sulphate (freshly prepared) and a drop of ferric chloride solution. Warm the mixture for two or three minutes, then cool it, and then just acidify with concentrated hydrochloric acid. The solution becomes bluish-green, and gradually a precipitate of " Prussian blue " (ferric ferro-cyanide) separates out.

In this test during the fusion sodium cyanide is formed from part of the nitrogen of the albumin, and this, treated with ferrous sulphate and ferric chloride and warmed, is converted to " Prussian blue." The production of this coloured compound therefore indicates the presence of *nitrogen* in the substance that is being tested.

Sulphur

Experiment 6. Note that in the following tests a negative result for " loosely combined sulphur " does not prove that sulphur is absent from the substance under test. A fusion

test is essential to determine absolutely the presence or absence of sulphur.

(i.) Add 2 drops of a solution of neutral lead acetate to a few cubic centimetres of sodium hydroxide solution. A precipitate of lead hydroxide is first formed and then redissolves. Add a very little powdered albumin to this solution and warm. The mixture turns black. Part of the sulphur in the albumin molecule has been split off to form sulphide, and this reacting with lead ions has given insoluble black lead sulphide. The sulphur capable of reacting in this way is spoken of as "*loosely combined sulphur.*"

(ii.) Fuse some of the albumin-magnesium powder-sodium carbonate mixture as in Experiment 5 (ii.), and after breaking the tube in water and filtering add to the filtrate a *freshly prepared* solution of sodium nitroprusside. A reddish-violet colour appears. The sulphur of the albumin has been converted into sodium sulphide, and sulphides give this colour reaction with the nitroprusside.

(iii.) Take enough dried albumin to cover $\frac{1}{2}$ in. of a penknife blade, place it in a porcelain crucible, then add $\frac{1}{2}$ in. of stick potassium hydroxide and about as much potassium nitrate as albumin. Heat strongly. Cool. Treat with water and filter. Acidify the filtrate with hydrochloric acid. Add barium chloride (4 or 5 drops). A white precipitate of barium sulphate is produced. The sulphur present in the albumin has been oxidised to sulphate.

Phosphorus

Experiment 7. Fuse some casein, or some nucleo-protein, or some lecithin (all of which contain phosphorus in organic combination) just as in Experiment 6 (iii.). Cool, treat with water and filter. Mix 3 c.c. of the filtrate in a test-tube with 2 c.c. of concentrated nitric acid and 4 or 5 c.c. of ammonium molybdate solution, and warm gently for some time. A yellow crystalline precipitate of ammonium phospho-molybdate gradually separates along the side of the tube. The formation of this precipitate indicates the presence of **Phosphorus** in the original substance.

Iodine

Experiment 8. Mix about as much desiccated thyroid tissue as will be held on a silver threepenny or five-cent

piece with five or six times its weight of anhydrous sodium carbonate in a porcelain crucible, add 2 drops of concentrated sodium hydroxide and heat over a Bunsen flame in the fume-chamber, gradually increasing the heat to redness. After ten minutes cool and place in a beaker containing 15 to 20 c.c. of distilled water. Warm. Filter. Transfer part of the filtrate to a test-tube, slightly acidify with concentrated hydrochloric acid, add 1 c.c. of chloroform and then 2 or 3 drops of chlorine water. Shake up and allow the layers to separate. The chloroform layer will become pinkish-violet in colour.

The fusion liberates iodine from organic combination to form sodium iodide. Chlorine displaces iodine from this salt, and iodine in chloroform is coloured as described. Excess of chlorine must be avoided, since it forms with iodine colourless iodine chloride.

Iron

A simple test for the detection of iron is given in Exercise XII., Experiment 9.

EXERCISE II

THE CARBOHYDRATES

(Four or five Three-hour Periods)

THE important groups of carbohydrates that will be studied are the simple sugars (monosaccharides), typified by *glucose*, the complex sugars (disaccharides), typified by *cane sugar* and *lactose*, and the polysaccharides, typified by *starch* and *cellulose*.

In each of these divisions it is necessary to study also other compounds closely related to the types—fructose, galactose, and arabinose, related to glucose, maltose, related to lactose, and glycogen, the dextrins, and inulin, related to starch.[1]

Glucose

The tests for glucose depend upon both chemical properties and physical properties. On account of its easy oxidisability, it brings about certain "reductions" and is hence a reducing sugar. It reacts with phenylhydrazine to form a characteristic yellow crystalline "osazone," which can be identified under the microscope. It is decomposed by certain ferments in yeast with production of ethyl alcohol and carbon dioxide gas, so that the evolution of gas serves as a test for it. It rotates the plane of polarisation of polarised light, and can therefore be tested for in the polarimeter. It is readily soluble in water.

[1] Before and while carrying out the tests in this Exercise the chapters on carbohydrates in a theoretical text book should be consulted, *e.g.* Cameron's "Biochemistry," 5th edit., Chapter IV.

The following tests should be carried out with a 1 per cent. solution of glucose :—

General Test for Carbohydrates (Molisch's Test)

Experiment 1. Add to 5 c.c. of glucose solution in a test-tube 2 drops of the special *Molisch's reagent* (made by dissolving 5 gm. of α-naphthol in 100 c.c. of ethyl alcohol). Shake. Pour carefully down the side of the inclined test-tube 5 c.c. of concentrated sulphuric acid. With care scarcely any mixing takes place.

A reddish-violet colour is produced at the surface between the two solutions. (A greenish colour is to be neglected.)

This test is given by all soluble carbohydrates, and a similar colour is developed on the surface of insoluble carbohydrates under appropriate conditions.

Reduction Tests

Copper solutions are reduced with precipitation of cuprous oxide (red or yellow according to variations in the experimental conditions), certain silver solutions are reduced with precipitation of metallic silver, and certain bismuth solutions with precipitation of metallic bismuth. Such results are due to the ease with which glucose and related sugars can be oxidised. This is usually attributed to the presence (or potential presence) of an aldehyde or ketone radical in the carbohydrate molecule.

Trommer's Test

Experiment 2. To 3 c.c. of glucose solution in a test-tube add an equal volume of 40 per cent. sodium hydroxide. Then add drop by drop a *very dilute* solution of copper sulphate. A deep blue solution is produced. Continue adding copper sulphate until there is the faintest turbidity. Warm nearly to boiling. A yellowish-red precipitate of cuprous oxide separates.

Reduction of Fehling's Solution

FEHLING'S SOLUTION. This solution decomposes fairly rapidly, so that its two parts must be kept separately. Part I

consists of 34·65 gm. crystallised copper sulphate, $CuSO_4$, $5H_2O$, dissolved in water, and water added to 500 c.c. Part II consists of 125 gm. of potassium hydroxide and 173 gm. Rochelle salt (sodium potassium tartrate) dissolved in water, and water added to 500 c.c.

Experiment 3. Mix 1 c.c. of each of the two parts of Fehling's solution in a test-tube. Note the appearance of a deep blue colour, due to the formation of a complex copper tartrate. Add an equal quantity of water. Boil. No change of colour and no reduction should take place. If any does occur the Fehling's solution has decomposed and must be replaced by fresh solution. If the solution is proved to be satisfactory, then—

As before, mix equal quantities of the two solutions (1 c.c. of each) in a test-tube and add 2 c.c. of the glucose solution. Boil. A yellow or brownish-red precipitate of cuprous oxide is produced rapidly, the colour of the precipitate depending on the rapidity of the reduction.

Reduction of Benedict's Solution

BENEDICT'S SOLUTION. This has the following composition: 17·3 gm. copper sulphate, 173 gm. sodium citrate, 100 gm. anhydrous sodium carbonate, dissolved in water, and water added to 1 litre. The substitution of citrate for tartrate gives a much more permanent solution.

Experiment 4. Transfer 5 c.c. of Benedict's solution to a test-tube and add exactly 8 drops (using a small piece of glass tubing or a pipette as a dropper) of the glucose solution. Boil for two minutes. Allow to cool slowly. A precipitate gradually forms throughout the solution, red, yellow or green, depending upon the concentration of the glucose solution used. In absence of a reducing substance the solution remains clear.

Reduction of Nylander's Solution

NYLANDER'S SOLUTION. This is prepared by heating 2 gm. bismuth subnitrate and 4 gm. Rochelle salt with 100 c.c. of 10 per cent. potassium hydroxide solution, cooling and filtering.

Experiment 5. Add exactly 10 drops of Nylander's solution to 5 c.c. of glucose solution in a test-tube, and heat for five minutes on a boiling water-bath. The solution gradually darkens and turns black, due to precipitation of metallic bismuth. (Note that albumin also produces this effect.)

Reduction of Barfoed's Solution

BARFOED'S SOLUTION. This is prepared by dissolving 4·5 gm. of crystalline copper acetate in 100 c.c. water (filtering if necessary), and adding 1 c.c. of 50 per cent. acetic acid. It keeps only for a short time.

Experiment 6 (after Hinkel and Sherman). To 1 c.c. of the glucose solution in a test-tube add 5 c.c. of Barfoed's reagent. Place in the boiling water-bath for 3·5 minutes. A red precipitate of cuprous oxide forms.

Formation of an Osazone

Experiment 7. Two parts of phenylhydrazine hydrochloride (by weight) are intimately mixed with three or four parts of sodium acetate. Place just sufficient of this **Phenylhydrazine Mixture** in a test-tube to fill the rounded bottom of the tube, then add 5 c.c. of the glucose solution, and after shaking, filter into a second tube and heat on the boiling water-bath for thirty minutes. A yellow crystalline precipitate gradually forms. Allow to cool slowly. The precipitate increases. Transfer a trace to a slide, cover with a clean coverslip and examine under the microscope. Compare the appearance of the crystals with the photographs in Plate I.

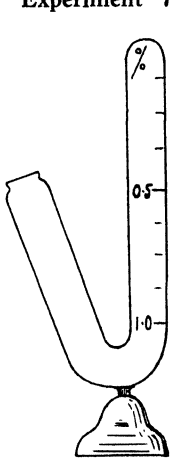


FIG. 1. Einhorn saccharimeter.

Note. When glucose is only present in small amount the appearance of the osazone is markedly affected by other constituents (impurities). This is well shown by the difference between (*d*) and (*e*), Plate I. Crystals resembling (*e*) are frequently seen

when the osazone is prepared from urines containing only a trace of glucose.

DEMONSTRATION.—Fermentation of Glucose

Experiment 8. Rub up in a mortar 20 to 25 c.c. of the sugar solution with 1 or 2 gm. of Fleischmann's yeast or some similar dried yeast preparation until the consistency of the mixture resembles milk. Transfer to an Einhorn saccharimeter (Fig. 1) and invert this so that the closed end is completely filled with liquid. Restore the saccharimeter to normal position so that the longer tube remains filled. Place in an incubator at 37° C. and allow to remain overnight. Gas has then collected in the closed limb of the apparatus, forcing most of the liquid back into the bulb. Add dilute sodium hydroxide until the open limb is filled, close the opening with the thumb and invert several times. The gas should be almost completely absorbed, and on removing the thumb the liquid should almost completely fill the closed limb. This absorption indicates that the gas consisted almost entirely of carbon dioxide, formed from glucose by fermentation.

The student should repeat this test himself.

DEMONSTRATION.—Fermentation of Glucose

Experiment 9. Into a 500 c.c. flask pour 250 c.c. of 10 per cent. glucose solution. Add several grams of a dried yeast preparation and a little commercial peptone (to afford nutriment to the yeast). Fit with a cork and bent tube leading to a second, smaller flask, containing a saturated solution of barium hydroxide. The tube should dip below the surface of this solution. Close the second flask with a two-hole cork and connect through the second hole with a soda-lime tube (to prevent entry of carbon dioxide from the atmosphere). Place in an incubator at 37° C. for from twenty-four to forty-eight hours. During this period carbon dioxide steadily bubbles into the smaller flask, forming a precipitate of barium carbonate. After the allotted time has elapsed decant off the clear liquid from the larger flask into a distillation flask and distil, collecting the first 25 c.c. of the distillate.

Test this for ethyl alcohol : (*a*) Add to 2 or 3 c.c. 1 drop of

sodium hydroxide solution and a few drops of iodine solution (in potassium iodide) and warm; the odour of iodoform should be detectable and iodoform crystals should separate. Examine these under the microscope and identify by comparison with crystals of iodoform. (*b*) To 5 c.c. of the distillate add a few drops of potassium bichromate solution and a drop or two of dilute sulphuric acid and heat. The odour of acetaldehyde should be detectable, changing to that of acetic acid, while the colour should change to a dirty green.

Note. The iodoform test is also given by aldehydes and other compounds.

DEMONSTRATION.—Optical Activity of Glucose

On account of the optical activity of glucose, it rotates the plane of polarisation of light to the right. From this it follows that when plane polarised light, formed by passage of light rays through a pair of Nicol prisms, is passed through a solution of glucose, the plane in which the light is vibrating is rotated **clockwise.** A second pair of Nicol prisms, placed in corresponding position to the first, but behind the solution, will therefore no longer allow the maximum amount of light to pass through unless they are also rotated clockwise to the right, and to the same extent as the solution has rotated the plane of light. However, since the measurement of maximum

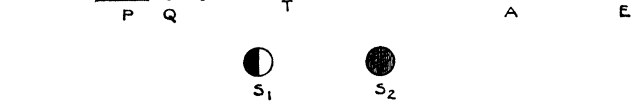


Fig. 2. Rough diagram of a polarimeter. P, polarising Nicol prism; Q, quartz plate covering *one-half* of the field; T, glass sugar tube closed by glass plates; A, analysing (rotating) Nicol prism; E, eyepiece. The ocular lenses are not shown.

S_1, view at position of unequal shadow; S_2, view at position of equal shadow.

intensity of light is extremely difficult, a device has been introduced into the polariscope whereby one half of the field of light is retarded one half wave-length. With

this arrangement, when one half of the field admits maximum light the other half admits none. By rotation a position of " equal shadow " of the two halves can be fixed very accurately in relation to a circular scale. Fig. 2 shows a schematic arrangement of the polarimeter.

Experiment 10. The demonstrator will show that a tube containing water gives an " equal shadow " position corresponding to zero on the scale. A tube containing 1 per cent. glucose is then introduced, and the second pair of Nicol prisms is rotated until the " equal shadow " position is again attained. The reading is carefully noted. Now a tube of the same length containing 2 per cent. glucose is introduced, and a second reading taken and noted.

It is convenient at this stage to measure also the corresponding rotation produced by tubes containing 1 per cent. cane sugar and 1 per cent. fructose.

The *specific rotation* of such compounds is defined as that rotation expressed in degrees which is produced by 1 gm. of the compound under measurement dissolved in water to make 1 c.c. of solution, this 1 c.c. of solution forming a column exactly 1 decimetre in length. It can be calculated from the formula

$$(\alpha)_D = \alpha/pl,$$

where α is the observed rotation, p is the number of grams of the compound dissolved in 1 c.c. of solution, l is the length of the tube in decimetres and $(\alpha)_D$ is the specific rotation for sodium light. (It varies slightly for different wave-lengths.)

The specific rotations of the three compounds examined are—

d-Glucose	$+ 52{\cdot}2°$,
d-Fructose	$- 92{\cdot}0°$,
Sucrose	$+ 66{\cdot}5°$.

FRUCTOSE

Carry out Experiments 1 to 8 with a 1 per cent. solution of fructose. Positive results will be obtained in every test.

Specific Tests for Fructose

Experiment 11. SELIWANOFF'S REACTION. To 5 c.c. of the special (Seliwanoff's) reagent (0·5 gm. of resorcinol

dissolved in a litre of 10 per cent. hydrochloric acid) add a few drops of the fructose solution. **Boil for not more than thirty seconds.** A red colour develops, and then a brown-red precipitate separates. (Dilute solutions of fructose do not give the precipitate.) Decant off the solution and add alcohol. The precipitate dissolves to a red solution.

Repeat this test with 1 per cent. glucose solution, and note that no red colour develops during thirty seconds' boiling, but that longer boiling does produce it.

Experiment 12. DIPHENYLAMINE TEST. To 2 c.c. of the sugar solution add 1 c.c. of concentrated hydrochloric acid and 3 drops of a 20 per cent. alcoholic solution of diphenylamine. Place in a boiling water-bath for not more than 2 minutes. An intense blue colour indicates the presence of fructose. (With prolonged boiling, glucose also gives a blue colour.)

Galactose

Carry out Experiments 1 to 8 with a 1 per cent. solution of galactose. Positive results will be obtained in the first seven experiments. Show that Seliwanoff's test gives a negative result.

Experiment 13. MUCIC ACID TEST. Take 25 c.c. of the galactose solution in a small evaporating basin, preferably of glass, and add 5 c.c. of concentrated nitric acid. Heat on the boiling water-bath in the fume-chamber until the volume is reduced to 5 or 6 c.c. A white precipitate slowly forms. Stir it with a glass rod and note that it is composed of hard and gritty crystals. Galactose has been oxidised to insoluble, crystalline mucic acid. The test is specific for galactose and compounds which yield galactose on hydrolysis.

Note. If a porcelain basin is used, transfer the concentrated liquid to a test-tube, and set aside. If galactose (or lactose) was present in the original solution crystals of mucic acid should separate out while the liquid is cooling.

Arabinose (a Pentose)

Carry out Experiments 1 to 8 with a 1 per cent. solution of arabinose and note that positive results are given in every test except Experiment 8. Repeat Experiments 11 and 13; negative results are obtained.

Experiment 14. BIAL'S REACTION (*Sumner's Modification*). The modified Bial's reagent consists of 6 gm. of orcinol in 200 c.c. of 95 per cent. ethyl alcohol, to which 40 drops of 10 per cent. ferric chloride have been added.

To 2 c.c. of arabinose solution add 5 c.c. of concentrated hydrochloric acid, and 15 drops of the reagent. Heat gently to the boiling point. Cool. The solution becomes deep blue-green, and a green flocculent precipitate may form. This test is specific for pentoses and glycuronic acid.

Experiment 15. THE ANILINE TEST.[1] To 2 c.c. of arabinose solution add 2 c.c. of glacial acetic acid, and 5 drops of pure (redistilled) aniline. Heat the mixture just to boiling and allow to stand for 2 minutes. A bright red coloration appears. This test is specific for pentoses, and is not given by glycuronic acid.

Experiment 16. THE β-NAPHTHOL RING TEST. The reagent consists of 0·3 gm. of β-naphthol dissolved in 100 c.c. of concentrated sulphuric acid. Take about 3 c.c. of this reagent in a test-tube, and *carefully* pour down the sides of the tube an equal volume of arabinose solution. At the junction of the liquids a coloured ring is formed almost immediately. This is deep blue for pentoses. A brownish-green ring may be formed with hexoses and glycuronic acid.

DEMONSTRATION.—Glycuronic Acid

Repeat Experiments 1 to 8 and 14 with a solution of a glycuronate.

Experiment 17. NAPHTHORESORCINOL TEST. To 2 c.c. of glycuronate solution add 2 c.c. of concentrated hydrochloric acid, and 15 drops of 1 per cent. alcoholic solution of naphthoresorcinol. Boil for 1 minute, then allow to stand for 4 minutes, cool, and extract with ether. The ether layer shows a marked reddish violet colour. This test is specific for glycuronic acid and glycuronates.

Galactans and Pentosans[2]

Experiment 18. Take sufficient agar-agar to fill the

[1] White and Green, *Trans. Roy. Soc. Can.*, 1932, XXVI., Sect. V., 145.

[2] Tests for these polysaccharides can conveniently be carried out at this stage.

rounded bottom of a test-tube. Add 4 c.c. of water and 1 c.c. of concentrated hydrochloric acid, and hydrolyse by heating on the boiling water-bath for twenty minutes. Cool, and neutralise with dilute sodium hydroxide (testing with litmus paper). Show that galactose is present in the hydrolysed solution, utilising Benedict's test and the mucic acid test.

Experiment 19. Hydrolyse a small amount of gum arabic by similar treatment, and show that the diluted and neutralised hydrolysate contains a pentose, utilising Benedict's, Bial's and the aniline tests. This pentose is arabinose.

THE DISACCHARIDES

Sucrose or Cane Sugar

Repeat Experiments 1 to 8 with a 1 per cent. solution of cane sugar. Note that Molisch's and the fermentation tests are positive, while the reduction and osazone tests are negative. In the reduction tests *prolonged* boiling may give some reduction through partial hydrolysis of the sucrose.

Repeat Seliwanoff's and the diphenylamine tests. Prolonged boiling will give a markedly positive reaction, since after hydrolysis both fructose and glucose are present.

Experiment 20. Specific Colour Test. This is based on the reaction between sucrose and diazo-uracil.[1] Add 1 c.c. of 1 per cent. sodium nitrite solution to 5 c.c. of an amino-uracil solution (0·2 per cent. of 5.amino-uracil in *N*/20 hydrochloric acid). Mix (diazo-uracil is formed). To 2 c.c. of the mixture add 5 c.c. of a 1 per cent. sucrose solution and 3 drops of 10 per cent. sodium hydroxide, and allow to stand. In a few minutes a clear blue colour develops. (The reaction is also given by raffinose, although somewhat more faintly. Glucose, fructose, maltose, lactose, galactose,

[1] Raybin, *J. Amer. Chem. Soc.*, 1933, LV, 2603. The test has been slightly modified by one of the present authors (F.D.W.). 5.Amino-uracil is now available commercially. It can be prepared by converting uracil into nitro-uracil (Johnson and Matsuo, *J. Amer. Chem. Soc.*, 1919, XLI, 782), and reducing to the amino-compound (Bogert and Davidson, *ibid.*, 1933, LV, 1667).

arabinose, inulin, and dextrin give only a yellow to brown colour.)

DEMONSTRATION.—**"Inversion of Cane Sugar"**

Experiment 21. Make up 100 c.c. of a 5 per cent. solution of sucrose. Measure the rotation. Take 50 c.c. of this solution (accurately measured) in a shallow porcelain dish, add 2 c.c. of concentrated hydrochloric acid and heat on the water-bath for forty-five minutes. Cool, exactly neutralise with 10 per cent. sodium hydroxide, transfer to a 50 c.c. graduated flask, with rinsings, and make up to the original 50 c.c. volume. Again measure the rotation. Calculate from the results whether or not the change represents complete transformation of the sucrose to glucose and fructose.

Then, with the hydrolysed solution, carry out Benedict's, Fehling's, Barfoed's and Seliwanoff's tests, and show that these are all positive.

The student himself should hydrolyse a little sucrose on the small scale with acid, cool, neutralise and carry out the tests just enumerated.

Lactose

Experiment 22. Make up a 1 per cent. solution of lactose. Note the hard, gritty feel of the crystals and their relative insolubility as compared with glucose and fructose.

Repeat Experiments 1 to 8 with this solution. Note that positive results are obtained with all these tests except Barfoed's and the fermentation test. *Barfoed's test is not given by disaccharides.* A good baker's yeast (free from foreign strains) does not ferment lactose. Note the microscopic appearance of the lactosazone and compare it with the photograph in Plate I.

Repeat the mucic acid test (Experiment 13). A positive result is obtained. Lactose is first hydrolysed to glucose and galactose, and these are oxidised respectively to saccharic and mucic acids. The saccharic acid remains in solution.

Maltose

Experiment 23. Repeat experiments 1 to 8 with a 1 per cent. solution of maltose. Note that all the tests are positive except Barfoed's. Note the microscopic appearance of the osazone and compare it with the photograph in Plate I.

Note. Glucosazone crystals commence to separate from the hot solution, but lactosazone and maltosazone crystals only commence to separate when the solution is allowed to cool. This fact helps to differentiate between them.

POLYSACCHARIDES

Starch

Experiment 24. Scrape a raw potato, stir up the scrapings vigorously in a little water and strain through cheese cloth into a small beaker. The starch granules rapidly settle. Decant off the liquid, wash with cold water, and again decant off the liquid. Shake up the residue with water, transfer a

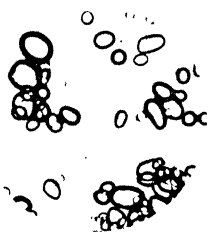


FIG. 3A.

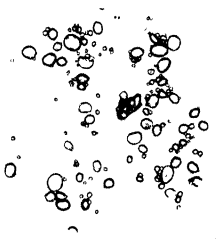


FIG. 3B.

FIG. 3. Photo-micrographs of starch granules, × 90. A, from potato ; B, from wheat.

little to a slide and examine under the low power of the microscope. Make a sketch of the granules. Compare with a little rice and wheat starch suspended in water and transferred to slides. Note that each has a characteristically different appearance. Compare with the photo-micrographs in Fig. 3.

Experiment 25. Place a few granules of starch on a porcelain tablet and add a drop of diluted iodine solution (iodine in potassium iodide). The granules are coloured blue.

(Repeat this test with agar-agar, a galactan, and gum arabic, a pentosan, and note that no blue colour is given, indicating the specific nature of this test for starch.)

Experiment 26. Carry out Molisch's test with some starch granules. The positive test for carbohydrates is obtained.

Experiment 27. Treat some starch granules with cold water. Decant off the liquid and test it with iodine solution. It is only coloured yellow (due to the iodine). Starch is insoluble in cold water. Warm starch granules with water. Then pour off the supernatant liquid and test this with iodine solution. A blue colour results. Warm. The colour disappears. Just as soon as it has disappeared cool the solution. The colour reappears.

Starch is soluble in hot water, giving an opalescent solution. It reacts with iodine to give the so-called "iodide of starch." Note that if the blue "iodide of starch" is heated too long the colour does not reappear on cooling, due probably to an interaction between the iodine and water.

Experiment 28. Make up a stiff starch paste by stirring starch granules with warm water. Take 10 c.c. in a small beaker, add 4 drops of concentrated hydrochloric acid and heat on the water-bath. Then, at minute intervals, remove a drop of the solution to a porcelain tablet and test with iodine solution. The blue colour is gradually replaced by a port-wine tint, and finally no colour is given. At this stage cool, add 10 per cent. sodium hydroxide drop by drop until the solution is alkaline to congo red paper, but still acid to litmus, and test a portion with Seliwanoff's reagent; note absence of fructose. To the remainder add 10 per cent. sodium hydroxide drop by drop until neutral to litmus, and then test portions with Benedict's solution and with the phenyl-hydrazine test. Note that there is a reducing sugar present, and that both maltosazone and glucosazone crystals may be seen under the microscope.

PROPERTIES OF COLLOIDS [1]

Certain colloidal properties can be conveniently demonstrated by comparing a sugar such as sucrose or glucose and a colloid such as starch. Note that the first is crystalline and its solution is clear, while the second is amorphous and its solution is opalescent.

[1] *Cf.* Cameron's "Biochemistry," 5th edit., Chapter II., p. 31.

DEMONSTRATION.—Turbidity

Experiment 29. Fill two Erlenmeyer flasks respectively with 1 per cent. solutions of starch and of sucrose. Make a small pinhole in a piece of cardboard and in a dark room place an electric light on one side of the cardboard and the two solutions in turn on the other. The beam of light is visible in the starch solution and invisible in the sugar solution (Tyndall's phenomenon).

Diffusion

Experiment 30. Make up a 0·05 per cent. solution of iodine in 0·1 per cent. potassium iodide. Dissolve in this sufficient agar to make a 2 per cent. solution. Half fill two test-tubes with the solution and allow them to set to a jelly. Pour on to the surface of one 5 c.c. of 1 per cent. starch solution and on to that of the other 5 c.c. of 1 per cent. copper sulphate solution to which ammonium hydroxide has been added in excess just sufficient to give a deep blue-coloured solution. Set the tubes vertically in a rack for two or three hours or longer. Note the slow development of a blue colour, passing downwards in the tube containing starch, the iodine starch reaction indicating *the slow rate of diffusion of the colloid*, while the movement of the blue copper crystalloidal solution is much more rapid.

DEMONSTRATION.—Dialysis

Experiment 31. Collodion bags are prepared by dissolving some collodion (*e.g.*, solid celloidin) in a mixture of equal parts of absolute alcohol and ether to give a thick syrup. Pour 3 or 4 c.c. of this into a test-tube and rotate the tube in a horizontal position. After the whole of the interior is covered by a film of the solution pour off the remainder. Continue rotation until the collodion film is fairly firm and most of the ether and alcohol has evaporated, then gently detach the film round the rim of the tube, pour in a little distilled water between the film and the glass and rotate with gentle pulling until the bag is detached.

Two-thirds fill a collodion bag with a 1 per cent. solution of starch, making certain of the absence of pinholes. No

starch should leak from the bag. Suspend in a dilute solution of iodine in potassium iodide in a beaker. On standing there is no development of blue colour in the iodine solution for some hours (the starch does not dialyse to any appreciable extent), but, on the other hand, the starch within the bag does turn blue (the iodine can pass through).

Two-thirds fill a second tube with 1 per cent. glucose solution and suspend it in distilled water in a beaker. After thirty minutes test the water for reducing sugar. It will be found that the glucose has dialysed through the bag.

Dextrins

These are intermediate in composition between starch and maltose and are formed from starch by partial hydrolysis.[1]

Experiment 32. Dissolve some powdered dextrin in water. Note that it dissolves easily in hot water to a clear solution. To some of the solution add iodine solution. Note the formation of a brownish-red colour. Carry out Molisch's test with a little of the solution. A positive result is obtained.

Test a little of the solution with Benedict's reagent. There will be no definite reduction if pure dextrin is used.

Hydrolyse a little of the solution with acid (exactly as for starch). Note that after a very short interval no colour is given with iodine. After neutralisation test with Benedict's solution (positive result) and prepare an osazone (glucose-azone).

Glycogen

Experiment 33. Take a little glycogen (that has been prepared from liver or muscle), note its amorphous character, and dissolve it in hot water. The solution is opalescent. To a few cubic centimetres add iodine solution. Note that the same colour is produced as with dextrins. Hydrolyse a little of the solution with acid and note that the hydrolysed solution gives a positive Benedict's test and yields glucosazone.

[1] Some commercial dextrins are not completely free from starch or reducing sugar. They can be purified from starch by making a concentrated aqueous solution and adding an equal volume of 95 per cent. ethyl alcohol, which precipitates the starch.

Inulin

Inulin is a white amorphous powder, prepared from dahlia roots.

Experiment 34. Make a solution of inulin in hot water. Cool. Add iodine solution. Note the yellow colour. To an equal volume of water in another test-tube add the same amount of iodine as was added to the inulin. Note that the same depth of yellow colour is produced and that this is **due simply to the dilution of the iodine solution.**

Note that Molisch's test is positive, while Benedict's test is negative.

Hydrolyse some of the inulin solution. Neutralise. Note that Benedict's test is now positive, that the phenylhydrazine test yields glucosazone, and that Seliwanoff's and the diphenylamine tests are positive for fructose. **Inulin hydrolyses to fructose.**

Celluloses

Cotton-wool, filter paper and (white) blotting paper are composed of almost pure cellulose.

Experiment 35. Take a few shreds of filter paper and add 10 c.c. of 50 per cent. sulphuric acid. Note that the filter paper slowly dissolves without application of external heat.

Take a portion of this solution, cool, and dilute with an equal quantity of water. **Amyloid** separates out as a gummy precipitate. Add iodine solution. A brown or blue colour is produced.

Take a second portion, allow to stand ten or fifteen minutes, dilute with water, and boil thirty minutes. Cool, neutralise, and test with Benedict's, the phenylhydrazine and Seliwanoff's tests. Note that **glucose is produced by the hydrolysis of cellulose.**

Experiment 36. To a little cotton-wool in a test-tube add Cross and Bevan's reagent (two parts of concentrated hydrochloric acid and one part of zinc chloride crystals by weight). Stir with a glass rod. The cellulose dissolves. Reprecipitate by addition of 95 per cent. ethyl alcohol.

Tests for Unknown Carbohydrates

A positive Molisch test indicates that carbohydrate is *probably* present.

The following scheme can be used to identify unknown carbohydrates :—

If the unknown is a solid note whether it is crystalline (suggesting sugars) or amorphous. Make a solution in water, noting whether heat is necessary.

If the unknown is a solution (or after the solution has been prepared) note whether it is clear or opalescent, acid, neutral or alkaline. If opalescent, starch or glycogen is suggested ; if alkaline, neutralise with dilute hydrochloric acid.

Test a cubic centimetre in a test-tube with 1 or 2 drops of iodine solution.

(A) A blue colour indicates starch. Confirm by absence of reducing properties and then, after hydrolysis, presence of reducing sugar.

(B) A red colour indicates dextrins if the solution is clear, or glycogen if the solution is opalescent. Confirm by reduction tests after hydrolysis. (Agar-agar, whose solution is also opalescent, gives a port-wine tint with excess of iodine, but under the conditions stated no colour is produced with it.)

(C) If no colour is given with iodine, sugars or inulin may be present. Test with Benedict's solution.

(i.) A negative result suggests sucrose, a pentosan, a galactan, or inulin. Hydrolyse and apply Seliwanoff's and the diphenylamine tests. A positive result indicates inulin or sucrose. Taste the original solution. If it is sweet sucrose is indicated (confirm by the specific colour test), if tasteless inulin.

Note. **In tasting a solution put a drop on the tip of the tongue and then immediately spit it out and wash out the mouth with water.**

If Seliwanoff's test is negative apply tests for pentoses. If these are positive a pentosan is indicated ; if negative, then a galactan is present.

(ii.) A positive result with Benedict's test indicates reducing sugars. Test the original solution with Barfoed's reagent.

(*a*) If the result is negative lactose or maltose may be present. Apply the mucic acid test. A positive result indicates lactose, a negative, maltose. Confirm by preparing the osazone and examining it under the microscope. A positive maltosazone indicates maltose. Confirm by hydrolysing some of the original solution, when a fresh phenylhydrazine test should yield glucosazone.

(*b*) If the result is positive glucose, fructose, galactose or pentoses may be present. Differentiate by successive applications of Seliwanoff's, Bial's, the aniline, and the mucic acid tests until a definite conclusion can be drawn, and confirm by an osazone test.

Note. **The scheme only includes those sugars of biochemical importance.**

EXERCISE III

SALIVA AND AMYLASE

(Three Three-hour Periods)

THE study of saliva affords a convenient method of introduction, not only to the nature of a typical digestive juice, but also to the nature of enzyme action.[1]

Preparation of Saliva. Chew a small piece of paraffin wax; the act of chewing stimulates a flow of saliva. Collect 20 or 30 c.c. in a beaker. Filter a portion. Since the filtration frequently takes a considerable amount of time, carry out the following tests with filtered or unfiltered saliva as directed :—

Experiment 1. Transfer sufficient unfiltered saliva to a small clean urinometer tube to nearly fill it, and measure the specific gravity with the urinometer. (It should lie between 1·002 and 1·008.) Test the saliva with litmus paper, phenolphthalein paper, and congo red paper. (If these paper strips are not available add 1 drop of the corresponding **indicator** solution to 1 c.c. of saliva in a test-tube.) The saliva will react alkaline to congo red, acid to phenolphthalein, and neutral or very faintly acid to litmus, owing to the fact that these indicators change colour at very different degrees of hydrogen-ion concentration.

Use up the saliva in tests calling for unfiltered saliva.

Experiment 2. To 2 c.c. of filtered saliva in a test-tube add 1 drop of dilute acetic acid. A white amorphous precipitate of the glucoprotein **Mucin** separates. This

[1] The student should read, before and while carrying out this Exercise, the portions of a theoretical text-book dealing with the nature of enzyme action and the composition and action of saliva, *e.g.*, Cameron's "Biochemistry," 5th edit., Chapter III., Chapter IV., pp. 95–6, and Chapter IX., p. 210

protein aids, by virtue of its viscous nature, in the mastication and balling together of solid food prior to swallowing. It contains carbohydrate radicals.

Experiment 3. *For this and the following two experiments use filtered saliva, one volume diluted with four volumes of water.*

To 2 c.c. of saliva add an equal volume of 40 per cent. sodium hydroxide and 1 or 2 drops of very dilute copper sulphate. A purplish-red colour is formed, due to the protein **Mucin.** (This is an example of a positive biuret test; see Exercise V.)

Experiment 4. To 2 or 3 c.c. of saliva add a few drops of Millon's reagent (see Exercise V.). A light yellow precipitate forms. Warm. The colour gradually changes to red. This also is due to the presence of a protein, **Mucin.**

Experiment 5. Add to 1 c.c. of saliva 2 drops of dilute nitric acid and 1 drop of silver nitrate. A white precipitate is formed, due to presence of **chloride** ions. On addition of a little ammonia the precipitate dissolves.

Experiment 6. Add to 1 c.c. of filtered but undiluted saliva 1 c.c. of concentrated nitric acid and 2 c.c. of ammonium molybdate solution, and warm gently. The **Phosphate** present forms a yellow crystalline precipitate of ammonium phosphomolybdate. (*Cf.* Exercise I., Experiment 7.)

Experiment 7. Add to 1 c.c. of filtered but undiluted saliva 2 or 3 drops of dilute hydrochloric acid, and then 2 drops of barium chloride solution. A white precipitate indicates the presence of **Sulphate.**

Experiment 8. To 5 c.c. of filtered, undiluted saliva in a small porcelain dish add 3 drops of dilute ferric chloride and 2 drops of dilute hydrochloric acid. A red coloration is produced. This is due to the formation of ferric **thiocyanate.** Add a drop of mercuric chloride solution; the colour disappears, since colourless mercuric thiocyanate is formed.

Repeat, using instead of saliva 5 c.c. of very dilute (0·1 per cent.) disodium hydrogen phosphate. Note the difference in result and how it differentiates between thiocyanate and phosphate.

Rapidity of Passage of Ions into Secretions and Excretions

Experiment 9. Swallow whole the small gelatin capsule provided, which contains 0·2 gm. of potassium iodide. The purpose of taking the iodide in this way is to introduce it into the stomach without contact with the saliva.[1] Then immediately rinse out the mouth with water and test for iodide as indicated in the following paragraph. At successive two-minute intervals again rinse out the mouth and test for iodide until a positive test is obtained. Note the time that has elapsed since swallowing the capsule.

Iodide Test. Mix 1 c.c. of 1 per cent. sodium nitrite solution and 1 c.c. of dilute sulphuric acid in a test-tube. Add 1 or 2 c.c. of saliva rinsed from the mouth as above and 1 c.c. of starch solution. If iodide is present in the saliva free iodine will be liberated by the nitrous acid, and will immediately develop a blue colour with the starch.

This test is rendered more complete if the urine is also examined. Empty the bladder before swallowing the iodide capsule, and then empty it at ten or fifteen-minute intervals, and test the successive samples of urine for iodide. Note the time which elapses before iodide is excreted.

Compare the relative times obtained by yourself and by several fellow-students. Considerable variations may be found.

Note. If the capsule of iodide is swallowed shortly after a meal, the positive reaction in both saliva and urine may be considerably delayed.

Digestion Experiments with Saliva

Experiment 10. Add 10 drops of filtered saliva to 25 c.c. of 1 per cent. starch solution (so-called " soluble starch " should be used for making this solution) and shake up. At one-minute intervals remove 1 drop of the solution to a porcelain plate and add to it 1 drop of iodine solution. At first a blue colour forms. Note the time taken until a blue colour is no longer formed (when a brownish-red colour will be seen) and until no colour at all is given (remembering that the iodine solution is itself coloured).

At the end of the experiment compare these times with those obtained by several other students. If the experi-

[1] These gelatin capsules can be made to order by any pharmacist.

ments have been carried out properly and in an exactly parallel manner, any differences found are due to the varying amounts of amylase in the different salivas.

Note that the opalescence of the starch disappears before the blue iodine reaction ceases to be given. When the addition of iodine ceases to produce a colour, test some of what was the starch solution by Benedict's test, and note that reduction occurs. Carry out an osazone test, and note that **Maltosazone** crystals are present.

Experiment 11. The previous experiment was carried out at room temperature (about 20° C.). Ascertain roughly the limits of temperature at which the action of amylase upon starch can occur, and the effect of temperature upon the rate of the action, as follows :—

Place 5 c.c. of 1 per cent. starch solution in each of four test-tubes. Cool one by placing it in a beaker through which is passing a stream of water from the cold water tap. Note the temperature of the water in the beaker. Keep the second at room temperature, noting that temperature. Keep the third in an incubator at about 37° C. (noting the actual temperature), and keep the fourth in the water-bath at a temperature of 75°. Add 2 drops of filtered saliva to each tube. Shake and test with iodine each three or four minutes, exactly as in Experiment 10. Note the time of disappearance of the blue (starch) colour and of the red (dextrin) colour for each tube. (If more convenient, these four tests can be carried out one after the other, but portions of the same sample of saliva must be used.)

It should be found that most rapid action takes place in the tube at 37°, the action being less rapid the lower the temperature (and less rapid at temperatures above 50° if the effects at these higher temperatures are measured), while at 75° the enzyme is destroyed and no action occurs.

Most enzymes are decomposed rapidly at specific temperatures between 70° *and* 100° *C., definitely below the temperatures at which the solutions containing them boil, and more slowly at somewhat lower temperatures ; cooling their solutions towards* 0° *C. slows their catalytic action markedly. For each enzyme there is a specific temperature* (*its optimum temperature*) *at which its catalytic*

power is at a maximum. The optimum temperature for salivary amylase lies between 40° and 50° C.

Influence of *p*H on the Action of Salivary Amylase [1]

Experiment 12. To each of four test-tubes add respectively (*a*) 2 c.c. of 0·4 per cent. hydrochloric acid, (*b*) 2 c.c. of 0·1 per cent. lactic acid, (*c*) 2 c.c. of distilled water, and (*d*) 2 c.c. of 1 per cent. (anhydrous) sodium carbonate. These give respective *p*H values of approximately 1, 5, 7 and 9. Add to each tube 2 c.c. of 1 per cent. starch solution and 2 c.c. of unfiltered saliva, and shake the whole well; then place in the water-bath at 37° C. for fifteen minutes. Remove. Exactly neutralise with acid or alkali as necessary, using litmus as indicator. Divide the contents of each tube into two parts. To one add 1 drop of dilute hydrochloric acid and iodine solution, and to the other apply Benedict's test. It will be found that the tube containing distilled water will show most action, those with lactic acid and sodium carbonate may show slight action, and that with hydrochloric acid will show no action.

The activity of salivary amylase is greatest at pH 7 : the range of activity extends from pH 4 to pH 9. The enzyme is decomposed when these limits are much exceeded.

Experiment 13. Saliva is stated to act more rapidly when diluted with water. Test this statement as follows :—

Take a test-tube containing 6 c.c. of saliva and three test-tubes containing 5 c.c. of distilled water each. Transfer 1 c.c. of saliva from the first to the second tube, shake the latter thoroughly, then transfer 1 c.c. from it to the third, and shake that tube. The concentration of saliva in the respective tubes will be 1, 1/6, 1/36, 1/216. Add 5 c.c. of 1 per cent. starch solution to each tube (so that the dilutions are doubled in each case) and stir up thoroughly. Place on the water-bath at 40°. Carry out tests every two minutes on each tube with iodine solution on a porcelain plate, as described in Experiment 10. Note the time of disappearance

[1] Before performing this experiment the student should read Chapter II., pp. 22 *et seq.*, in Cameron's "Biochemistry," 5th edit., or some similar chapter, dealing with hydrogen-ion concentration, in some other theoretical text-book.

of the blue and of the red colour reactions. Apply your results to the statement as to dilution. Amplify your conclusions by applying, after thirty minutes, Benedict's test to 2 c.c. from each of the tubes.

Pancreatic Amylase

Experiment 14. Repeat experiments 10 to 13, using, instead of saliva, a fresh aqueous extract of pancreas. *Note that pancreatic amylase behaves in a very similar manner to salivary amylase.*

Intestinal Sucrase

Experiment 15. Add to 5 c.c. of 1 per cent. sucrose solution 1 c.c. of a special extract of intestinal mucosa prepared as described below. Make up a similar second tube, but boil the intestinal extract and cool before adding it. Place both tubes on the water-bath at 37° C. for two hours. Then boil, filter off coagulated protein, and test the filtrates for reducing sugar. The first tube should contain it; the other should not. Why is this?

Preparation of the Intestinal Extract. Minced mucosa of pig's intestine is treated with three volumes of 2 per cent. sodium fluoride, and the mixture allowed to stand at room temperature for twenty-four hours, then strained through cotton-wool. (The fluoride acts as a preservative.)

EXERCISE IV

LIPIDES AND THEIR DERIVATIVES

(Two Three-hour Periods)

LIPIDES or lipoids are insoluble in water, and can be extracted from plant and animal tissues by *fat solvents*, such as alcohol, ether and chloroform.

Simple lipides include the fats, the waxes and the cholesterol esters. Complex lipides include the phosphatides (lecithins, cephalins and sphingomyelins), glucolipides (*e.g.*, cerebrosides), sulpho - lipides, etc. Derived lipides are those compounds obtained from lipides by hydrolysis, which still possess similar physical properties. The experiments in this Exercise deal especially with fats.[1]

Experiment 1. Take a little mutton suet in a test-tube and add 3 or 4 c.c. of ether. Shake. Note that the mutton suet, consisting almost entirely of fat, almost completely goes into solution. Pour a little of the clear solution on to a watch-glass and allow the ether to evaporate. Solid fat remains behind. Transfer a little of this to a test-tube, add 2 c.c. of water, and warm. The fat melts to an oil which floats on the surface of the water, but does not dissolve. Add 2 c.c. of alcoholic sodium hydroxide and continue heating. The oil drop gradually disappears, leaving a clear solution of **Soap**. Shake up this soap solution, and note its characteristic behaviour, the rapid formation of a froth on account of the lowered surface tension.

Experiment 2. Add to some of the remaining solution of fat prepared in Experiment 1 an equal volume of alcohol, pour 2 drops on a microscope slide and allow them to

[1] In connection with this Exercise the student should read the chapters devoted to lipides in a theoretical text-book, *e.g.*, Cameron's "Biochemistry," 5th edit., Chapter V.

evaporate. Examine under the microscope the radiating needles of fat crystals left on the slide. Sketch their appearance.

Experiment 3. Pour a drop of the ether solution on to a piece of filter paper. After the ether has evaporated a characteristic, transparent " grease spot " is left on the paper.

Experiment 4. Take some pure olive oil (a vegetable fat); test it with litmus, congo red, and phenolphthalein papers (or solution); note that a drop of it will mix completely with a drop of ether or of alcohol, but not with water. (These tests can be made on a watch-glass.) Shake up some of the olive oil with water in a test-tube. A (temporary) emulsion is produced. On standing the oil separates and rises above the water. Add 2 drops of 0·5 per cent. sodium carbonate solution and again shake. A better emulsion is produced. The formation of a trace of soap has facilitated emulsion formation.

Fats hydrolyse to glycerol and fatty acids such as palmitic and stearic acids, or, in presence of alkali, their corresponding soaps. This is shown by the following series of tests :—

Experiment 5. *Carry out in a fume-chamber all parts of this test requiring the application of heat.*

Add to a few drops of glycerol in a dry test-tube some powdered potassium hydrogen sulphate. Shake up. Warm gently. A peculiar, unpleasant, and irritating odour, due to **Acrolein,** is observed. Acrolein is an unsaturated aldehyde formed from a molecule of glycerol by loss of two molecules of water (abstracted by the potassium hydrogen sulphate). Repeat the test with olive oil, with butter, and with lard. The acrolein smell is given with all three. *This reaction is specific for glycerol, and is given by fats because they are glycerol compounds.*

Experiment 6. Pour a little glycerol into a clean, dry test-tube. Note its thick, viscous appearance. Taste a drop and notice the sweet taste. Transfer several drops to watch-glasses, and note that glycerol mixes completely with water and with alcohol, but not with ether nor with chloroform. Transfer a drop to filter paper and notice the appearance of a grease spot. Wash with water and dry the

paper. The spot has disappeared. Contrast this with the behaviour of olive oil similarly treated.

Experiment 7. Apply Benedict's test to glycerol and note the result.

Experiment 8. Dissolve a little palmitic acid in a 2 : 1 alcohol-ether mixture, transfer a drop to a slide, allow the ether to evaporate, and examine the slide under the microscope. Sketch the feathery crystals.

Experiment 9. Take 2 or 3 c.c. of a 0·2 per cent. solution of palmitic acid in 95 per cent. ethyl alcohol, in a test-tube, add an equal volume of alcoholic sodium hydroxide, and warm for five minutes. Then pour into a beaker containing 10 c.c. of water and boil on the water-bath over a **low flame** (*alcohol vapour easily catches fire*) until the alcohol is driven off. Pour back the solution into a test-tube and cool under the tap. Add sodium chloride crystals until saturation is produced. The excess of crystals sinks to the bottom of the tube, but the **soap** formed rises to the top and is said to have been **salted out.** Skim off this solid sodium soap, add 5 c.c. of water, and shake up. The soap dissolves. Pour half of the solution into another test-tube. Add to one tube a few drops of calcium chloride solution and to the other a like amount of magnesium sulphate solution. In both cases insoluble soaps (of calcium and magnesium respectively) separate.

Experiment 10. Apply the acrolein test to palmitic acid. Note that no smell of acrolein is produced.

Experiment 11. Apply the grease spot test to palmitic acid and note the result.

The above tests characterise glycerol and palmitic acid (a higher fatty acid). They will therefore be employed in the following **saponifications.**

Saponification of Bayberry Tallow (from Wax Myrtle) [1]

Experiment 12. Pour 30 c.c. of water into an evaporating basin. Add 1 c.c. of 40 per cent. sodium hydroxide and then

[1] If bayberry tallow is not available substitute cacao butter (containing a large proportion of tristearin) or some other suitable plant fat. The results will vary slightly with the varying proportions of the different fats present.

as much powdered bayberry tallow as would cover a florin (or a fifty-cent piece). Boil for fifteen minutes, keeping the volume constant by adding more water from time to time. After this period the fat should have entirely passed into solution and no oil drops should be visible. If this is not the case boil longer. When *saponification* is complete pour off one-third of the soap solution into a test-tube (A). Allow the rest to cool and then add concentrated hydrochloric acid until litmus paper shows that the solution is just acid. A white precipitate gradually forms, rises to the surface and forms a cake. This consists chiefly of palmitic acid (B). The residual liquid (C) contains glycerol.

(i.) Salt out the soap solution (A), redissolve it in water and prepare an insoluble calcium soap from it.

(ii.) Remove the cake of fatty acid (B), break it up, wash it with water, then transfer it to a test-tube, dissolve in 95 per cent. alcohol by warming, filter through a dry paper and allow to crystallise. Examine some of the crystals under the microscope. Redissolve in alcohol-ether mixture, transfer a drop to a slide and again examine microscopically.

(iii.) Evaporate off the water from a little of (C) and test the greasy residue for glycerol by the acrolein test. The test will not be given unless all the water has been driven off.

Hydrolysis of Butter

Experiment 13. Heat about 4 gm. of butter with excess of alcoholic sodium hydroxide; a clear yellow solution results. Pour into 10 c.c. of water. No oil drops should be seen if heating has been prolonged sufficiently. Heat the liquid on the water-bath **carefully** until all the alcohol has been driven off. Acidify with dilute sulphuric acid. Again heat. Note the unpleasant smell of butyric acid and other volatile fatty acids. An oily layer forms on the surface. Transfer this to a test-tube. Warm it **very** gently in the fume-chamber until the volatile fatty acids have been driven off. Cool. The remaining oil does not solidify, since it contains a good deal of oleic acid. Carry out an acrolein test. It should be negative.

Tests for Unsaturated Linkages

Fats and fatty acids and similar compounds, if they contain unsaturated linkages, unite with iodine, decolor-

ising it, and the amount of iodine taken up can be used to determine the number of double bonds in such compounds. Such fats and fatty acids reduce osmic acid to a black osmium oxide.

Experiment 14. Dissolve a small amount of oleic acid in chloroform, and add 2 or 3 drops of Hübl's iodine solution (made by dissolving 2·6 gm. of iodine and 3 gm. of mercuric chloride in 100 c.c. of 95 per cent. ethyl alcohol). Shake up. The iodine colour disappears. Repeat, using palmitic acid instead of oleic acid. The iodine colour does not disappear. Now repeat, using as nearly as possible corresponding amounts of olive oil, butter, suet and lard in four different dry test-tubes. A reasonable comparison of volume may be made by warming the three latter fat-mixtures, so that there are then four liquids for the volume comparison. Add equal volumes of chloroform to each and then drop by drop the Hübl iodine solution, shaking after each addition, until the iodine colour no longer disappears. Note that different amounts of the iodine solution are required in each case, indicating that this test can be used quantitatively.

Experiment 15. Add a drop or two of osmic acid solution to a few drops of olive oil in a test-tube. A blackening occurs. Repeat, using pure palmitic acid, instead of olive oil, and warming. No blackening results.

Since most of the naturally occurring animal and plant fats are mixtures containing unsaturated fatty acid radicals, a micro-test depending on this reaction is often used to demonstrate the presence of fat in tissues.

DEMONSTRATION.—Determination of a Melting Point

Experiment 16. *Comparison of the melting points of tristearin and tripalmitin.*

Transfer a little powdered tristearin into a drawn-out thin-walled capillary tube previously closed at one end. Transfer a little powdered tripalmitin into a second, similar tube. Pack down with very thin glass rods. Affix by

rubber bands to a thermometer, so that the closed ends of the tubes are adjacent to the mercury bulb of the thermometer. Place in a beaker half full of water, with the open ends of the tubes well above the surface of the water, and heat gently, stirring the water. The general arrangement is sketched in Fig. 4. Note the temperature at which the solid in each tube melts to an oil.

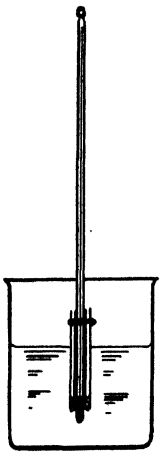


FIG. 4. Apparatus for determination of melting points.

Tripalmitin melts at 65·5° and tristearin at 71·6° C.

This method is used for the determination of most melting points. Sulphuric acid or some other suitable liquid is employed for substances whose melting points are in the neighbourhood of or above 100° C.

DIGESTION OF FATS

Pancreatic Lipase

Experiment 17. Demonstrate the presence of lipase in extract of pancreas (glycerol extract will do) by transferring to a test-tube 10 c.c. of milk, 10 drops of (blue) litmus solution, and 5 c.c. of extract, and to a second tube the milk and litmus and 5 c.c. of extract that has been previously **boiled,** and cooled, and neutralised if necessary to litmus paper with dilute sodium hydroxide. Keep the tubes on the water-bath for fifteen to thirty minutes. The first tube gradually changes to red, due to production of free fatty acid by the lipase.

Experiment 18. Pancreatic lipase, or an *esterase* accompanying it, will also decompose esters of the lower fatty acids.

To a test-tube add 3 c.c. of water, 2 c.c. of ethyl butyrate and as much litmus powder as will go on the tip of a penknife (or 10 drops of litmus solution). Add 4 c.c. of glycerol extract of pancreas. Neutralise. Make up an exactly similar tube, but boil and cool the pancreas extract before adding it. Keep on the water-bath for fifteen minutes or longer as necessary. Note the gradual change of colour in the first tube, due to the production of free butyric acid.

Waxes

Experiment 19. Test a little beeswax or some other wax by the acrolein test. Note that the result is negative, since the wax is not a glyceride. Heat a little of the wax with sodium hydroxide solution. Note that it does not pass into solution. Waxes are saponified with much greater difficulty than fats.

Lecithins

Experiment 20. Carry out the acrolein test with a **trace** of lecithin. A positive result should be obtained. Test with osmic acid ; blackening occurs.

Hydrolyse a little lecithin with alkali, heating for thirty minutes on the water-bath. Pour off some of the liquid into a test-tube, note that it behaves like a soap solution, salt out the soap, redissolve it in water and prepare an insoluble calcium soap.

Carry out a test for phosphorus on some more of the liquid with nitric acid and ammonium molybdate (*cf.* Exercise I., Experiment 7). A positive result should be obtained.

(The choline radical is present in the lecithin, but choline cannot easily be tested for unless it is isolated in pure condition.)

EXERCISE V

AMINO-ACIDS AND PROTEINS

(Three Three-hour Periods)

PROTEINS, the essential nitrogen-containing compounds of the diet, are composed of very complex molecules built up of combinations of a large number of amino-acids. Certain of these amino-acids are characterised by specific colour tests. Different groups of proteins are differentiated to a great extent by differences in solubility and other physical properties. Acid or alkaline hydrolysis gradually breaks down the complex protein molecule through the stages of less complex *proteoses* and *peptones* to mixtures of the amino-acids.[1]

Certain essential and frequently used tests will first be described.

THE BIURET TEST. To 2 c.c. of the solution under test add an equal volume of 40 per cent. sodium hydroxide, mix the two solutions, and add 2 or 3 drops of very dilute copper sulphate solution (made by diluting $\frac{1}{2}$ c.c. of dilute copper sulphate with sufficient water to fill a test-tube). Shake up. If the result is positive a purplish-red to pink colour will develop. If a blue colour develop it should be carefully contrasted with that of copper sulphate itself in alkaline solution by carrying out a control test with water instead of the test solution, and unless there is a definite difference the result should be considered negative.

MILLON'S TEST. Add a few drops of Millon's solution to 5 c.c. of the test solution and warm. If the result is positive a white precipitate may be produced, and this, or the solution, turns red on warming. If a solid is being tested which

[1] In connection with this exercise the student should read the chapters on proteins and amino-acids in a theoretical text-book (*e.g.*, Cameron's "Biochemistry," 5th edit., Chapter VI.).

is insoluble in water proceed as above, suspending the solid in 5 c.c. of water. Millon's test is given by hydroxy-benzene derivatives. Used with material of biochemical significance it indicates the presence of tyrosine or tyrosine radicals.

Millon's Reagent is prepared by dissolving mercury (one part by weight) in two parts of concentrated nitric acid and after solution is complete diluting the result with two volumes of water.

Xanthoproteic Test. Add to 2 c.c. of the test solution a few drops of concentrated nitric acid. If the test is positive a white precipitate may be produced, which turns yellow, and may dissolve on warming over the Bunsen flame. Proteoses, peptones, and certain amino-acids give no precipitate, but only the yellow colour. After cooling, on addition of ammonia or sodium hydroxide until the reaction is alkaline, the colour changes to orange.[1] A positive test indicates the presence of phenyl groups.

Tests for Tryptophane or Tryptophane Radicals. (i.) *The Adamkiewicz-Hopkins Reaction as modified by Winkler.*[2] Add to 2 c.c. of the solution under test several drops of glyoxylic acid solution and 1 drop of 5 per cent. copper sulphate, and mix. Pour carefully down the side of the test-tube an equal volume of concentrated sulphuric acid. If the test is positive a reddish-violet colour develops just above the zone of contact of the two liquids.

Note. If more convenient the mixed aqueous solution can be allowed to flow slowly on to the surface of the sulphuric acid from a pipette. In all tests of this type the student should employ the method which in his experience gives him the best result, *i.e.*, the least degree of mixing of the two liquids.

Glyoxylic acid solution is prepared by reducing a saturated solution of oxalic acid with sodium amalgam.[3]

[1] Cole has drawn attention to the fact that with such lipides as oleic acid and olive oil colour changes similar to those with proteins are produced when this test is applied.

[2] *Zeitschr. f. physiol. Chem.*, 1934, CCVIII., 50.

[3] Benedict (*J. Biol. Chem.*, 1909, VI., 51) has suggested a useful modification: Place 10 gm. of magnesium powder in a 500 c.c. Erlenmeyer flask, cover with distilled water, with shaking, and pour in slowly 250 c.c. of cold saturated solution of oxalic acid. Magnesium oxalate is precipitated, and magnesium glyoxalate remains in solution. The reaction is rapid and much heat is liberated, hence cool under the tap. When the reaction appears complete, shake and filter. Add a little acetic acid to the filtrate, to prevent decomposition of the gloyxalate.

(ii.) *Acree-Rosenheim Formaldehyde Reaction as modified by Cole.*[1] Add to 1 c.c. of the test solution 1 drop of very dilute formaldehyde (a 1 in 500 dilution of commercial formalin, which is 40 per cent. formaldehyde). Add 1 drop of 10 per cent. mercuric sulphate in sulphuric acid. Mix. Run in slight excess of concentrated sulphuric acid and shake gently. In presence of tryptophane or tryptophane radicals a deep violet or purple colour develops.

NINHYDRIN TEST. Add to 5 c.c. of the test solution (which must be neutral, or at most very slightly acid) $\frac{1}{2}$ c.c. of 0·1 per cent. solution of ninhydrin (triketohydrindene hydrate) and boil for one minute. Development of a blue colour indicates a positive result.

Note. In precipitation tests, to saturate completely with a reagent shake up with crystals of that reagent until it is quite evident that crystals of the reagent remain undissolved. Compounds such as ammonium sulphate are very soluble. To make a half-saturated solution add to the solution under test an equal volume of saturated solution of the reagent in water.

DEMONSTRATION.—THE PROPERTIES OF INDIVIDUAL AMINO-ACIDS

Glycine

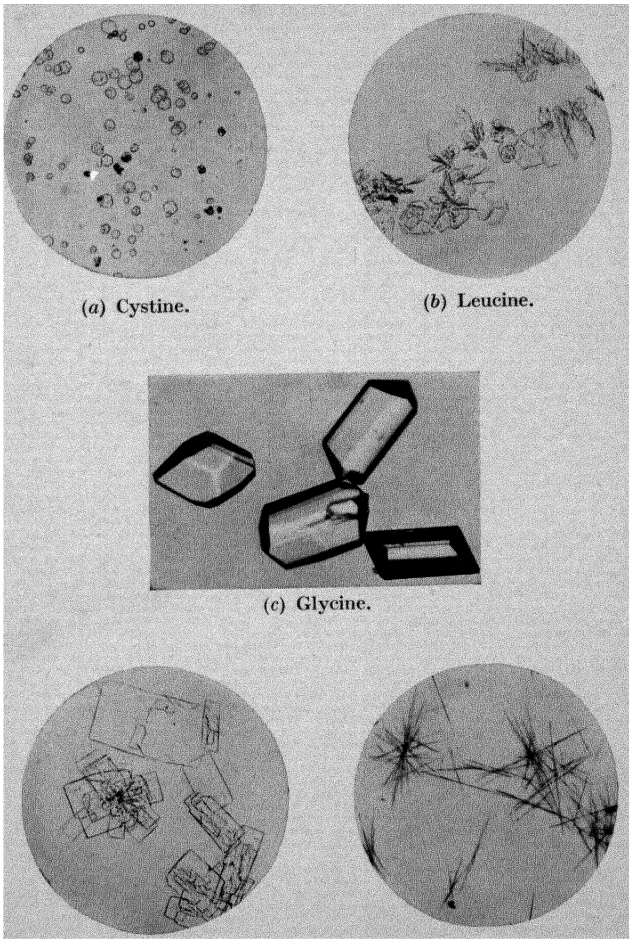
Experiment 1. Observe the crystal form under the microscope. Compare with the photo-micrograph in Plate II. Observe that glycine is very soluble in water, and test the reaction with both red and blue litmus paper. Each is changed in colour. Glycine solutions are *amphoteric.*[2]

Note that the biuret, Millon's, the xanthoproteic, the glyocylic acid, and the formaldehyde tests are negative, while the ninhydrin test is positive. Note, however, that in the biuret test a deep blue colour is formed, and that with Fehling's and Benedict's solutions the same colour results, although no reduction takes place on warming. This is due to the formation of blue copper-glycine.

[1] " Practical Physiological Chemistry," 9th edit., Heffer, Cambridge, 1933.

[2] This can be demonstrated by placing strips of red and blue litmus paper side by side on a microscope slide, with a crystal of glycine touching both. Moisten the crystal with 1 or 2 drops of water. *Slight* colour changes are seen adjacent to the crystal.

PLATE II



(*a*) Cystine.

(*b*) Leucine.

(*c*) Glycine.

(*d*) Aspartic acid.

(*e*) Tyrosine.

Photo-micrographs of recrystallised amino-acids, to illustrate their differing crystal habits. *Magnification* × 45.

[*To face p.* 40.

Alanine

Experiment 2. Carry out the same tests as with glycine and note the results.

Glutamic Acid

Experiment 3. Carry out the same tests as with glycine and note the results.

Cystine

Experiment 4. Carry out the same tests as with glycine and note the results. Cystine dissolves in slightly alkaline solution. Test also for sulphur and note that sulphur is present.

Phenylalanine

Experiment 5. Carry out the same tests as with glycine and note the results.

Tyrosine

Experiment 6. Carry out the same tests as with glycine and note the results. Tyrosine dissolves in slightly alkaline solution. Test also with the special phosphomolybdotungstate reagent (see Exercise XXVIII.), and note the development of a blue colour. This result is believed to be specific for tyrosine, and to be given by tyrosine radicals in proteins.

Experiment 7. The *amphoteric nature* of amino-acids can well be illustrated by an experiment with tyrosine. Place a small amount in a test-tube, add distilled water, and note that the tyrosine does not go into solution. It is one of the most insoluble of the amino-acids. Add 0·5 *N* hydrochloric acid, drop by drop. The tyrosine dissolves, due to the formation of a soluble hydrochloride. After solution is complete carefully add 0·5 *N* sodium hydroxide drop by drop. The tyrosine is reprecipitated at neutrality, and then again goes into solution, owing to the formation of a soluble sodium salt. It can thus react either as base or as acid, that is, it is *amphoteric*.

Histidine

Experiment 8. Carry out the same tests as with glycine and note the results. Bring the histidine into solution with the aid of a little hydrochloric acid. In addition, carry out Knoop's test (specific for histidine) and Pauli's test.

Knoop's Test. To a few cubic centimetres of histidine solution add bromine water until the solution has a slight yellow tinge (excess bromine) and heat. The yellow colour disappears and is succeeded by a claret colour. A dark precipitate may also separate. The test is sensitive to one part in a thousand.

Pauli's Test. Add to a trace of histidine hydrochloride in a test-tube 2 c.c. of 10 per cent. sodium carbonate, and to this 4 c.c. of the sodium carbonate in which has been dissolved somewhat more diazo-benzene-sulphonic acid than the amount of histidine taken. A dark red colour results. This is given by all substances containing the iminazolyl group and, also by tyrosine; *Totani's modification* permits differentiation. To the red solution add concentrated hydrochloric acid drop by drop until the solution is distinctly acid, and then zinc dust. Allow the resulting reduction to proceed for fifteen minutes. Pour off the upper clear solution into a test-tube and add half its volume of 25 per cent. ammonia (ammonia of sp. gr. 0·88 diluted with 3 parts of water). A characteristically golden colour is given by histidine, while with tyrosine the colour is rose-red.

Arginine

Experiment 9. Carry out the same tests as with glycine and note the results. In addition, carry out Sakaguchi's test. To a solution of arginine add a trace of alcoholic solution of α-naphthol, 2 or 3 drops of sodium hypochlorite solution, and a little sodium hydroxide. A red colour is produced. This is stated to be given by arginine in dilution of one part in a million, and also to be a delicate specific test for the presence of arginine radicals in proteins.

Citrulline

Experiment 10. *Fearon's Test.*[1] To 2 c.c. of a dilute solution of citrulline, or of a protein such as egg albumin, add 5 c.c. of concentrated hydrochloric acid and 4 drops of a 3 per cent. aqueous solution of diacetyl-monoxime. Heat the mixture just to boiling, allow to cool for two minutes, and then add two drops of 1 per cent. potassium persulphate.

If a solution of citrulline has been taken, a carmine colour will develop and deepen on standing. If a solution of a

[1] Fearon, *Biochem. J.*, 1939, XXXIII, 902.

protein containing citrulline radicals has been taken a reddish purple colour will develop. In this case it is desirable to carry out a control test, omitting the diacetyl-monoxime. With albumins and other proteins containing carbohydrate radicals heating with concentrated hydrochloric acid liberates furfuraldehydes which combine with tryptophane radicals to form violet pigments. The reddish purple (combination) colour can be easily distinguished from the violet of the control.

Note. Excess of persulphate must be avoided since a very slight excess of this oxidising agent prevents colour development or at most gives only a transient colour.

Tryptophane

Experiment 11. Carry out the same tests as for glycine and note the results. In addition, add to a dilute solution of tryptophane bromine water drop by drop. A pink colour develops which changes to yellow if excess of bromine water is added. This test is specific for free tryptophane.

The above experiments should demonstrate the following facts :—

The biuret test is not given by amino-acids.

The ninhydrin reaction is given by amino-acids.

The xanthoproteic reaction is given by amino-acids containing a phenyl group (phenylalanine, tyrosine and tryptophane).

Millon's test is given by tyrosine as a red coloration. (It is then called Hoffmann's reaction.)

The glyoxylic acid and formaldehyde tests are specific for tryptophane and tryptophane radicals.

Pauli's test is given by tyrosine and histidine. Totani's modification or Millon's test differentiates between them.

TESTS ON PROTEINS

Experiment 12. Prepare a solution of egg-albumin as follows : Shake up vigorously one volume of egg-white with ten volumes of water and filter off the precipitate of ovoglobulin which forms. (Since the filtration requires a considerable amount of time, it is convenient to prepare the filtrate in bulk for a whole class.) Carry out the following tests with the filtrate :—

Note that the biuret, Millon's, the xanthoproteic and the

glyoxylic acid test, and also the ninhydrin test are all positive.

Half saturate 10 c.c. of the solution with ammonium sulphate. Note that no precipitation takes place. Add more **crystals**, little by little, until saturation is complete. Albumin is precipitated as a flocculent precipitate, easily distinguished from excess of crystals of the sulphate. Filter off a little, suspend the precipitate in water, shake and observe that it redissolves. *Albumins are soluble in water and in half-saturated solutions of ammonium sulphate, but are precipitated by saturation with ammonium sulphate.*

Saturate 10 c.c. of the solution with sodium chloride crystals. Note that no precipitation of protein occurs. Add 2 or 3 drops of dilute acid and observe the result.

Coagulation Test

Experiment 13. Boil 5 c.c. of the albumin solution in a test-tube. The protein coagulates and will no longer redissolve. Cool and test the suspended coagulum with Millon's reagent. Notice that on warming it reddens.

Precipitation Test

Experiment 14. Add mercuric chloride solution, drop by drop, to 2 c.c. of the egg-albumin solution in a test-tube. Note that a precipitate forms and does not redissolve with excess of the reagent. Repeat, adding sufficient of the reagent to give a definite precipitate. Then add a few drops of 10 per cent. sodium chloride. The precipitate redissolves.

Add dilute solutions of lead acetate, silver nitrate, copper sulphate, ferric chloride and barium chloride each to separate 2 c.c. portions of the albumin solution, and note the results.

Add to 2 c.c. of the solution picric acid solution drop by drop and note the formation of a precipitate.

Add, similarly, trichloracetic acid solution and note that it also precipitates protein. Repeat with tannic acid; a precipitate is formed.

In the same way test with phosphotungstic acid, phosphomolybdic acid and potassium-mercuric iodide (in presence of a little dilute hydrochloric acid); all of these precipitate protein.

These are examples of agents useful for the removal of proteins from solution, and are frequently used both to obtain

a protein from its solution and to remove proteins so that other constituents of the solution can be estimated (**as in blood analyses**).

To 5 c.c. of the egg-albumin solution add 10 drops of dilute acetic acid, shake up and then add potassium ferrocyanide drop by drop. A precipitate forms. This is a very delicate test for soluble protein.

NITRIC ACID TEST (*Heller's test*). Transfer 5 c.c. of concentrated nitric acid to a test-tube and pour on to the surface very carefully, so that mixing does not occur, 2 or 3 c.c. of the egg-albumin solution. A white layer of coagulated albumin appears at the interface between the two liquids.

This is an important test for albumin and is of value as a clinical test. It is somewhat more delicate if the nitric acid is replaced by a mixture containing nitric acid and saturated magnesium sulphate in the proportions of 1 to 5 (Robert's reagent).

Precipitation by Alcohol. Add to 3 c.c. of the solution 95 per cent. ethyl alcohol, 2 or 3 drops at a time, and note what occurs.

Experiment 15. Test solid egg-albumin for phosphorus and for sulphur by the methods described in Exercise I. Positive results will be obtained for sulphur, negative for phosphorus.

DEMONSTRATION.—**Temperature of Coagulation**

Experiment 16. Place 5 c.c. of the egg-albumin solution in a test-tube and immerse a thermometer in the solution. Lower the test-tube into a beaker half filled with water until the top of the albumin solution is below the level of the water. Immerse this beaker within a second, also partly filled with water, supporting the smaller beaker within the other on corks. Apply heat to the larger beaker cautiously and so raise the temperature of the inner beaker slowly and steadily. Note the temperature at which coagulation occurs.

EXAMPLE OF A GLOBULIN

Preparation of Edestin

Experiment 17. Digest 5 gm. of hemp-seed previously rubbed up in a mortar with 10 c.c. of 5 per cent. sodium chloride solution in a beaker on the water-bath at 60° C. for

half an hour. Filter the **hot** solution through a paper moistened with **hot** 5 per cent. sodium chloride solution. Place the filtrate on the **hot** water-bath and allow the whole to cool slowly and spontaneously and then stand overnight (two days if necessary). The globulin crystallises out very slowly. Pour off the liquid from the crystals and carry out the following tests with them.

Properties of Edestin, a Typical Globulin

Examine the crystals under the microscope. They belong to the cubic system and occur chiefly as octahedra. *Cf.* with Fig. 5.

Test their solubility. They are not soluble in water, but are soluble in dilute sodium chloride solution. With their solution carry out the biuret, Millon's, the xanthoproteic, glyoxylic and ninhydrin tests; they all give positive results.

FIG. 5. Photo-micrograph of edestin crystals, × 135.

With the solution in sodium chloride try the effect of half-saturation with ammonium sulphate; the globulin is precipitated.

Try the coagulation and nitric acids test with the solution.

Ascertain the effect of complete saturation with sodium chloride.

Ascertain what occurs when the dilute sodium chloride solution of the globulin is further diluted with several volumes of water.

EXAMPLE OF A GLUTELIN AND OF A PROLAMIN

Experiment 18. Mix thoroughly 20 gm. of wheat flour with a little water in an evaporating dish. Knead the stiff dough which results. Then cover it with water and allow to stand for thirty minutes. Then pour off the water and knead the ball of dough under running water over a sieve or cheese-cloth until it is freed from starch (as indicated by the iodine test applied to a little water expressed from the ball). Yellow fibrous **Gluten** remains. Add about

100 c.c. of 70 per cent. alcohol to this in a flask and allow to stand for twenty-four hours, occasionally shaking it. Filter.

Filtrate. Evaporate to dryness in a porcelain basin on the water-bath. Powder the residue of **Gliadin,** a **prolamin.** Demonstrate that it is soluble in 70 per cent. alcohol, but is less soluble in 95 and in 30 per cent. alcohol. Test the solution with the various colour tests, precipitation tests, and coagulation tests.

Residue. Treat with more 70 per cent. alcohol, and allow to stand for an additional twenty-four hours to extract any remaining gliadin. The residue from this second treatment is crude **Glutelin.** Rub it up in a mortar with 0·2 per cent. sodium hydroxide, filter the solution through a wet pleated filter and neutralise the filtrate carefully with 0·2 per cent. hydrochloric acid. (Excess of acid will redissolve the precipitate which forms.) Filter off the precipitate, wash it several times with 70 per cent. alcohol and then with water, and then study its solubility in water, acid, sodium carbonate, and carry out with its solution the different colour tests.

(*Note.* Several students can conveniently combine to carry out the initial parts of these preparations and then individually carry out the tests on the products.)

COAGULATED PROTEIN

Experiment 19. Take some egg-white, coagulate it by heating, and carry out on the solid the various solubility tests and colour tests and note the results.

EGG-YOLK

Experiment 20. Take a little egg-yolk, note its characteristic appearance, study its solubility reactions, and ascertain if it is possible to separate any of its constituents by salting out. If so, determine their nature (albumin, globulin, etc.) as far as possible, and carry out tests for glycerol and phosphorus on the different fractions in an endeavour to ascertain in which is the **Ovo-vitellin,** said to be a complex lecitho-nucleo-protein characteristic of egg-yolk.

Hydrolysis with mineral acids or with baryta for a period of several hours breaks down the protein molecule

through the stages of "metaprotein," proteoses and peptones, and simple polypeptides, to amino-acids. The results are similar to those obtained with enzymic actions dealt with in Exercise VI. Tests for proteoses and peptones are included in that Exercise.

EXERCISE VI

GASTRO-INTESTINAL DIGESTION OF PROTEINS

(Two Three-hour Periods)

GASTRIC, pancreatic and intestinal juices can be obtained in various ways, *e.g.*, by fistulae, by stomach tube, etc. For class experimental purposes useful extracts can be made with normal saline (plus preservative) or with glycerol. Such extracts, of course, do not give the true composition of the juices, but qualitatively they represent the juice plus the material used for extraction.

Experiments 1 to 7 and 9 to 11 of this Exercise should be carried out with extracts previously prepared by steeping respectively the minced gastric mucosa, pancreas, and intestinal mucosa of hogs in glycerol for several days.[1]

DEMONSTRATION.—Action of Pepsin

Experiment 1. Take about 200 gm. of lean beef, mince it, transfer it to a large flask, add 1,000 c.c. of 0·4 per cent. hydrochloric acid and 100 c.c. of glycerol extract of stomach mucosa. (No other antiseptic than the acid is necessary.) Place the flask in an incubator at 37° C. for ninety-six hours. During this period shake it occasionally and then transfer 2 or 3 c.c. to a test-tube ; test this small amount for the presence of " free hydrochloric acid " with Töpfer's reagent (prepared by dissolving 0·5 gm. of dimethylaminoazobenzene in 100 c.c. of 95 per cent. ethyl alcohol). A characteristic pinkish-red colour should be produced at each such test.

[1] Read the corresponding chapter on protein digestion in a theoretical text-book (*e.g.*, Cameron's " Biochemistry," Chapter VI., pp. 160 *et seq.*).

If it is not given add 5 drops of 10 per cent. hydrochloric acid, shake and test again, repeating the addition if necessary until the test is positive. (*As digestion proceeds and the number of free amino-groups increases, these unite with the acid, giving the so-called "combined hydrochloric acid," and the degree of acidity of the digest is thereby much reduced.*)

At the end of the ninety-six hours filter through a large pleated filter paper. The filtrate consists chiefly of a mixture of **Proteoses** and **Peptones.** Carry out the following tests with it :—

(i.) Saturate 50 c.c. of the filtrate with ammonium sulphate. *Proteoses* are precipitated. Collect the sticky precipitate on a rubber-tipped glass rod, transfer it to a small glass basin and dissolve it in a little water. Add some barium carbonate and boil. Barium sulphate is precipitated (from the adherent ammonium sulphate). Filter this off along with the excess of barium carbonate, concentrate the filtrate to small bulk and test it as follows (*preferably side by side with the tests on the peptone solution prepared as described below*) :—

Note that the biuret test yields a slightly different colour from that given by proteins.

Millon's, the glyoxylic, the xanthoproteic and the ninhydrin tests are positive.

Concentrated nitric acid gives only a very slight precipitate (due to the so-called "primary proteoses," which are probably the most complex of the proteoses).

The solution does not coagulate on warming.

Picric acid gives a precipitate which dissolves on warming and reappears on cooling. Potassium ferrocyanide and acetic acid give a precipitate.

(ii.) Concentrate the filtrate from proteose to 20 c.c., filter again if necessary (ammonium sulphate will have separated, since it is more soluble in hot than in cold water), remove sulphate with barium carbonate as above and again concentrate the filtrate (to half-volume). Carry out the same tests with it as with proteose.

Note the **pink** biuret test.

Note that picric acid and other precipitating reagents do not produce a precipitate, with the exception of phosphotungstic and tannic acids.

Note that nitric acid gives no precipitate, and warming produces no coagulation.

Note that potassium ferrocyanide and acetic acid give no precipitate.

The different results obtained with protein, proteose and peptone are in agreement with the idea of a gradual breakdown of a very complex molecule to simpler ones.

Experiment 2. Sufficient of the combined proteose-peptone solution should have been prepared to furnish each student with 20 c.c. The student will saturate this with ammonium sulphate, remove the proteose, dissolve it in water and carry out on the proteose and peptone solutions thus obtained (without removing excess of sulphate) the following tests, using not more than 1 c.c. of solution for each: the biuret test, Millon's, the glyoxylic, acetic acid and potassium ferrocyanide, coagulation, nitric acid and picric acid tests. (Note that in presence of excess of sulphate no precipitate may be formed, and the result is sometimes indefinite.)

Experiments 1 and 2 show that in presence of acid a glycerol extract of stomach mucosa containing the enzyme *pepsin* decomposes meat protein with formation of proteoses and peptones. Subsequent tests will show that in absence of the glycerol extract, or in presence of boiled glycerol extract, no such decomposition takes place, indicating that the action is enzymic. More rigorous and extended experiments would be necessary to prove that only proteoses and peptones, and not amino-acids, are liberated by gastric digestion.

DEMONSTRATION

Experiment 3. Make up the following mixtures in five test-tubes :—

(i.) 3 c.c. of glycerol extract of stomach plus 3 c.c. of water.

(ii.) 3 c.c. of glycerol extract of stomach plus 3 c.c. of 0·4 per cent. hydrochloric acid.

(iii.) 3 c.c. of glycerol extract of stomach plus 3 c.c. of 0·5 per cent. sodium carbonate.

(iv.) 3 c.c. of *boiled* glycerol extract of stomach plus 3 c.c. of 0·4 per cent. hydrochloric acid.

(v.) 3 c.c. of water plus 3 c.c. of 0·4 per cent. hydrochloric acid.

Add a piece of the protein fibrin (formed from fibrinogen during the clotting of blood) to each tube. Place the tubes in an incubator or on the water-bath at 37° for half an hour. Note that in (i.) and (iii.) there is no change, while in (iv.) and (v.) the fibrin has swelled up to a translucent mass (**imbibition** in presence of acid). In (ii.) a large part or all of the fibrin has passed into solution. This tube therefore represents a condition under which digestion takes place. Boiling the glycerol extract decomposes the enzyme, so that in (iv.) only the effect of the acid is seen.

Filter each tube and apply the biuret test to each *filtrate* as confirmatory test of the above conclusions.

DEMONSTRATION

Experiment 4. Make up the following mixtures in three test-tubes :—

(i.) 3 c.c. of glycerol extract of stomach plus 3 c.c. of 0·4 per cent. hydrochloric acid.

(ii.) 3 c.c. of glycerol extract of stomach plus 2·9 c.c. of 0·4 per cent. hydrochloric acid plus 2 drops of concentrated hydrochloric acid (hence a stronger acid mixture).

(iii.) 3 c.c. of glycerol extract of stomach plus 2 drops of 0·4 per cent. hydrochloric acid plus 2·9 c.c. distilled water (hence a weaker acid mixture).

Add a piece of fibrin to each solution and heat on the water-bath at 37° for half an hour. Note that most digestion has taken place in tube (i.) which, after mixture represents a concentration of 0·2 per cent. hydrochloric acid (actually a little less than this through the formation of combined acid).

DEMONSTRATION.—Optimum Temperature for Pepsin Action

Experiment 5. Into each of four test-tubes transfer 3 c.c. of glycerol extract of stomach, 3 c.c. of 0·4 per cent. hydrochloric acid and a piece of fibrin (giving the optimum acidity conditions.) Keep the first tube in a beaker through which cold (tap) water is running. Note the temperature. Keep the second tube at room temperature and note that temperature. Keep the third tube in a water-bath at 40° C., and the fourth in another water-bath at 60° to 70° C. Note

that of the four tubes most action takes place in that kept at 40°. The optimum temperature for pepsin activity is close to 40°.

After the student has seen Experiments 3 to 5 demonstrated he should repeat them himself. If he does not get correct results, his technique will obviously have been faulty.

Comparative Effects of Different Acids on Peptic Digestion

Provided the correct *pH* be attained, the nature of the acid is of secondary importance for peptic digestion, unless its acid radical produce some toxic action.

Experiment 6. To a series of test-tubes add 2 c.c. of glycerol extract of stomach mucosa and a small piece of fibrin, and 2 c.c. of one of the following acid solutions: N/10 HCl, N/10 HBr, N/10 H_2SO_4, N/10 HNO_3, " combined HCl " (made from N/10 HCl and peptone), N/10 acetic acid, N/10 lactic acid and N/10 oxalic acid. Put the series of tubes in a water-bath at 38° for half an hour and compare the results.

In considering the results, remember that the strengths of mineral acids used will give approximately the same *pH* values, while the combined acid and the organic acids will be much more weakly acid (and therefore have a higher *pH*). Read the chapter on hydrogen-ion concentration in a theoretical text-book in connection with this experiment.

Rennin

Commercial rennet can be used for the following tests. This is prepared from calf's gastric mucosa, and is used domestically in the preparation of junket.

Experiment 7. Take 5 c.c. of fresh milk in a test-tube. add 10 drops of a rennet preparation, shake up and place on the water-bath at 38° for a few minutes. Note that a clot forms, so solid that the tube can be turned upside down without the contents shifting. (Casein has been changed to calcium paracaseinate.) Leave the tube standing at 38° for one hour. At the end of this time the clot has contracted, and an almost clear liquid the " whey," has been expressed.

The clotting of milk requires both the presence of calcium ions and that of the enzyme in the rennet solution. This is demonstrated in the following experiment :—

Experiment 8. Take three test-tubes, each containing 5 c.c. of fresh milk. To (*a*) add 1 drop of rennet (diluted in accordance with the instructions on the commercial preparation used), to (*b*) 1 c.c. of dilute ammonium oxalate and 1 drop of rennet, and to (*c*) 1 drop of boiled rennet. Keep all three tubes at 38° for ten minutes. There will be a clot in (*a*), but none in (*b*) or (*c*). Boiling has destroyed the enzyme in (*c*), while ammonium oxalate has removed the calcium ions in (*b*) through formation of insoluble calcium oxalate. To (*b*) add 1 c.c. of dilute calcium chloride. A flocculent precipitate is formed, indicating that the rennin has acted in this solution, but the (soluble) product does not form a clot unless calcium ions are present.

DEMONSTRATION.—Action of Trypsin

Experiment 9. Transfer to a 500 c.c. flask 40 gm. of commercial casein and 200 c.c. of 1 per cent. sodium carbonate solution. Add 50 c.c. of a glycerol extract of pancreas and 20 c.c. of a glycerol extract of intestinal mucosa. Add a few cubic centimetres of chloroform and also of toluene (to prevent bacterial action), shake up, and **loosely** cork the flask. Place it in an incubator at 37° for, preferably, eight days. Add more toluene from time to time.

At the end of the stated period pour off the supernatant liquid into a smaller flask, raise it to the boiling point and then add glacial acetic acid drop by drop with shaking until the reaction is acid to litmus paper. Cool and filter. The undecomposed protein is filtered off.

Carry out a parallel control, omitting extract of pancreas.

Make the following parallel tests on the two filtrates :—

(i.) Take 20 c.c. and saturate with ammonium sulphate. A small precipitate of proteose may form. Remove this as usual and dissolve it in water. Apply the biuret test to this and to the filtrate and note that the results indicate that both proteose and peptone are still present in the tryptic digest (carried out in glass vessels, with accumulation of the products of digestion).

(ii.) To 5 c.c. add bromine water drop by drop. A pink colour develops, which disappears when more bromine water

is added. This indicates the presence of free tryptophane (*cf.* Exercise V., Experiment 10).

(iii.) To 10 c.c. add 20 drops of concentrated sulphuric acid and 20 c.c. of 10 per cent. mercuric sulphate in 5 per cent. sulphuric acid. Allow to stand for ten minutes. A yellow precipitate forms, a mercuric salt of tryptophane. Filter it off and wash it with water on the filter paper. Test portions of it by the glycocylic acid test (positive), the xanthoproteic test (positive) and Millon's test (negative, only a yellow colour).

(iv.) To the filtrate from (iii.) apply Millon's test. A positive test is obtained (without heating, since the filtrate contains sulphuric acid), due to the presence of free tyrosine.

(v.) Concentrate 100 c.c. of filtrate to 20 c.c. over the Bunsen flame. Filter hot into a beaker. Allow to stand twenty-four hours. Crystals separate out. Examine them under the microscope and identify leucine and tyrosine by comparison with the photo-micrographs of Plate II. Cystine crystals may also be seen. (Tyrosine crystals may separate from the unconcentrated solution ; they will probably not be typical in form, but can be identified by washing, dissolving in a trace of alkali, and applying Millon's test.)

The above experiment demonstrates that tryptic digestion of casein during several days produces proteose, peptone, and at least the three amino-acids tryptophane, tyrosine and leucine. More elaborate procedures are necessary to identify the other amino-acids present in the digested fluid, and cannot profitably be included in this course.

Under the conditions of the experiment trypsin and chymotrypsin are present. The control experiment shows that the peptidases of the intestinal juice cannot digest casein.

Carry out the following experiments with an old glycerol extract of pancreas (which will contain trypsin ; if it is acid it should be neutralised before it is used) :—

Experiment 10. Ascertain the *most favourable pH* for tryptic digestion by the following tests. Add to five test-tubes :—

(i.) 3 c.c. of extract and 3 c.c. of water (*pH* about 7).

(ii.) 3 c.c. of extract and 3 c.c. of 0·5 per cent. sodium carbonate (*pH* about 8).

(iii.) 3 c.c. of extract and 3 c.c. of 1 per cent. sodium carbonate (*pH* about 9).

(iv.) 3 c.c. of extract and 3 c.c. of 0·5 per cent. lactic acid (*pH* about 5).

(v.) 3 c.c. of extract and 3 c.c. of 0·4 per cent. hydrochloric acid (*pH* about 1).

Add to each tube a piece of fibrin and keep them on the water-bath for thirty minutes at 37° C. It will be found that digestion takes place best in that tube to which has been added 0·5 per cent. sodium carbonate. Note that in certain tubes no digestion takes place. Why is this ?

Experiment 11. To ascertain *the most favourable temperature* for tryptic digestion make up the following series of test-tubes :—

(i.) Take 3 c.c. of extract, 3 c.c. of 0·5 per cent. sodium carbonate, add a piece of fibrin and keep in a beaker through which cold water is running. Note the temperature.

(ii.) Make up a similar mixture and keep at room temperature. Note it.

(iii.) Make up a similar mixture and keep on the water-bath at 40° C.

(iv.) Make up a similar mixture and keep on a second water-bath at 75° C.

After thirty minutes note the degree of digestion in each and draw the appropriate conclusions.

EXERCISE VII

BILE [1]

(One Three-hour Period)

The chief constituents of bile are of three types : (*a*) the bile salts, (*b*) cholesterol and (*c*) the bile pigments.

Fresh ox bile should be employed in the following experiments :—

Experiment 1. Note the colour, appearance, and taste of bile. To what is this taste due ?

Experiment 2. Note the reaction of fresh bile. It may be slightly acid, slightly alkaline, or neutral, so test with litmus, phenolphthalein, and congo red papers.

Remember that congo red changes at pH 4, litmus at pH 7, and phenolphthalein at pH 9. Such tests therefore only roughly indicate the reaction of a solution.

Experiment 3. Add dilute acetic acid. If a precipitate forms, what does it indicate ? Examine it to ascertain if possible what is its nature. (The precipitate with ox bile is a nucleoprotein. With human bile both nucleoprotein and mucin are precipitated.)

Experiment 4. Boil some bile and see what happens.

Experiment 5. Test bile for inorganic constituents such as chloride, sulphate and phosphate (see Experiments 5, 6 and 7, Exercise III.).

DEMONSTRATION.—Preparation of Bile Salts

Experiment 6. Mix 40 c.c. of ox bile with 10 gm. of animal charcoal, and stir up to a paste. Evaporate to

[1] Cameron's "Biochemistry," 5th edit., Chapter V., pp. 117–121, 135–137, Chapter IX., p. 211, and Chapter XIV., p. 317, should be read at this stage.

dryness on the water-bath with stirring. Powder the residue in a mortar, transfer to a 250 c.c. flask, add 70 c.c. of 95 per cent. ethyl alcohol and boil on the water-bath for twenty minutes. Cool and filter into a dry beaker. Add ether to the filtrate just until there is a permanent cloudiness. Cover with a glass plate and allow to stand over night at, if possible, somewhat below room temperature. A crystal mass of bile salts will have separated. Filter off, dry in air, and perform the following tests with these crystals side by side with the same tests on pure sodium glycocholate and sodium taurocholate, and contrast the results :—

(i.) Dissolve or suspend a trace in 10 c.c. of warm water in a test-tube, add 2 drops of a 20 per cent. solution of sucrose,

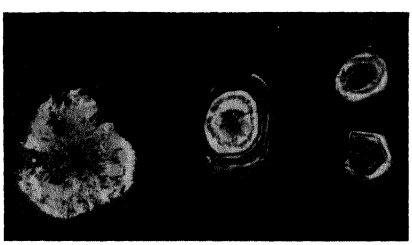


FIG. 6. Photograph of cross-sections of human gall-stones, natural size.

cool and run in below 5 c.c. of concentrated sulphuric acid. Gently shake. A deep purple colour develops as the two liquids mix. (*Pettenkofer's test*).

(ii.) Dissolve a trace in 20 c.c. of warm water in a beaker. Sprinkle the surface of the liquid with a little flowers of sulphur. The powder falls through the liquid. Run a parallel test with water. The sulphur stays at the surface (*Hay's test*, illustrating the fact that bile salts lower the surface tension of a liquid).

(iii.) Dissolve a trace in 4 c.c. of syrup (85 per cent.) phosphoric acid, and place on the boiling water-bath for four minutes. Cool, and add 1 c.c. of 0·6 per cent. aqueous solution of vanillin. A deep rose colour develops, which is completely discharged by the addition of 8 c.c. of water.[1]

[1] Chabrol et al., *Compt. rend. soc. biol.*, 1934, CXV., 834.

This demonstration shows that these results are due to the glycocholate and taurocholate, the " bile salts." The student should now repeat the tests (i.) to (iii.) of this experiment with fresh ox bile, using not more than 3 c.c. of bile for each test. Positive results will be obtained due to the presence of the " bile salts."

Cholesterol

Samples of biliary calculi (gall - stones), whole and sectioned, should be shown to the student. The appearance of typical sections is shown in Fig. 6.

Experiment 7. Suspend a few crystals of pure cholesterol in a drop of water on a microscope slide and examine under the low power of the microscope. Note the re-entrant angle frequently seen. Sketch the crystals. Compare with the photo-micrograph in Plate III.

Experiment 8. SALKOWSKI'S TEST. Dissolve a few crystals of cholesterol in 2 c.c. of chloroform, and add 2 c.c. of concentrated sulphuric acid. The chloroform layer shows colour changes from bluish-red through cherry-red to purple. The acid shows a green fluorescence.

Experiment 9. LIEBERMANN-BURCHARDT TEST. Dissolve a few crystals of cholesterol in 2 c.c. of chloroform in a dry test-tube, add 10 drops of acetic anhydride and 2 drops of concentrated sulphuric acid, and shake. The solution turns red, then blue, and finally bluish-green. *Absence of water is essential.*

This test forms the basis of an important quantitative method for the estimation of cholesterol.

Experiment 10. Take a few crystals of cholesterol in a dry evaporating basin, add 2 or 3 drops of concentrated sulphuric acid, and note that the crystal edges turn red. Add a drop of iodine solution and note the change of colour to violet, green, blue and finally black.

Note that these tests for cholesterol cannot be carried out with bile itself on account of other interfering constituents, especially water. They can be applied to powdered gall-stones.

A partial separation can perhaps be effected as follows, depending upon the percentage of cholesterol present in the bile :—

Experiment 11. Take a test-tube **nearly** half full of bile, add an equal volume of ether, shake vigorously, allow to stand until the two layers are sharply separated, pour off 4 c.c. of ether (being careful to pour off no water), allow this ether to evaporate, and test the residue as in Experiment 9.

Bile Pigments

These consist chiefly of bilirubin and biliverdin, and on the proportion in which these are present in any bile depends its actual colour. Various colour tests can be used for these " pigments," depending upon the fact that they oxidise to highly coloured compounds. The following is typical :—

Experiment 12. Take 5 c.c. of concentrated nitric acid in a test-tube. Dilute a little bile with two volumes of water and run in the diluted liquid upon the top of the acid with a pipette. Various coloured rings form at the junction —green, blue, violet, red, reddish-yellow. Repeat with bile diluted 1 in 10, 1 in 100, and 1 in 1,000, in order to ascertain how delicate this test is (*Gmelin's test*).

Repeat the test with a drop of bile on a porcelain tile, adding a drop of **yellow** nitric acid to it.

DEMONSTRATION.—Effect on Lipase Action

Experiment 13. The following mixtures are made up in large test-tubes :—

(i.) 1 c.c. of olive oil plus 19 c.c. of water.

(ii.) 1 c.c. of olive oil plus 10 c.c. of glycerol extract of pancreas plus 9 c.c. of water.

(iii.) 1 c.c. of olive oil plus 2 c.c. of bile plus 17 c.c. of water.

(iv.) 1 c.c. of olive oil plus 2 c.c. of bile plus 10 c.c. of glycerol extract of pancreas plus 7 c.c. of water.

(v.) 2 c.c. of bile plus 10 c.c. of glycerol extract of pancreas plus 8 c.c. of water.

These measurements should be made accurately, the con-

tents of each tube carefully stirred, 1 or 2 drops of toluene added to each, and then the set placed in an incubator at 37° for twenty-four hours. Then add 2 drops of phenolphthalein to each tube and titrate with shaking against N/10 sodium hydroxide. Record the amounts of alkali necessary for each tube. The larger the amount, the greater the amount of free acid that will have been liberated. What do the results indicate ?

EXERCISE VIII

BACTERIAL ACTION [1]

(One Three-hour Period)

Experiment 1. Take 200 c.c. of water, suspend it in 50 gm. of fresh minced lean meat, 10 c.c. of glycerol extract of pancreas, and 10 c.c. of glycerol extract of intestinal mucosa, and then inoculate with a little fresh faeces. **Add no preservative.** Close the Erlenmeyer flask containing the above with a cork through which passes a tube leading into dilute acid in a smaller flask ; allow the whole to stand in an incubator at 37° for forty-eight hours. Then filter through pleated filter paper in the open air. Concentrate the filtrate to one-fifth volume at 40° to 45° C. under diminished pressure. *Keep the distillate.* Carry out the following tests on the concentrated filtrate. Remember that bacteria, acting upon amino-acids, tend to produce mainly phenols and related compounds, and amines.

(*a*) Tests for phenols, hydroxy-aromatic acids, and imidazolyl compounds :—

(i.) *Hanke and Koessler's Test.* A special p-diazobenzene-sulphonic acid reagent is used, prepared from sulphanilic acid and nitrous acid (see Appendix I.).

To 5 c.c. of 1 per cent. sodium carbonate add 2 c.c. of the special reagent. One minute later add 1 c.c. of the bacterial filtrate. A red colour will develop, the shade depending upon the relative proportions of the various phenols and other compounds present.

(For comparison repeat this test with a 0·1 per cent. solution of pure phenol.)

(ii.) Apply Millon's test. A positive result will be obtained, due to tyramine (and any residual tyrosine).

(iii.) To 5 c.c. of the concentrated filtrate add several drops of bromine water. No red colour should develop, since all

[1] In connection with this demonstration Chapter XIII. of Cameron's "Biochemistry," 5th edit., should be read carefully.

the tryptophane should have been destroyed. A white precipitate will form, due to bromine derivatives of cresols and similar compounds.

(*b*) Tests for putrefaction amines. Take three-fourths of the concentrated fraction in a separating funnel. Make alkaline with sodium carbonate. Shake well with half the volume of amyl alcohol. Discard the aqueous layer. Treat the amyl alcohol extract with 1 per cent. sodium hydroxide and allow to stand until the two layers separate. Drain off the aqueous layer and carry out the following tests with it :—

(i.) To 1 c.c. add picric acid solution. A crystalline picrate will separate, due to the presence of one or more amines (basic compounds).

(ii.) To 1 c.c. apply Millon's test. A positive test indicates presence of a compound with a parahydroxyphenol nucleus (tyramine, since tyrosine is not extracted by amyl alcohol).

(iii.) To 1 c.c. apply the ninhydrin test. It will be negative, indicating the absence of amino-acids.

(*c*) Tests for indole and skatole. *Use the distillate.*

(i.) *Herter's Test.* To 10 c.c. add 2 drops of 2 per cent. β-naphthaquinone sodium monosulphonate and 2 c.c. of 10 per cent. sodium hydroxide. Allow to stand fifteen minutes and then shake with 2 c.c. of chloroform. A pink red colour appears in the chloroform, due to indole.

(ii.) To 10 c.c. add 1 c.c. of 5 per cent. alcoholic solution of p-dimethylaminobenzaldehyde and 1 c.c. of concentrated hydrochloric acid. Indole gives a red and skatole a blue colour. The observed colour will be predominantly red.

(iii.) To 5 c.c. add a few drops of nitric acid and then a few drops of sodium nitrite. Indole gives a red colour, skatole a white turbidity. Both effects should show.

(iv.) To 5 c.c. add 1 c.c. of 5 per cent. p-dimethylaminobenzaldehyde in 10 per cent. sulphuric acid and heat just to boiling. The resulting purplish-blue colour indicates skatole. Side by side with the above, similar tests should be carried out with pure indole and pure skatole. Note the odours of these compounds.

EXERCISE IX

QUALITATIVE EXAMINATION OF MILK

(One Three-hour Period)

MILK contains representatives of all the classes of food constituents. Its *pH* value closely approximates to that of the blood from which it was secreted. Its quantitative composition varies from animal to animal and to a much greater extent from species to species; qualitatively milks show little variation.[1]

Carry out the following tests with fresh or with pasteurised milk :—

Experiment 1. Put 1 or 2 drops of milk on a microscope slide and examine under the low power of the microscope. Then cover with a cover-slip and examine under the high power. Sketch what you see.

Experiment 2. Measure the specific gravity of milk with an urinometer. For normal fresh cow's milk the figures vary between 1·028 and 1·034. Compare with some skimmed milk (from which most of the fat has been removed). The specific gravity of the latter is slightly greater. Why ?

Experiment 3. Test some milk with red and with blue litmus paper, with phenolphthalein and with congo red paper. In each case put a drop of milk on the paper, and wash it off with 2 drops of distilled water. Blue litmus paper is usually turned red, and red litmus paper is turned blue. Milk is **amphoteric.**

Milk contains four proteins, of which casein is present in largest amount. The following experiments illustrate some of the properties of these compounds :—

[1] In connection with this Exercise the student should read the section on milk (Chapter X., p. 239) in Cameron's "Biochemistry," 5th edit.

Experiment 4. Take 3 c.c. of milk in a test-tube ; add 6 c.c. of water, and then 2 drops of glacial acetic acid ; shake thoroughly. **Casein** separates as a flocculent precipitate. As it settles it carries down the fat globules along with it.

Experiment 5. Transfer 10 c.c. of milk to a small beaker. Add to it 10 c.c. of saturated ammonium sulphate solution (thus producing half-saturation with ammonium sulphate). Casein is precipitated and carries down fat. Filter.

(i.) Treat the precipitate with 10 c.c. of water ; the casein does not dissolve. Add 3 or 4 drops of dilute sodium hydroxide ; the suspension dissolves in great part. Extract the fat by shaking up the solution with 5 c.c. of ether, allowing the liquids to separate and pouring or pipetting off the ether into a small beaker. Repeat, adding the two ether portions together. (Allow the ether to evaporate off away from all flames. An oil drop will be left. Carry out an acrolein test with it. The positive result will confirm fat.) After the fat has been extracted a clear solution of casein is left. Carry out the following tests with it :—

(*a*) To 2 c.c. add a drop of glacial acetic acid and shake. The casein is reprecipitated. Add 2 or 3 drops of dilute sodium hydroxide. The casein redissolves.

(*b*) To 1 c.c. apply Millon's test. The result is positive.

(*c*) To 1 c.c. apply the glyoxylic-acid test. The result is positive.

(*d*) To 1 c.c. apply the xanthoproteic test. The result is positive.

(*e*) Apply to 2 c.c. the test for phosphorus. Phosphorus is present.

(ii.) Take the filtrate from the casein precipitate and boil. The protein **Lactalbumin** coagulates. (Compare this with the scum formed on boiling 5 c.c. of milk in a test-tube. This scum contains some coagulated protein, plus other solid material formed by surface evaporation.) Filter off the lactalbumin, suspend it in water, and apply Millon's test. A positive result should be obtained.

(iii) Apply Benedict's test to the filtrate from lactalbumin. A positive result will be obtained, due to **Lactose.**

Note. Whereas the filtrate gives a positive reaction on boiling for thirty seconds with Benedict's reagent, it gives no reaction with Fehling's solution, owing to the fact that it is half saturated with ammonium sulphate.

The solutions used in the last experiment contained much ammonium sulphate. Hence for further tests on milk-whey proceed as follows :—

Experiment 6. Take 20 c.c. of milk in a beaker, add 20 c.c. of water, and then add dilute hydrochloric acid, **drop by drop,** until a definite maximum flocculent precipitate has formed. *Too much acid* must not be added, since the precipitate redissolves in excess of acid. Allow the precipitate to settle and add one more drop of acid. If more precipitate forms shake, allow to settle, add another drop, and so on, until addition of a drop of acid produces no further precipitate. Filter. (The precipitated casein can be tested as before if desired.)

Concentrate the filtrate in an evaporating basin to half-bulk. **Lactalbumin** and **Lactoglobulin** coagulate to a scum. Filter them off. (They can be tested as to protein nature.)

FIG. 7. Micro-photograph of lactose crystals from milk, × 135.

Continue to evaporate down to 3 or 4 c.c. on the water-bath. Crystals of calcium phosphate separate. Cool and filter. (Did the calcium phosphate exist as such in the milk ?)

(i.) Examine the crystals under the microscope and sketch them. Then dissolve them in dilute nitric acid, divide the solution into two equal portions, to one add a drop of ammonium oxalate (a precipitate of calcium oxalate indicating calcium) and to the other apply a phosphate test.

(ii.) Concentrate the filtrate to a syrup. Allow it to stand overnight. Notice then the hard, gritty crystals of **Lactose** that have separated. Compare them under the microscope with the photo-micrograph in Fig. 7. Remove the crystals, dissolve them in a trace of water, and confirm lactose by reduction and osazone tests (using micro-procedures).

Milk is the chief secretion in the body containing citric acid. Carry out the following tests to demonstrate the presence of this acid in milk :—

Experiment 7. Add 2 c.c. of a freshly prepared 5 per cent. solution of **Metaphosphoric Acid** (a protein precipitant) to 10 c.c. of milk in a beaker and shake. Add 3 c.c. of Denigès' reagent (see below). Again shake and filter. Boil the filtrate in a test-tube and then add 2 per cent. potassium permanganate **drop by drop.** About 6 or 8 drops are sufficient to produce at first a turbidity and then a white precipitate. This result is due to the citrate present.

Denigè's reagent is prepared by dissolving with aid of heat 50 gm. of red or yellow mercuric oxide in a mixture of 200 c.c. of concentrated sulphuric acid and 1 litre of distilled water.

Consider the results that were obtained in the experiment on butter (Exercise IV., Experiment 13) *as compared with the experiments on milk.*

Experiments on the clotting of milk by rennin have already been performed (Exercise VI., Experiments 7 and 8).

EXERCISE X

SCHEME FOR THE EXAMINATION OF UNKNOWN PROTEINS AND PROTEIN DERIVATIVES

(One or more Three-hour Periods)

CARRY out investigations of several unknown solids and solutions, using the following scheme. This scheme is fragmentary. A complete analysis to differentiate between all the classes of proteins, the different types of proteoses, and the different amino-acids, is outside the scope of this course. However, the types of procedure, based upon the differences in properties of proteins that have already been brought out in various experiments, are exemplified, so that the exercise forms a useful repetition from a different angle.

(I.) If the unknown is a solid, test its solubility in water, in dilute alkali, in 1 per cent. sodium chloride solution and in 70 per cent. alcohol.

(*a*) If it is insoluble in water, but soluble in 70 per cent. alcohol, suspect a *prolamine*. Confirm by protein colour tests and insolubility in 95 per cent. alcohol.

(*b*) If the substance is insoluble in water and in alcohol, but soluble in dilute alkali, suspect *casein* or *cystine*. Confirm by neutralising the solution (when casein or cystine should be precipitated), " protein " colour tests (which should be positive for casein and negative for cystine), a test for phosphorus, positive for casein, negative for cystine, and a test for sulphur (positive for both). Examine under the microscope. Cystine may be crystalline or amorphous ; casein is amorphous.

(*c*) If the substance is insoluble in water, but soluble in 1 per cent. sodium chloride, suspect a *globulin*. Confirm by diluting part of the solution, when globulin should be precipitated, by half saturating another portion with ammonium sulphate (which precipitates globulins) and by the " protein " colour tests, which should all be positive.

(*d*) If the substance is insoluble in all the above solvents, it may be a *scleroprotein*. Confirm by the " protein " colour tests, carried out on the solid. (Remember, however, that coagulated proteins and certain other groups will also be included here.)

(*e*) If the substance is soluble in distilled water it may be an albumin, a protamine, a histone, a proteose, a peptone or an amino-acid. If it is crystalline, then it is probably an amino-acid, but remember that some proteins are crystalline. Test further as indicated below (III.).

(II.) If the unknown is a solution, test the reaction. Test for the presence of chloride and for alcohol (by smell).

(*f*) Alcohol, if present in marked amount, suggests a solution of a *prolamine*. Evaporate off most of the alcohol. The prolamine will be precipitated. Suspend a little in some water and a little in 95 per cent. alcohol. It should be insoluble in both solvents. Suspend a little in 70 per cent. alcohol. It should dissolve. Confirm its protein nature by the colour tests.

(*g*) If chloride is present in definite amount, a *globulin* may be present (but albumins, proteoses, etc., are not excluded). Dilute the solution with distilled water. Globulin, if present, will be precipitated. Confirm as under (I.) (*c*).

(*h*) If the solution is alkaline, carefully neutralise. Casein or cystine, if present, will be precipitated. Confirm as under (I.) (*b*). Protamines and histones, naturally alkaline, will not be precipitated. See under (III.) (*m*).

(*i*) If the solution is neutral and does not contain marked amounts of either alcohol or chloride, proceed as under (III.).

(III.) Examination of an aqueous solution of the unknown. Take a portion and try the biuret test. **Note that the biuret test will only permit the following differentiations when it is very carefully controlled. Therefore great care must be exercised in considering the results of tests employed subsequent to the biuret test.**

(*j*) A **negative** test suggests that *amino-acids* may be present. Test portions of the solution with Millon's reagent (positive for *tyrosine*), with the glyoxylic acid reagent (positive for *tryptophane*), with the xanthoproteic test (positive for *tyrosine*, *phenylalanine* and *tryptophane*), and with the ninhydrin test (positive for amino-acids, but not exclusive). Other amino-acids cannot easily be differen-

tiated, but addition to a portion of the solution of a few drops of sodium nitrite solution and of dilute hydrochloric acid should give a marked effervescence if amino-acids are present (though this also is not an exclusive test for amino-acids).

(*k*) A **pink** biuret test indicates *peptones.* Confirm by testing a portion of the solution with potassium ferrocyanide and acetic acid. No precipitate is produced by peptones. Picric acid does not precipitate peptones, but phosphotungstic acid does. Warming does not coagulate peptones.

(*l*) A **purplish-pink** biuret test suggests *proteoses.* In this case potassium ferrocyanide and acetic acid give a precipitate, concentrated nitric acid gives only a very slight precipitate, picric acid gives a precipitate which dissolves on warming and reappears on cooling, and application of heat to the unknown solution produces no coagulation of proteoses.

(*m*) A **purple** biuret test suggests *proteins.* Such may be *albumins*, *protamines* or *histones.* Test the reaction. If the solution is alkaline protamines or histones are suggested. Saturate a portion of the solution with ammonium sulphate. Albumin will be precipitated ; protamines and histones will not. Warm a portion of the solution. Albumin will be coagulated ; protamines and histones will not. The glyoxylic-acid test will be negative for protamines.

(*n*) A **blue-pink** biuret test suggests *gelatine.* A **blue** colour does not rule out gelatine in very dilute solution. In such a case evaporate some of the solution to small bulk and allow to cool slowly. If it *gels* confirm gelatine by a negative tryptophane test, positive xanthoproteic, doubtful Millon's, positive ninhydrin, and absence of coagulation on warming.

EXERCISE XI

QUALITATIVE EXAMINATION OF FOODSTUFFS

(One or two Three-hour Periods)

EACH examination is in the nature of a small piece of research on material in which usually more than one compound is present.

Each of the following should be tested : (*a*) a plain soda-biscuit ; (*b*) a piece of carrot ; (*c*) a piece of raw meat. In addition one or more of the following may be tested as time permits : (*d*) a piece of plain (not milk) chocolate ; (*e*) a piece of potato ; (*f*) a piece of apple ; (*g*) a piece of banana.

Grind or mince up the material thoroughly and in each case carry out the following examination :—

(A) Boil the foodstuff with five times its volume of water for ten minutes and filter hot.

(i.) *Residue.* Test portions for carbohydrate (including cellulose), fat (ether solubility and acrolein test) and protein (colour tests).

(ii.) *Filtrate.* Test portions for soluble carbohydrates, soluble proteins and their derivatives, and for salts.

(B) Boil the foodstuff with five times its volume of 10 per cent. hydrochloric acid for thirty minutes, keeping the volume constant by adding water from time to time. Cool, filter, neutralise exactly with dilute sodium hydroxide, filter off any precipitate, and test separately the residue, the precipitate and the filtrate, as under (A), noting carefully any differences from the results found under (A). Explain how the hydrolysis with acid has resulted in such differences and compare the action of the acid with that of digestion processes.

EXERCISE XII

BLOOD [1]

(Three Three-hour Periods)

The Clotting of Blood

DEMONSTRATION

Experiment 1. (A) Anaesthetise a rat with chloroform. Rapidly open up the abdomen and thorax and sever the great veins on the right side. Allow the blood to drain into a test-tube. Time the period before clotting occurs. Allow the clotted blood to stand and notice that after some time the clot contracts and clear yellowish serum exudes.

(B) Anaesthetise a rat, open up and sever the veins as in (A), but drain the blood into a test-tube in which have previously been placed 2 drops of 20 per cent. potassium oxalate solution. Rotate the tube as the blood flows in. Note that no clot forms.

(C) Proceed as in (A), but collect the blood in a test-tube containing a trace (about 20 mg.) of powdered sodium fluoride. Rotate the tube as the blood flows in. Note that no clot forms.

(D) Proceed as in (A), but drain the blood in a test-tube containing 2 drops of saturated solution of sodium citrate, rotating the tube. Again no clot forms.

(E) Proceed as in (A), but collect the blood in a small bottle containing a glass bead and shake up the bottle immediately. The blood remains unclotted, the "clot" being now white stringy material (*fibrin*) adherent to the bead.

Note. Formation of a clot requires the presence of various compounds and of calcium ions. Oxalate and fluoride precipitate calcium as insoluble calcium salts.

[1] Read the section on blood in Chapter IX. of Cameron's "Biochemistry," 5th edit.

Citrate reduces calcium-ion concentration to a negligible minimum, since calcium citrate is practically un-ionised.

Hence if to the test-tubes in Experiment 1 (B), (C) and (D), after a reasonable time has elapsed to demonstrate that clotting has not occurred, 2 drops of saturated solution of calcium chloride are added, it will be found that the normal clots form. On the other hand, addition of the calcium chloride to the " defibrinated " blood in the bottle causes no clot to form, since in this case fibrinogen, not calcium, was removed.

The student will carry out the following experiments on oxalated ox or dog blood in which clotting has been prevented by drawing the blood into sufficient 20 per cent. potassium oxalate (1 c.c. to 100 c.c. blood) with continuous shaking. (Excess oxalate interferes with some of the following experiments.)

Experiment 2. Add to 10 c.c. of oxalated blood 10 drops of calcium chloride solution. A firm clot forms. Allow to stand. After one hour the clot will have contracted sufficiently to permit the pouring off of 2 or 3 c.c. of *serum*. To this serum add an equal volume of saturated solution of ammonium sulphate (producing half-saturation). A cloudiness indicates the precipitation of " serum globulin." Filter it off, suspend it in 1 per cent. sodium chloride and shake. It dissolves. Confirm the presence of protein by colour tests.

Take the filtrate from the globulin and completely saturate it with ammonium sulphate crystals. A further precipitate of " serum albumin " is seen as a cloudiness. Filter this off, suspend it in water (before it dries) and note that it redissolves. Confirm its protein character by colour tests.

Doubt has recently been thrown upon the existence of more than one globulin and more than one albumin in blood plasma, and no useful purpose is to be gained in this course by attempted differentiations.

Experiment 3. Examine a drop of oxalated blood under the microscope. Notice the red cells, which singly appear reddish-yellow, and the fewer and larger white cells. Add 2 drops of distilled water. The cells rupture (*haemolysis*), the

coloured material (*haemoglobin*) being liberated and diffusing through the solution. This is the process of **laking.**

Experiment 4. Centrifuge 10 c.c. of oxalated blood for fifteen minutes. Only partial separation of cells and "oxalated plasma" will have been effected in this short period. Pour off the clear upper layer. Add 2 drops of saturated solution of calcium chloride to it. Allow it to stand. A **colourless clot** forms. The compounds required for clotting and the process of clotting have nothing intrinsically to do with the red cells.

On standing this clot also shrinks, and serum exudes. This can be hastened by stirring up the clot with a glass rod. A white mass of *fibrin* adheres to the rod. Transfer this mass to a test-tube and wash it with water. Then try on separate minute portions of it the various colour tests, and so demonstrate its protein nature.

The serum can be tested as in Experiment 2.

DEMONSTRATION TO ILLUSTRATE HOWELL'S THEORY OF THE CLOTTING PROCESS

Experiment 5. (A) Centrifuge 50 c.c. of oxalated blood at 3,000 revolutions per minute for fifteen minutes. Transfer 20 or more cubic centimetres of the oxalated plasma to a small beaker. Add to it two volumes of a saturated solution of sodium chloride.[1] *Fibrinogen is precipitated.* Centrifuge it off, wash it with half-saturated sodium chloride solution, and recentrifuge, and then transfer it to a beaker containing 20 c.c. of 1 per cent. sodium chloride solution. It redissolves. Add 20 c.c. of the saturated sodium chloride solution. The fibrinogen is reprecipitated. Once more filter, wash, and dissolve in 1 per cent. sodium chloride solution. On standing it will be observed that **this solution of fibrinogen does not clot.**

(B) Whip some freshly drawn blood until 10 or 15 gm. of **fibrin** strings have collected on the twigs used for whipping. Collect this fibrin and knead it in water to remove adherent red cells, etc. Squeeze it dry between filter paper, cut it up into small pieces with scissors, transfer it to a small beaker, and cover it with 8 per cent. sodium chloride solution. Then

[1] Some samples of sodium chloride contain calcium salts. The sodium chloride used in this experiment must obviously be free from calcium.

place it in a refrigerator for forty-eight hours. Finally, squeeze the liquid through cheese-cloth. A viscous solution of **Thrombin** is obtained. Observe that this does not clot on standing.

(C) Centrifuge 50 c.c. of oxalated blood as in (A), and obtain 20 c.c. of plasma. Heat this carefully **exactly** to 54° C. A precipitate of **Fibrinogen** is formed (by heat coagulation). Decant and centrifuge. To the filtrate add acetone until a precipitate forms. Collect this precipitate on a filter, wash with acetone, and dry ; then extract with 10 c.c. of 2 per cent. sodium carbonate. The solution contains **Prothrombin.**

With the material obtained in (A), (B) and (C) carry out the following tests :—

(i.) Add to 2 c.c. of the fibrinogen solution a few drops of calcium chloride solution. No clot forms.

(ii.) Add to 2 c.c. of the fibrinogen solution a few drops of thrombin solution and 2 drops of calcium chloride solution. A clot forms.

(iii.) Add to 2 c.c. of the fibrinogen solution a few drops of thrombin solution, but no calcium chloride. Does a clot form ?

(iv.) Add to 2 c.c. of the fibrinogen solution a few drops of prothrombin solution. No clot forms. Add 2 drops of calcium chloride (ignore the calcium carbonate precipitate). A clot forms.

(v.) Add to a few drops of thrombin solution 2 drops of calcium chloride solution. No clot forms. Now add 2 c.c. of the fibrinogen solution. A clot is produced.

These results demonstrate that fibrinogen plus thrombin in the presence of calcium ions produces fibrin (the clot mesh), while fibrinogen and prothrombin do not give a clot. Once prothrombin and calcium ions have reacted, the result (thrombin) will clot with fibrinogen.

Inorganic Constituents, etc.

Experiment 6. Take 10 c.c. of serum obtained from centrifuged, defibrinated blood, remove proteins by boiling and adding 2 drops of dilute acetic acid and filter. Test the protein-free filtrate as follows :—

(i.) Add a few drops of dilute nitric acid and 2 drops of silver nitrate to 1 c.c. in a test-tube. A white precipitate of silver chloride is produced.

(ii.) Take 2 c.c. and test for phosphate.

(iii.) To 2 c.c. add 1 c.c. of saturated solution of ammonium oxalate. A white cloudiness (calcium oxalate) indicates presence of calcium.

(iv.) To 4 c.c. add 4 drops of Benedict's solution and boil for one minute. A slight but definite reduction should be observed, due to the presence of glucose.

Carry out the following three experiments with defibrinated blood :—

Experiment 7. Moisten red and blue litmus papers with 10 per cent. sodium chloride (to facilitate absorption of blood by osmosis) and then place on each a drop of blood. It should show a very slightly alkaline reaction.

Experiment 8. Determine roughly the specific gravity of blood with a urinometer.

Experiment 9. Take 5 c.c. of defibrinated blood in a porcelain crucible and evaporate it to dryness on the water-bath. Then heat it in the fume-chamber to redness until most of the carbon has been burned off. Cool, dissolve in dilute hydrochloric acid, filter and test the filtrate for iron with ammonium thiocyanate (development of a red colour indicates iron).

This test can also be carried out with the red cells (after centrifuging) and with plasma or serum. The first will give a positive result, the others a negative, indicating that the iron is contained in the red cells.

Tests for Blood

There are various tests for blood, more or less specific, which are especially of importance *from a medico-legal standpoint.* Certain of them involve spectroscopic examinations, and these will be referred to later.

Carry out the following tests with a mixture of one part of blood in 100 parts of water :—

The Guaiac Test

Experiment 10. Add to 5 c.c. of diluted blood in a test-tube a freshly prepared 2 per cent. alcoholic solution of

guaiac (resin) drop by drop from a pipette until a turbidity is just produced. Then add dilute hydrogen peroxide drop by drop. A blue colour results. Repeat with diluted blood which has previously been boiled for from fifteen to twenty seconds. The test is still positive.

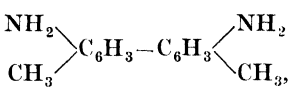
Now repeat, using instead of blood 3 drops of milk in 5 c.c. of water. A blue colour will probably be produced *before* the addition of hydrogen peroxide *provided the milk is fresh.* It may not be given by pasteurised milk, and is not given after the milk solution has been boiled for fifteen seconds. Verify these statemeuts.

Provided the guaiac test is carried out in a delicate and specific manner, it is a very definite test for blood even in dilutions of 1 *to* 100,000 *or less.* It can thus, with due precaution, be used as a medico-legal test.

The blue reaction is often due to an **oxidase** or **peroxidase** present in the material tested, and is actually brought about under varying conditions by a large number of animal and vegetable materials. Since it is given by boiled blood, in which of course any enzymes present have been decomposed, these are not the active factor as far as blood is concerned. In blood haemoglobin causes the reaction. Iron-containing derivatives of haemoglobin are also active in the production of this colour.

The Orthotolidin Test

Experiment 11. To 1 c.c. of a freshly-prepared 4 per cent. solution of orthotolidin,



in glacial acetic acid add 1 c.c. of the diluted blood and 1 c.c. of 3 per cent. hydrogen peroxide. A bluish colour develops and may persist for several hours. (Old solutions of orthotolidin give a dirty green, instead of a blue colour, rendering the test unreliable.)

This is one of the most delicate and satisfactory tests for blood in faeces, urine, blood-stains, etc.

The Benzidine Test

Experiment 12. Take 3 c.c. of a freshly-prepared saturated solution of benzidine, $NH_2 . C_6H_4 . C_6H_4 . NH_2$, in glacial acetic acid and add 2 c.c. of the blood solution, and **then** 1 c.c. of 3 per cent. hydrogen peroxide. A blue or green colour develops.

This is also a very delicate test for blood. It is produced by catalysis of the peroxide by haemoglobin, the oxygen then oxidising the benzidine to a blue compound.

The Haemin Test

Experiment 13. Place **one** drop of **undiluted** blood on a microscope slide, add **one** drop of water, stir up to produce laking, add **half** a drop of 1 per cent. sodium chloride solution, and hold 6 in. above the top of a small Bunsen flame until the mixture has evaporated to dryness. Cover with a cover-slip, then run underneath the slip **one** drop of glacial acetic acid, and warm **just as before,** until formation of gas bubbles **just** commences. Cool slowly, and examine under the microscope. Characteristic small dark red, rhombic crystals of **Haemin** should be seen. Sketch them. Compare with the photo-micrograph in Plate III.

The Haemochromogen Test [1]

Experiment 14. TAKAYAMA'S SOLUTION.—Mix together 3 c.c. of 10 per cent. sodium hydroxide, 3 c.c. of pyridine, 3 c.c. of saturated solution of glucose and 7 c.c. of water. The solution should be freshly made up.

Add to 2 or 3 drops of blood on a microscopic slide 2 drops of Takayama's solution, cover with a slip and watch under the microscope. Salmon-pink crystals (shallow rhombs) appear in between one and six minutes. They gradually change in colour to green-brown, dark red and finally pink. Sketch them and compare with the photo-micrograph in Plate III.

Haemoglobin and its Derivatives

The experiment of laking blood under the microscope and the production of a clear plasma or serum and a coloured mass of cells by centrifuging oxalated or

[1] Kerr and Mason, *Brit. Med. J.*, 1926, I., 134.

defibrinated blood have demonstrated that the red colouring matter is localised in the red cells. While many of the properties of this compound can be studied on blood itself, yet it is desirable to prove by direct comparison that at any rate some of them as demonstated with blood are really due to haemoglobin as demonstrated directly upon haemoglobin.

Preparation of Haemoglobin on the Microscopic Scale

Experiment 15. Add ether, drop by drop, to a few cubic centimetres of defibrinated *dog's* blood in a test-tube until laking is complete. (This laking, as produced by ether, is due to solution of the lipide envelope of the cell in ether.) Add a **small pinch** of **finely powdered** ammonium oxalate. Shake very gently for two or three minutes to dissolve the oxalate. Allow the tube to stand in contact with ice or snow for one hour. Shake gently and transfer a drop of the blood to a slide. Examine under the microscope and sketch the crystals. (If no crystals are seen spread the drop in a thin layer. Crystals should commence to separate within five minutes.)

Haemoglobin crystals vary in form from species to species. Contrast the crystals obtained in Experiment 15 with those in the photo-micrograph in Plate III.

The amount of *oxyhaemoglobin* prepared in the last experiment is much too small for chemical examination. Carry out the following parallel experiments with (*a*) a solution of good commercial haemoglobin and (*b*) defibrinated blood. Satisfy yourself that the results are the same, and are therefore due to the haemoglobin.

Experiment 16. Prepare haemin crystals as in Experiment 13.

Experiment 17. Add to some blood or haemoglobin solution in a test-tube a drop or two of ammonium sulphide, warm gently and notice a rapid change in colour from scarlet red to a dark purplish-red. Shake up with air. The original colour gradually reappears. The oxyhaemoglobin was reduced by the sulphide to " reduced " haemoglobin, which

later absorbed oxygen from the air again to form oxyhaemoglobin.

Experiment 18. Pass a slow stream of coal-gas (which contains carbon monoxide) through 3 c.c. of blood or haemoglobin solution in a test-tube (carrying out the operation in a fume-chamber) by means of a bent glass tube attached to rubber tubing from a gas tap. Notice the change of colour to **cherry-red,** due to formation of *carboxy-haemoglobin.*

Experiment 19. Dilute 1 c.c. of blood or haemoglobin solution with ten volumes of water in a test-tube. Note the resulting colour. Add 3 drops of 10 per cent. potassium ferricyanide, shake and note the change in colour to brownish-red due to the production of methaemoglobin.

Experiment 20. Add 2 drops of blood or haemoglobin solution to 5 c.c. of concentrated sulphuric acid in a test-tube, shaking thoroughly. A (Burgundy) wine-red solution of *haematoporphyrin,* which contains no iron in its molecule, results.

Experiment 21. Add 3 c.c. of glacial acetic acid to 6 c.c. of blood (or haemoglobin solution) in a test-tube and **then** 3 c.c. of ether. Shake thoroughly. *Haematin* is produced and dissolves in the ether layer to a red solution.

Repeat this test without the acetic acid. No haematin is produced, haemoglobin is not soluble in ether, and the ether layer remains uncoloured.

Since in all the above experiments the same results will be obtained whether whole blood or a haemoglobin solution is used, these results obtained with blood must be due to its haemoglobin content.

Spectroscopic Examination of Haemoglobin and its Derivatives

White light is made up of a mixture of coloured radiations of continuously varying wave-length. Passage of such light through a prism breaks it up into its constituents, which thereby form a " spectrum "—a rainbow band—showing a colour series from red, through yellow,

PLATE III

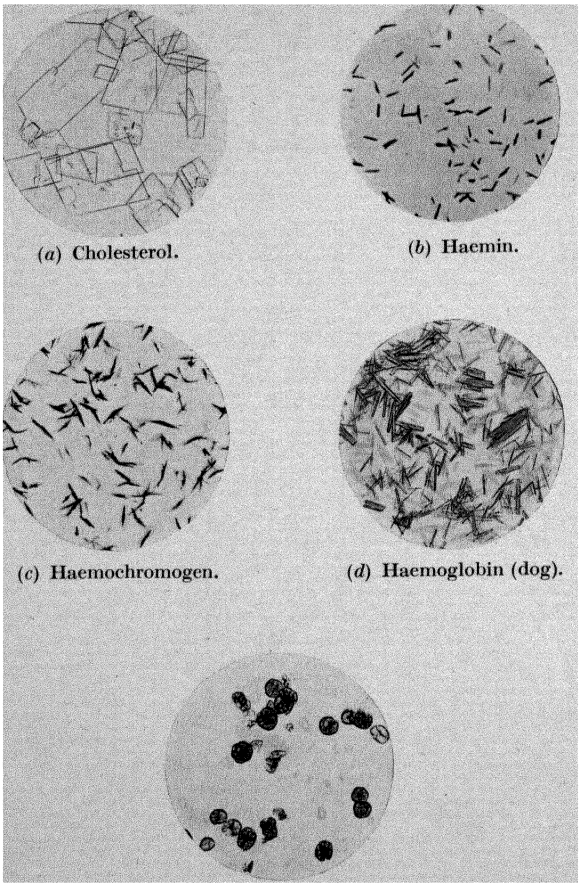


Photo-micrographs. (*a*) Cholesterol, × 67·5 ; (*b*) haemin from human blood, × 135 ; (*c*) haemochromogen from human blood, × 45 ; (*d*) haemoglobin from dog's blood, × 90 ; (*e*) haemoglobin from rat's blood, × 90.

Crystals obtained by the procedures described in the text.

[*To face p.* 81 .

green and blue, to violet. Certain compounds have a special property of absorbing rays in various regions of this spectrum, so that if solutions of these compounds are placed between the source of light and the prism of the **spectroscope**, in place of a continuous band of changing colour at certain parts **black** bands appear, **absorption bands.** The positions of such bands can be definitely fixed in larger spectroscopes, and the series of wave-lengths of the absorbed light measured. In smaller spectroscopes approximations to these positions can be determined by noting the type of colour absorbed.

Haemoglobin and its derivatives show very little absorption bands.

DEMONSTRATION (*with small groups*)

Experiment 22. The instructor will first explain the essential points of the spectroscope and illustrate the continuous spectrum. He will then show that certain metals, such as sodium, calcium, etc., give line spectra in perfectly definite regions of the spectra corresponding to definite wave-lengths, and point out how it is possible to calibrate the spectroscope roughly with a series of such lines corresponding to known wave-lengths.

The following tests will then be made, using (i.) a 1 in 30 solution of blood and (ii.) a 0·5 per cent. solution of haemoglobin. Both solutions must be well shaken up with air before use to ensure that almost all the haemoglobin is present in the oxidised form. The solutions can be examined conveniently in small test-tubes.

(*a*) Note that at the dilutions used a single dark band in the yellow-green and green is visible. Dilute further with distilled water. The band breaks up into two bands in the yellow-green and green. the one nearer the red end of the spectrum being narrower and darker. With continued dilution the bands grow fainter and fainter, but even at such dilution that a red colour is barely perceptible the bands are still just seen. These are the two characteristic bands of oxyhaemoglobin.

If a spectroscope fitted with a cup and plunger is available

the same effect is more conveniently demonstrated by gradually raising the cup so that the layer of liquid is gradually decreased (and so the number of effective molecules of oxyhaemoglobin is gradually decreased).

(*b*) Dilute the solutions to such an extent that the two dark bands of oxyhaemoglobin are only just separated. Then add to 5 c.c. of each (blood and haemoglobin) 2 drops of ammonium sulphide and warm. Note the change in colour

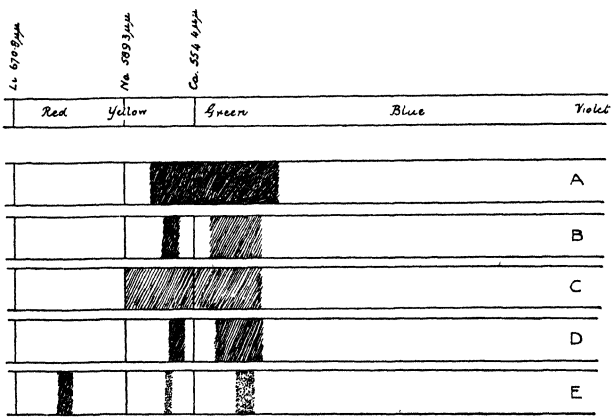


FIG. 8. Absorption bands of haemoglobin and its derivatives, as seen through a spectroscope with diluted blood in a test-tube 15 mm. in diameter. The blood used contained 12 gm. of haemoglobin per 100 c.c. For A the blood was diluted 1 : 33 with water, and for B–E 1 : 50. A and B, oxyhaemoglobin ; C, (reduced) haemoglobin ; D, carboxyhaemoglobin ; E, methaemoglobin.
The shading roughly indicates the density of the bands.

(darker and more purple). The spectroscope now shows a single band, spread more widely over the same region and not so dark. Shake up with air. The two bands reappear. The single diffuse band is that of (" reduced ") haemoglobin.

(*c*) Take solutions at the same dilution as in (*b*) and pass through them a slow stream of coal-gas. Note the change of colour to cherry-red. The spectrum apparently is unaltered, but careful comparison shows that the absorption bands of carboxyhaemoglobin are both shifted slightly towards the violet end of the spectrum. Shake up with air. The cherry-

red colour persists. Carboxyhaemoglobin is a very stable compound.

(*d*) Take solutions at the same strength as in (*b*) and to 5 c.c. add 3 drops of freshly prepared potassium ferricyanide solution (10 per cent.). Note the change to the brownish-red colour of methaemoglobin. Examine in the spectroscope. A band just in the red is seen, and either one diffuse band or, on greater dilution, two faint bands lying somewhat nearer the blue end of the spectrum than those of oxyhaemoglobin. Add 2 drops of ammonium sulphide and warm. The spectrum now shows the absorption band of reduced haemoglobin only. Shake up with air. The two bands of oxyhaemoglobin appear.

This is an important test for medico-legal use. Old blood stains contain methaemoglobin. The series of changes just demonstrated always indicate the presence of haemoglobin and therefore almost certainly the presence of blood.

Fig. 8 shows in chart fashion the position and extent of the bands seen under the conditions laid down for this experiment.

The student should repeat Experiment 22 with diluted defibrinated blood and a small spectroscope.

Identification of Blood in Old Stains

Experiment 23. Take a piece of blood-stained rag that has been allowed to dry and to stand exposed to air for at least a week. Scrape off some of the dried blood, transfer the scrapings to a test-tube, add 1 or 2 c.c. of water, shake, filter if necessary and carry out suitable tests to demonstrate the presence of blood, using the smallest volumes possible.[1]

These tests should include the haemin test (a dried scraping can be used for this), the orthotolidin, benzidine, perhaps the haemochromogen and finally the spectroscopic test illustrating the changes with methaemoglobin.

An excellent modification of the haemin test, especially useful for dried blood scrapings, employs a reagent consisting of 1 gm. of crystallised magnesium chloride, 1 gm. of water, 5 gm. of 30 per cent. glycerol, and 20 gm. of glacial acetic acid.

A few fine particles of the suspected material are scraped

[1] Or cut up the rag into minute pieces, transfer these to a test-tube, and extract by shaking with a very small amount of water. Filter if necessary.

on to a slide, one drop of the reagent added, and the slide warmed for a few seconds over a small flame. If the test is positive characteristic crystals of haemin develop.[1]

Such tests, if positive, are conclusive for the presence of haemoglobin, from which there is strong presumption that the stains are due to blood. Such tests do not demonstrate the presence of human blood. A biological test can be employed to demonstrate this, but this test, involving agglutination of corpuscles by serum of the same species of animal, is outside the scope of this course.

[1] Bertrand, *Ann. chim. anal.*, 1932, p. 353 ; through *Analyst*, 1932, p. 664.

EXERCISE XIII

THE CHEMICAL COMPOSITION OF THE TISSUES

(Two Three-hour Periods)

MUSCLE, bone and tendon epithelial tissue and nerve tissue are here selected for examination. In addition a few tests on the endocrine secretions and related compounds are added.[1]

MUSCLE

Preparation of a Muscle Extract

Mince finely about ½ lb. of very lean beef, wash to remove blood and lymph as far as possible, and place the washed mass in a large beaker. Add 500 c.c. of 10 per cent. sodium chloride. Stir occasionally for several hours, then remove the meat by straining through cheese-cloth, and filter the extract through coarse filter paper. This extract should be used for a number of the following experiments, and will be referred to as the **prepared muscle extract.**

Experiment 1. Take 20 c.c. of the prepared muscle extract and saturate it with sodium chloride crystals. **Myosin** is precipitated. Filter. The filtration proceeds very slowly, and since myosin in contact with air rapidly changes to a more insoluble protein, *before filtration is complete* remove some of the myosin by scraping the side of the filter paper and test it as follows :—

(i.) Suspend a little in water and shake it up ; it is insoluble. Pour off the water and replace it by 1 per cent. sodium chloride. The myosin dissolves, being a globulin. (Dilute a few cubic centimetres of the original extract and observe that the globulin, myosin, is precipitated.)

(ii.) Carry out the biuret, xanthoproteic, glyoxylic and

[1] The theory corresponding to this chapter is given in Cameron's "Biochemistry," 5th edit., Chapter X.

Millon's tests with small portions of the solution in sodium chloride. Note that the results are all positive.

Creatine [1]

Experiment 2. Carry out the following tests with any good commercial preparation of creatine :—

(i.) Examine the crystals under the microscope. Sketch them. Compare with the photo-micrograph in Plate IV.

(ii.) Compare the solubility in cold and in hot water. Recrystallise from a hot solution and examine the crystals under the microscope.

(iii.) Test by Jaffé's reaction (*cf.* Exercise XV., Experiment 22).

(iv.) To 5 c.c. of creatine solution add 5 c.c. of saturated sodium carbonate solution and a few drops of alcoholic solution of diacetyl. A **pink** colour develops. *This is a specific test for creatine.*

DEMONSTRATION.—Carnosine

Experiment 3. Take about 20 gm. of lean meat, previously minced and washed, and place in a beaker with 20 c.c. of water. Stir thoroughly, and warm on the water-bath at 50° to 60° C. for fifteen minutes. Filter through muslin, and extract the residue again with 10 c.c. of water. Combine the two filtrates, boil, cool and filter from coagulated protein.

To 5 c.c. of the filtrate apply Pauli's test (p. 41). The positive result indicates iminazolyl compounds (histidine, or carnosine, β-alanyl-histidine) or tyrosine. Apply Millon's test; the negative result excludes tyrosine. Apply G. Hunter's modification of Knoop's test [2]: To 5 c.c. of the special extract in a separating funnel add bromine water in excess. Extract with chloroform until the aqueous layer is colourless, transfer it to a test-tube and heat to boiling. Non-appearance of a brownish-red colour excludes histidine. To confirm carnosine hydrolyse 5 c.c. of extract with 5 c.c. concentrated hydrochloric acid, boiling fifteen minutes; cool, neutralise, and apply the modified Knoop's test. The result should be positive.

[1] For a convenient method of preparation of creatine, see Hunter's "Creatine and Creatinine" ("Monographs on Biochemistry"), Longmans, London, 1928, p. 56.

[2] G. Hunter, *Biochem. J.*, 1922, XVI., 637, 640.

Glutathione

Experiment 4. To 5 c.c. of the prepared muscle extract add ammonium sulphate crystals until saturation is produced, and then 5 drops of freshly prepared sodium nitroprusside solution and 2 c.c. of concentrated ammonia. The mixture turns purple. *This reaction is given by the sulphydryl group*,—SH, and in this case the positive result is due to glutathione in the muscle extract.

Non-nitrogenous Constituents of Muscle

Inositol cannot be easily tested for unless it is isolated in pure condition.

Glycogen

Experiment 5. Grind up an oyster with sand in a mortar. Transfer to an evaporating basin, add water, and boil for twenty minutes. Faintly acidify with acetic acid and filter hot. Note that the filtrate is opalescent. (If canned oysters are taken, most of the glycogen is present in the liquid. Heat this on the water-bath for twenty minutes, acidify, and filter.)

(i.) To a portion of the solution apply the iodine test, carrying out a parallel test with an equal volume of water. The red colour is seen (given by both glycogen and erythrodextrin (*cf.* Exercise II., Experiments 32 and 33).

(ii.) Test a portion with Benedict's reagent. No reduction should be seen.

(iii.) Hydrolyse a portion with a little acid, boiling for at least ten minutes. Neutralise and again apply reduction tests. Positive results should now be obtained. An osazone test can also be applied, and glucosazone should be obtained. The glycogen has hydrolysed to glucose.

(iv.) Test the effect of saliva on the solution. After fifteen minutes at 40° on the water-bath reducing sugar should be demonstrable.

(v.) Add to a portion of the solution four times its volume of 95 per cent. ethyl alcohol. Glycogen is precipitated. Let it settle, decant off the fluid, filter the residue, and apply the iodine test to it. A positive result should be obtained.

DEMONSTRATION.—Lactic Acid

Experiment 6. Chloroform a rat, dissect out sufficient muscle tissue, and make an extract of it.

To 3 c.c. of the extract add 1 drop of ferric chloride solution. A yellow colour should be produced, due to ferric lactate. Carry out, side by side with this test, a control with the same volume of water and a drop of ferric chloride. If in doubt, repeat with a very dilute solution of lactic acid, and note the degree of colour difference obtainable.

Apply *Uffelmann's reaction.* The special reagent is prepared by adding ferric chloride to a 1 per cent. solution of phenol until an amethyst-blue colour is obtained. To 5 c.c. of this reagent add 5 c.c. of the muscle extract. In presence of lactic acid a canary-yellow or greenish-yellow colour develops. The test is stated to be positive for concentrations of 0·01 per cent. and over.

Mineral Constituents

Experiment 7. Incinerate a few grams of fresh meat in a porcelain crucible in the fume-chamber until only a grey ash is left. Cool. Dissolve the ash in dilute hydrochloric acid. The flame test for potassium is positive. Addition of ammonium oxalate gives a white precipitate of calcium oxalate. Addition of nitric acid and ammonium molybdate and warming gives the yellow crystalline precipitate that indicates phosphorus. Chlorides, of course, cannot be tested for in this solution. (If a trace of ash is removed before addition of hydrochloric acid and dissolved in nitric acid, addition of silver nitrate will give a slight precipitate indicating chlorides.)

BONE

Experiment 8. Take a small piece of bone from which marrow has been removed. Warm it with three times its volume of dilute hydrochloric acid for thirty minutes on the water-bath, adding water from time to time. Most of the mineral constituents will have passed into solution at the end of this period, leaving gelatinous **Ossein** (said to be identical with or very similar to the **Collagen** of connective tissue, for which see Experiment 9). Suspend bits of the ossein in water and show that the protein colour reactions are positive.

Test portions of the solution for calcium (with ammonium oxalate) and phosphate (with nitric acid and ammonium molybdate). For magnesium add ammonia in excess and

filter from the precipitate produced. To the filtrate add disodium hydrogen phosphate; a white precipitate of ammonium magnesium phosphate should be produced on warming or standing. (The original effervescence indicated presence of carbonates, and this could be confirmed by holding over the dish a glass tube at the end of which was a drop of lime or baryta water.)

CONNECTIVE TISSUE

Experiment 9. The preparation of *tendomucoid* and of *collagen* should be carried out prior to their use by the class.

The *tendo Achillis* of an ox is taken in sufficient quantity, cleaned from adherent material, cut up into small pieces, and these washed in running water with pressure, so that as much as possible of soluble protein and salts is removed. The washed material is transferred to a flask, and six to eight times its volume of half-saturated lime water added. The flask is shaken occasionally during twenty-four hours. **Mucoprotein** (tendomucoid) has then passed into solution, and the residue consists largely of **Collagen.** The student should test these two preparations as follows :—

(i.) To 5 c.c. of the mucoprotein solution add dilute hydrochloric acid. Filter off the precipitated protein.

Note that it is insoluble in water, in 1 per cent. sodium chloride solution, and in alcohol, but is soluble in dilute alkali.

Apply the biuret, Millon's and the xanthoproteic tests. They should all be positive.

Hydrolyse a very little with hydrochloric acid, boiling on the water-bath until a dark brown solution results. Cool, neutralise, and test with Benedict's solution. A positive result for reducing sugar should be obtained.

(ii.) Test the impure preparation of collagen[1] as follows :—

Note that when it is digested with water for several hours **Gelatine** is formed, so that the tests should be carried out side by side with collagen and gelatine, and any differences noted.

Try the difference in solubility in water, in dilute acid, in

[1] Collagen, when shaken up with water, is changed in physical condition and appears to disintegrate. This may be mistaken for solution.

dilute alkali, in concentrated acid, and in concentrated alkali.

Apply Millon's, the biuret, and the glyoxylic acid tests.

Try also the coagulation and precipitation tests with gelatine solutions and contrast the results with those obtained with other proteins.

EPITHELIAL TISSUE

Experiment 10. Take some horn shavings as typical **Keratin** material. Note that they are insoluble in all ordinary solvents. Suspend portions in water, and demonstrate that the colour reactions for proteins are all positive.

NERVE TISSUE

Experiment 11. The preliminary part of this experiment should be carried out before the class meets.

A sheep's brain is minced finely, placed in a large, wide-mouthed bottle, and 500 c.c. of ether added. The corked bottle is allowed to stand in an ice-box for seventy-two hours. The ether extracts lecithin and cholesterol.

The student will test the ethereal extract as follows :—

(i.) Take 10 c.c. and add acetone until no further precipitation is produced. Lecithin is thrown down. Filter.

(ii.) Apply the osmic acid test to a trace of the precipitate. The result is positive, due to the presence of fatty acid radicals (*cf.* Exercise IV., Experiment 15).

(iii.) Apply the acrolein test (in the fume-chamber) to a trace of the lecithin. The positive test indicates presence of glycerol radicals.

(iv.) Apply the nitric acid-ammonium molybdate test for phosphate. A positive result is obtained. (It may be necessary to ash some lecithin for this test.)

(v.) Evaporate the ether-acetone filtrate on a water-bath containing boiling water, **but away from all flames.** Examine the residue under the microscope. Apply the acetic anhydride sulphuric acid test to it. A positive result should be obtained, indicating the presence of cholesterol.

THE ENDOCRINE SECRETIONS

Most of the endocrine compounds have not yet been isolated in pure form. Their properties can only be demonstrated by time-consuming experiments, the

majority of which require physiological technique, and have to be carried out on the dog, the rabbit or the rat. They cannot be properly included in this short course.

Adrenine (or adrenaline, or epinephrine) and thyroxine, the internal secretions respectively of the adrenal medulla and the thyroid gland, are now obtainable in pure crystalline form. Certain of their chemical properties can be conveniently demonstrated.

DEMONSTRATION.—Adrenine

Experiment 12. Dissolve a trace of adrenine hydrochloride crystals in water.

To 1 or 2 c.c. of the solution add a few drops of ferric chloride solution. A green colour develops, typical of compounds containing a catechol nucleus (1, 2 dihydroxybenzene derivatives). Repeat the test, using, instead of adrenine, a dilute solution of pyrocatechol.

To 1 or 2 c.c. of the solution add a few drops of dilute potassium persulphate solution, and warm. A rose-red colour develops, due to the oxidation of the adrenine. This test is stated to be so delicate as to give a positive reaction with a dilution of adrenine of one part in five million.

DEMONSTRATION

Experiment 13. Mince a few grams of fresh adrenal glands (obtained just previously from the slaughterhouse) and extract with 1 per cent. sodium chloride, boil and filter. Carry out the above tests for adrenine on the filtrate. Positive results should be obtained, indicating the presence of adrenine in the extract.

DEMONSTRATION.—Thyroxine

Experiment 14. Examine a few crystals of thyroxine under the microscope and note that they are definitely crystalline (though usually no good picture of the crystal form is obtained with commercial preparations).

Suspend them in water. They do not dissolve. Add a trace of alkali. The thyroxine dissolves rapidly. To this solution add 1 c.c. of 1 per cent. sodium nitrite, and then 2 or 3 drops of concentrated hydrochloric acid. A (lemon)

yellow colour develops. Add sufficient strong ammonia, or 10 per cent. sodium hydroxide, to make the reaction alkaline. The colour changes to rose-red (or a more brownish-red if the concentration is sufficient).

Carry out a parallel test with some crystals of di-iodotyrosine. Note that the solubility is similar, that nitrous acid produces an (orange) yellow colour, and that addition of alkali changes this to a colour identical with that given by thyroxine.

The presence of iodine in thyroid tissue has been proved (Exercise I., Experiment 8). Repeat, using a very little alkaline solution of thyroxine, and show that this also contains iodine. (The actual amount present is 65 per cent.)

DEMONSTRATION.—Iodothyroglobulin

Experiment 15. Mince up some fresh ox thyroids, treat with three or four times their volume of 1 per cent. sodium chloride, shake up occasionally for twenty minutes, and filter through cheese-cloth. Add to the filtrate an equal volume of saturated solution of ammonium sulphate. A white precipitate of iodothyroglobulin is thrown down. Filter through coarse filter paper. The filtration is very slow. Before it is complete scrape off a little of the moist precipitate and suspend it in 1 per cent. sodium chloride solution. It dissolves. Again add an equal volume of saturated solution of ammonium sulphate. The thyroglobulin is thrown down in purer form. Filter a small amount, wash with water, then alcohol, squeeze roughly dry between filter paper, and carry out a test for iodine on this preparation. A positive result should be obtained. (The iodine content is variable, from a trace up to 1·5 per cent. or more.)

EXERCISE XIV

RESPIRATION [1]

(One Three-hour Period)

Most of the demonstrable facts of respiration cannot easily be illustrated by simple experiments. A few important points can be proved by the following experiments :—

DEMONSTRATION.—Comparative Composition of Inspired, Expired, and Alveolar Air

Experiment 1. Take three simple graduated eudiometer tubes, fitted with glass stopcocks. Regrease the stopcocks if necessary. Fill the tubes completely with water and, with stopcocks closed, invert the tubes over water in glass beakers large enough to admit the fist.

By opening its stopcock admit air to one in convenient quantity (two-thirds fill the tube) and measure its volume. Introduce rapidly into the tube under water a small piece of stick sodium hydroxide (being careful to introduce no air along with it), close with the thumb and shake up the tube. Replace over water. Allow a few minutes to elapse (since the sodium hydroxide, dissolving, produces heat which causes gas expansion). Read the volume of gas in the tube again and note that there is little or no change, indicating that atmospheric air contains practically no carbon dioxide. Now introduce in the same way a pellet of compressed pyrogallol (conveniently compressed in a pastille press) and then a somewhat larger piece of sodium hydroxide and shake up as before, replace in the beaker, allow to cool, and read the new height of solution in the tube. The difference is due to oxygen absorbed by the alkaline pyrogallol solution.

Fill the second tube with a corresponding volume of expired air by breathing normally through the (opened) glass tap. Repeat the analytical procedures. Notice that a

[1] Read Chapter XI. in Cameron's "Biochemistry," 5th edit.

definite amount of carbon dioxide is now present, and that the oxygen content is less than in atmospheric air. The residual volume of nitrogen is about the same.

Fill the third tube to the same extent as the others with **alveolar** air by blowing into the tap at the **end** of a **forced** expiration. Analyse as before. Notice that still more carbon dioxide and still less oxygen are present.

Express the results as percentages. (For greater accuracy correct approximately for the difference in water levels, taking the barometric pressure as 760 mm. mercury and remembering that water is 2/27 as heavy as mercury, so that, for example, a difference of 100 mm. height between the level of water inside (higher) and outside a tube means that the volume of gas within the tube is too great by the fraction $2/27 \times 100/760$. Subtract the necessary fraction from each reading of volume.)

DEMONSTRATION.—Gases Present in Blood

Experiment 2. (i.) Shake up some distilled water with air, and then introduce 1 c.c. into a Van Slyke apparatus (Fig. 21). Create a partial vacuum by lowering the mercury reservoir until the total gas volume is 50 c.c., shake for a minute and then rapidly raise the reservoir to re-establish normal pressure. Note that no gas, or practically no gas, is liberated.

Repeat with water that has been saturated with carbon dioxide by bubbling that gas through it. A definite volume of gas is evolved. Note the amount. Compare it with the total gas liberated from water that has been saturated with alveolar air by blowing through the water at the end of a forced respiration. In this case only a trace of gas will be liberated, showing that *the gases carbon dioxide, oxygen and nitrogen are only dissolved in negligible amount under the (partial) pressure conditions in which they exist in alveolar air.*

(ii.) Instead of water, repeat with 1 c.c. of (oxalated) whole blood. A definite volume of gas results. Carefully introduce alkali through the cup (having the gas within under slightly reduced pressure), and show from the difference in measurement that the gas is not (or the major part of it is not) carbon dioxide. Then introduce in the same way strong alkaline pyrogallol solution, and show that this residual gas is oxygen, since it will practically all be absorbed.

(iii.) Wash out the apparatus and repeat, using 1 c.c. of oxalated plasma and acidifying with 1 c.c. of dilute sulphuric acid before creating the vacuum. Again a definite volume of gas is produced. Introduce a little alkali. The gas (carbon dioxide) is absorbed, leaving none, or but the merest bubble, behind.

Hence plasma carries a distinct amount of carbon dioxide in combination, but practically no oxygen. Oxygen is therefore carried by the cells, but in such a loose state of combination that it is given up to a vacuum.

(iv.) If desired, (ii.) can be repeated, using a 15 per cent. solution of oxyhaemoglobin. The same result as with (ii.) will be obtained, indicating that the *oxygen is actually carried by the haemoglobin of the cells.*

Note. This experiment is designed rather to stress the qualitative differences than the actual quantitative volumes of gas obtainable. Further precautions would be necessary to obtain accurate quantitative data.

DEMONSTRATION.—Illustration of Oxygen Absorption in Tissue Respiration [1]

Experiment 3. Locke's solution approximates in composition and osmotic pressure to the inorganic constituents of blood, with the addition of a somewhat larger amount of glucose than is present in blood. This resemblance facilitates the functioning of tissues suspended in the solution.

Make up a special Locke's solution containing in addition methylene blue (composition: 0·9 gm. NaCl, 0·042 gm. KCl, 0·048 gm. $CaCl_2$, 0·02 gm. $NaHCO_3$, 0·2 gm. glucose and 0·001 gm. methylene blue, dissolved in water and the solution made up to 100 c.c.). Methylene blue is an oxidised compound which fairly easily gives up oxygen, leaving a colourless "leuko-base," and this readily again takes up oxygen from air to produce the original coloured compound. Hence it can be usefully used as an agent for providing oxygen, and loss of its colour indicates that it has given up oxygen.

Prepare three tubes each 20 cm. long, 5 mm. internal bore, and closed at one end. Nearly fill each of these with the Locke's solution. Kill a rat by chloroforming, and imme-

[1] After W. Cramer.

diately dissect out the kidneys, liver and brain, mince these separately, squeeze them between filter paper as free of blood as possible and introduce about ½ gm. of each tissue into the three tubes respectively. By means of a thin glass rod assist the tissue to fall to the bottom of the tubes. Then add a drop of liquid petrolatum to each, sufficient to cover the surface of the fluid and prevent access of air (which cannot pass through this or similar paraffins). Nearly submerge the three tubes in a beaker of convenient size half filled with water at 37° C. Note that in the tube containing kidney tissue about fifteen or twenty minutes are required for complete disappearance of the blue colour; a somewhat longer time is necessary before the contents of the other two tubes become colourless. After decolorisation is complete remove some of the colourless liquid by pipette to a test-tube, shake it with air and note that the blue colour returns.

The tissue cells have abstracted oxygen from the methylene blue without otherwise affecting it. It should have been noticed that the loss of colour is first observed in the region adjacent to the cells, thereafter spreading upwards.

Consumption of oxygen is a function of the tissue cells themselves.

EXERCISE XV

THE COMPOSITION OF THE EXCRETA

(Two or Three-hour Periods)

THERE are five different channels of excretion: the liver, which excretes *bile*, the intestinal mucosa, the lungs, the skin and the kidneys.[1]

The *faeces* contain, along with undigested and unabsorbed food material, excreta from the liver and through the intestinal mucosa, material from the digestive secretions, and bile that has not been absorbed.

Examination of faeces is largely microscopic and non-chemical in nature.

Experiments in Exercise XIV. demonstrated the presence of carbon dioxide in the *expired* air. It is a matter of ordinary observation that expired air contains water vapour. (Water condenses from it immediately on any cold surface.)

Sweat cannot be obtained in large enough amount for class examination.

URINE

The following experiments will, except where otherwise stated, be carried out on mixed human urine recently collected and preserved by addition of a little toluene :—

Toluene is the best preservative of urine, and all urines, tested clinically or otherwise, should be always preserved by addition of toluene unless tests are applied immediately after the urine has been voided.

Experiment 1. Test the reaction of urine to congo red, red and blue litmus, and phenolphthalein.

[1] In connection with this Exercise read Chapter XIV. of Cameron's "Biochemistry," 5th edit., or the corresponding chapter on excreta in some other theoretical text-book.

Urine is never sufficiently acid to turn congo red blue, and only badly decomposed and fermented urine is sufficiently alkaline to turn phenolphthalein red.

Experiment 2. Measure the specific gravity of urine with a urinometer.

The Chief Inorganic Constituents of Urine

Experiment 3. Evaporate about 10 c.c. of urine on the water-bath in a fume-chamber until about 2 c.c. remain. Allow to cool slowly. Crystals of sodium chloride separate. Remove these to a microscopic slide and observe that they are mainly in the forms of small cubes and octahedra. Dry them with a small piece of filter paper; add a drop of distilled water to them on the slide and then single drops of dilute nitric acid and of silver nitrate solution. Note the white precipitate of silver chloride.

Did the sodium chloride pre-exist as such in the urine? Why does it crystallise out before other constituents?

Experiment 4. Add to 5 c.c. of urine in a test-tube 1 c.c. each of dilute nitric acid and dilute silver nitrate solution. Note the precipitate of silver chloride. Add slight excess of ammonia. The precipitate dissolves.

Experiment 5. Add to 5 c.c. of urine in a test-tube 2 drops of concentrated hydrochloric acid. A slight effervescence indicates the presence of carbonate.

Experiment 6. Add to 5 c.c. of urine in a test-tube 2 drops of concentrated hydrochloric acid (to prevent precipitation of phosphates) and 1 c.c. of barium chloride solution. A white precipitate of barium sulphate gradually separates. (It may appear merely as a cloudiness.)

Experiment 7. Test 3 c.c. of urine for phosphate with concentrated nitric acid and ammonium molybdate. A positive result should be obtained.

Experiment 8. The cations present in urine include sodium, potassium and ammonium. Flame tests will indicate sodium and potassium. Ammonium can be demonstrated by making a little urine alkaline with potassium carbonate and distilling, and then testing the **first** portion

of the distillate with Nessler's solution ; a brown coloration will indicate the presence of ammonia.

The Chief Organic Constituents of Urine

It is convenient to study the properties of most of these compounds on the pure compounds themselves, since frequently their concentrations in urine are too small to yield positive results to tests for them. In order of amount present these compounds are—urea, creatinine, uric acid, hippuric acid, oxalic acid, ethereal sulphuric acids (such as phenol- and p-cresol- and indoxyl-sulphuric acid), and in the lower animal sallantoine. Creatine is frequently, but not invariably, present. In bird's urine uric acid is present to a greater extent than urea.

Glucose and albumin are present in certain pathological urines. The amount of glucose in normal urines is not detectable by ordinary procedures.

The various procedures for the *preparation* of urea, uric acid, creatinine, etc., are too time-consuming for a course for medical students.

Urea

Experiment 9. Carry out the following tests with urea crystals :—

(i.) Examine a few crystals of urea *macroscopically* and then under the low power of the microscope. Add a few drops of 95 per cent. ethyl alcohol to the crystals on the slide and observe that they dissolve. As the alcohol evaporates they recrystallise. Sketch them. Compare with the photomicrograph in Plate IV.

(ii.) Place a few crystals of urea in a dry test-tube and heat gently. The urea melts (and the smell of ammonia can perhaps be detected). With further heating the melt solidifies and may partially sublime. Allow to cool, and add 1 c.c. of water, an equal volume of 40 per cent. sodium hydroxide, and a drop or two of very dilute copper sulphate. The deep red colour develops, produced by **biuret** itself.

Biuret is the principal product of the sublimation of urea at a low temperature. The biuret test (compare Exercise V.) *gets its name from this reaction of biuret.*

(iii.) Make up 1 or 2 c.c. of a concentrated solution of urea. Transfer 2 separate drops to different parts of a microscope slide. To 1 drop add a drop of concentrated nitric acid and to the other a drop of saturated solution of oxalic acid. Crystals, characteristically different in form, separate out in each double drop. These consist of urea nitrate and urea oxalate respectively. Sketch them as they appear under the low power of the microscope. Compare with the photo-micrographs of Plate IV. *This is an important characterisation test for urea.*

(iv.) To 3 c.c. of a strong solution of urea add a powdered tablet of **Urease** and keep at 37° C. for ten minutes. Then boil and note the odour of ammonia. Note that moistened red litmus paper held at the mouth of the tube is turned blue. Repeat, using 3 c.c. of water and a powdered urease tablet. No ammonia is liberated.

The enzyme urease decomposes urea with liberation of ammonia. This test is the basis of the present accurate clinical methods of urea estimation.

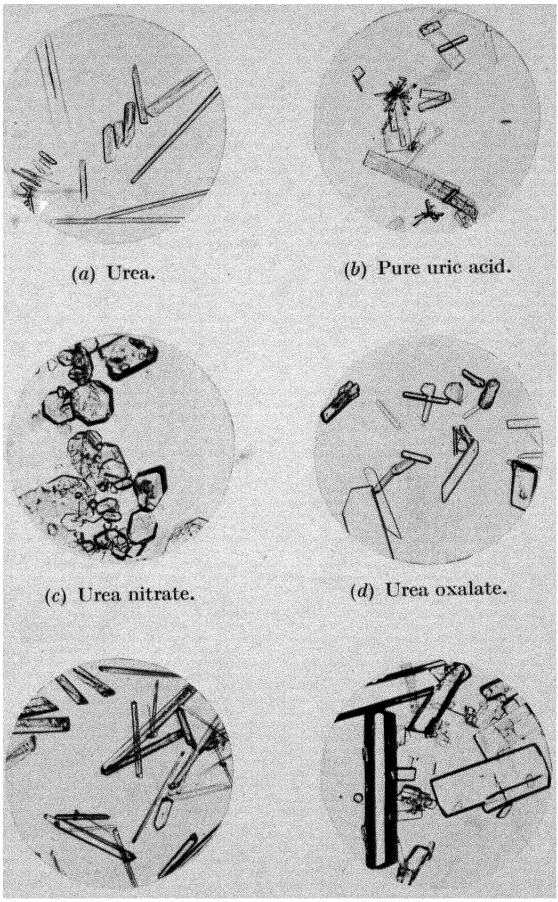
(v.) To 3 c.c. of a strong solution of urea add 2 c.c. of sodium hypobromite (previously prepared by addition of bromine to concentrated sodium hydroxide). Note the strong effervescence due to liberation of carbon dioxide and nitrogen gases. Write an equation for this change. It takes place almost quantitatively, and was largely used for urea determinations prior to application of the more accurate urease methods.

Experiment 10. Evaporate 25 c.c. of urine to 5 c.c. on the water-bath in a fume-chamber, and with this concentrated solution repeat Experiment 9, Tests (iii.), (iv.) and (v.). In carrying out (iv.) make the concentrated urine **just** alkaline to litmus with potassium carbonate solution before adding the urease. The positive results obtained in all three tests indicate the presence of urea in urine.

Uric Acid

Experiment 11. To 20 c.c. of water in a beaker add as much uric acid as will cover $\frac{1}{4}$ in. of a penknife. Warm to boiling and cool. There is no appreciable change. **Uric acid is very insoluble in water.** Now add 10 c.c. of 5 per cent. sodium carbonate, again boil and cool. Most of the uric acid will have dissolved. Pour off some of the clear

PLATE IV



(*a*) Urea.

(*b*) Pure uric acid.

(*c*) Urea nitrate.

(*d*) Urea oxalate.

(*e*) Hippuric acid.

(*f*) Creatine.

Photo-micrographs. (*a*) Urea, × 22·5 ; (*b*) pure uric acid, × 45 ; (*c*) urea nitrate, × 45 ; (*d*) urea oxalate, × 45 ; (*e*) hippuric acid, × 45 ; (*f*) creatine, × 45.

Crystals obtained by the procedures described in the text.

[*To face p.* 100.

liquid and make it just acid to litmus paper with dilute hydrochloric acid. A white precipitate of uric acid slowly separates. Transfer a few drops of the cloudy liquid to a microscope slide and examine under the microscope. Sketch the crystals and note their variability in size. Compare with the photo-micrograph in Plate IV.

Experiment 12. To a trace of solid uric acid in a porcelain evaporating basin add 2 or 3 drops of concentrated nitric acid. Evapo-ate on the water-bath in the fume-chamber until all trace of nitric fumes has ceased. A red or reddish-yellow deposit is left. Add a drop of very dilute ammonia (made by adding 5 drops of concentrated ammonia to a test-tube full of water). A purplish-red or reddish-violet colour is seen. Add a little sodium hydroxide. A blue-violet colour results. This is due to the formation of ammonium purpurate, "murexide," whence the test is known as the **Murexide test** for uric acid.

Experiment 13. Pour a little of the sodium carbonate solution of uric acid prepared in Experiment 11 on to a filter paper moistened with silver nitrate. A black stain is immediately seen, and is due to the formation of metallic silver. This is *Schiff's* test, and can be applied satisfactorily only in the absence of chlorides.

Experiment 14. Pour a little sodium carbonate solution of uric acid into a test-tube and add a few drops of Folin's uric acid reagent (a phosphotungstic acid reagent). A deep blue colour is produced. (This reaction is also given by adrenine.)

Experiment 15. To 5 c.c. of the sodium carbonate solution of uric acid add 2 drops of ammonia and then saturate with ammonium chloride crystals. A white amorphous gelatinous precipitate of ammonium urate separates.

No other organic substance present in biological fluids is precipitated under such conditions except soaps. This property can therefore be used to separate urates from urine. Hence :—

Experiment 16. Saturate 50 c.c. of urine with ammonium chloride (finely powdered crystals). Add 3 drops of strong ammonia and stir. Within a few seconds pour off from the excess of ammonium chloride into a second beaker. A

gelatinous precipitate of ammonium urate separates rapidly. Filter. Scrape the precipitate from the paper, transfer to an evaporating dish, add 3 drops of nitric acid and heat on the water-bath as in Experiment 12. Add successively ammonia and sodium hydroxide. A positive murexide test should result.

Experiment 17. Test 3 c.c. of urine with 2 c.c. of Folin's uric acid reagent. A blue colour should be obtained, due to the presence of uric acid.

Experiment 18. Apply the murexide test to some bird's guano. A positive result should be obtained.

Creatinine

Experiment 19. Dissolve a trace of creatinine in 5 c.c. of water (or take 5 c.c. of a 0·1 per cent. solution). Then add a few drops of a saturated solution of picric acid and then a few drops of 10 per cent. sodium hydroxide. A deep red coloration is produced, due to the production of red creatinine picrate. Add hydrochloric acid. The red colour disappears ; a yellow remains. This is *Jaffé's reaction*.

Experiment 20. To 5 c.c. of a dilute solution of creatinine add 5 drops of freshly prepared sodium nitroprusside, and then a 5 per cent. solution of sodium hydroxide, drop by drop, until a **ruby-red** colour is seen. Boil. The solution turns yellow. Add strong acetic acid. The colour becomes greenish-yellow, and a precipitate of Prussian blue may form (*Weyl's reaction*).

Experiment 21. Repeat Jaffé's and Weyl's tests, using for each 5 c.c. of urine. Exactly the same results should be obtained as with the creatinine solution, indicating that creatinine is a constituent of urine.

Creatine

Experiment 22. Apply Jaffé's test to a dilute solution of creatine. The result is negative.

Then heat 5 c.c. of creatine solution with 1 c.c. of dilute hydrochloric acid on the water-bath for ten minutes. Cool, add sodium hydroxide drop by drop until the solution is just alkaline to litmus paper, again cool, and repeat Jaffé's test.

The result is now positive. Creatine has been converted to creatinine.

Hippuric Acid

Experiment 23. Examine some hippuric acid crystals under the microscope and sketch their appearance. Compare with the photo-micrograph in Plate IV.

Experiment 24. To a few crystals of hippuric acid in a dry evaporating dish add 1 c.c. of concentrated nitric acid and evaporate to dryness on the water-bath in a fume-chamber. Transfer the dry residue to a test-tube and warm gently. The odour of nitrobenzene should be observed (similar to that of oil of bitter almonds).

Experiment 25. Heat a few crystals of hippuric acid in a dry test-tube. The crystals melt, solidify upon cooling, and on further heating turn red. Finally a white sublimate of benzoic acid is produced, hydrocyanic acid and benzonitrile being also formed (note the odour).

These experiments are in agreement with the fact that hippuric acid is benzoyl glycine, C_6H_5. CO . NH. CH_2 . COOH.

Ethereal Sulphates

Experiment 26. To detect the sulphate radical of ethereal sulphates, to 5 c.c. of urine add 1 c.c. of concentrated hydrochloric acid and 1 c.c. of barium chloride solution. Then filter off the barium sulphate formed from the inorganic sulphate of urine (*cf.* Experiment 6). Warm the filtrates. A further precipitate of barium sulphate gradually forms, as the acid hydrolyses the ethereal sulphates, liberating more inorganic sulphate.

One of the most important of the ethereal sulphates present in urine is indoxyl-sulphuric acid, and the amount of it in a sample of urine gives some clue to the extent of bacterial action going on in the intestine. The following tests can be employed for this compound, **Indican** :—

Experiment 27. Take 5 c.c. of urine in a test-tube, and add 5 c.c. of concentrated hydrochloric acid. Then add

2 c.c. of chloroform, and 2 or 3 drops of a strong solution of "bleach" (calcium hypochlorite). Close the tube with the thumb, and shake thoroughly. Allow the chloroform to separate. It will frequently be coloured a light or a deep shade of blue, varying with the amount of indican originally present, and due to the production of **Indigo** by oxidation of the indican by chlorine. Occasionally the colour will be red, due to formation of indigo-red. (With normal urine, however, the test is not infrequently negative.) This is *Jaffé's reaction for indican.*

Experiment 28. Shake up 10 c.c. of urine with 1 c.c. of a 5 per cent. solution of thymol in 95 per cent. alcohol. Add 10 c.c. of 0·5 per cent. solution of ferric chloride in fuming hydrochloric acid, again shake, and allow to stand for fifteen minutes. Add 4 c.c. of chloroform and shake gently and repeatedly. The chloroform layer develops an intense violet colour. This test (*Jolles' test*) is very delicate.

PATHOLOGICAL CONSTITUENTS OF URINE

Glucose

Experiment 29. Test a diabetic urine for glucose with Benedict's and Fehling's solution. The results are positive. Carry out an osazone test on the urine. Glucosazone crystals should be obtained.

Albumin

Experiment 30. Test the urine from a case of nephritis for albumin by the nitric acid method. A positive result should be obtained.

Try the coagulation test by nearly filling a test-tube with the **filtered** urine and warming the upper portion of the liquid in the tube gently. A varying degree of opalescence will develop. Add a drop of dilute acetic acid. The cloudiness persists. Normal urine will frequently give a similar result on warming, due to phosphates. These, however, redissolve on adding the acetic acid.

"Acetone Bodies"

Experiment 31. (i.) *Legal's Sodium Nitroprusside Test.*—Add a few drops of freshly prepared sodium nitroprusside solution to 5 c.c. of a diabetic urine in a test-tube and *then*

a little sodium hydroxide solution. A ruby-red colour will develop in presence of acetone. Creatinine also gives this result, if present in sufficient amount. Add excess of acetic acid. In presence of acetone the red colour is intensified, but if it is only due to creatinine it disappears, leaving a yellow colour (*cf.* Weyl's test).

(ii.) *Gerhardt's Test.* Add to 5 c.c. of urine in a test-tube dilute ferric chloride solution, drop by drop, until no more precipitate (ferric phosphate) is produced. Filter. In the presence of acetoacetic acid the filtrate is a Bordeaux wine-red in colour.

(iii.) *Le Nobel's Test.* Acidify 5 c.c. of urine with dilute acetic acid, add a few drops of freshly prepared sodium nitroprusside solution and pour in carefully concentrated ammonia to form an upper layer. A violet ring forms in presence of either acetoacetic acid or of acetone.

Note. Further experiments on pathological urines are given in Exercise XXV.

EXERCISE XVI

EXAMINATION OF UNKNOWN COMPOUNDS OF BIOCHEMICAL IMPORTANCE

(One or more Three-hour Periods)

No simple scheme of examination can cover any but the most important of these compounds. However, it is desirable that from time to time the student should refresh his memory concerning their properties by studying them from the point of view of analysis, which should better enable him to pick out their outstanding distinguishing characteristics.

The examination of an unknown permits utilisation of all physical characteristics, appearance, solubility, taste, etc. The colour of a solution, if any, should give a clue, although methods of colouring a solution in order to make analysis more difficult are not unknown. These are perhaps not altogether desirable (unless the colouring material added may occur naturally along with the compound that is being tested). **Pure water is one of the most misleading compounds to investigate.**

The student should work out some scheme for himself, utilising the notes that have already been given on the examination of unknown carbohydrates and proteins. The following additional notes should be consulted in drawing up such a scheme :—

Solubility is an essential distinguishing feature. The important solvents to consider are water, alcohol, ether, 1 per cent. sodium chloride, dilute alkali and dilute acid. These need not be employed in that order. The **appearance** of the substance, its crystalline or amorphous character, presence or absence of a greasy appearance, all should help to determine the treatment.

If a solution is distinctly acid or distinctly alkaline it should be at once neutralised, to ascertain if the acidity or the alkalinity is necessary for the solubility of whatever is present. *E.g.*, such neutralisation of an alkaline solution will precipitate casein, uric acid and cystine.

Molisch's Test should be employed at a very early period in the scheme, since a positive result strongly suggests the presence of carbohydrates or their derivatives (including glucoproteins), while if it is negative these are excluded.

The **Biuret Test** should also be employed early in the analysis, to detect or to exclude proteins and their derivatives (but not amino-acids).

Fats should present no difficulty through their characteristic appearance and solubilities. A positive acrolein test is necessary to distinguish them from fatty acids, but it of course does not necessarily indicate a fat.

Absence of any of the characteristic group tests necessitates employment of a number of single tests for amino-acids, urea, glycerol, creatinine, etc.

If difficulties are encountered with **colourless** solutions in water, which seem to give only negative results, it would seem obviously desirable to concentrate the solution to one-third or one-fifth its volume and repeat the tests.

Common sense is one of the best things to employ in work of this nature.

PART II

QUANTITATIVE AND CLINICAL PROCEDURES [1]

EXERCISE XVII

CLEANING, CARE AND STANDARDISATION OF APPARATUS FOR QUANTITATIVE WORK

(Two Three-hour Periods)

EACH student will require, as a minimum, the following graduated apparatus :—

Pipettes, 5, 10 and 25 c.c.

Graduated flask, with one mark (graduated for intake) at 100 c.c.

Burette with glass stopcock, 50 c.c., graduated to 0·1 c.c.

Additional apparatus for special experiments should be issued as required.

The student should see that the apparatus is in good condition, that the tips of pipettes and burette are unbroken, that the glass stopper of the flask and the tap of the burette fit properly, and that when this tap is properly greased and closed and the burette filled with water there is no leakage.

Greasing of Tap. Either vaseline or a special tap-grease [2]

[1] The second section of this book is intended for the training of medical students in certain of the simpler and more commonly used procedures of clinical chemistry. It is in no sense a manual of clinical chemical methods, and for information needing such a manual texts such as the following should be employed : Harrison, " Chemical Methods in Clinical Medicine," 2nd edit. (London, Churchill) ; Mattice, " Chemical Procedures for Clinical Laboratories " (Philadelphia, Lea and Febiger).

[2] Such a tap-grease can be made by heating together in a porcelain crucible on a sand-bath equal amounts of soft, black rubber and paraffin wax with twice the amount of vaseline, stirring until the rubber is dissolved.

can be used. Too much should be avoided. After greasing most of the surface of the **dry** barrel introduce this into the **dried** bore, and turn carefully until the whole of the inner surface is greased. Make sure that the bore of the barrel of the tap is not occluded. If grease gets into it beyond the smallest trace remove the barrel and remove the grease from its bore with a pin, then reinsert the barrel and again turn carefully till all streakiness has disappeared.

The Cleaning of Apparatus

All glass apparatus used for quantitative work requires proper cleansing of the interior surfaces to remove dirt. If dirt is present drops of liquid adhere, and the results obtained with such dirty apparatus are inaccurate.

CLEANING SOLUTION. Either concentrated sulphuric acid or else concentrated sulphuric acid saturated with potassium bichromate (chromic acid mixture) should be employed. A stoppered bottle containing at least 60 c.c. should be issued to each student. When not in use this should be kept stoppered. The solution can be used repeatedly, and should not be discarded (since it is moderately expensive), but poured back into the bottle for further use until either repeated use has appreciably diluted it with water or the solution is commencing to turn green (through reduction of the bichromate), when it is no longer an efficient oxidising agent.

In using this solution first rinse out the glassware that is to be cleaned with tap-water, then fill pipettes or burette and allow to stand filled for a few minutes. Thoroughly wash the flask, making certain that every part of the interior surface is in repeated contact with the cleaning mixture.

Be very careful that all water is drained off from the neighbourhood of the tap of the burette **before** adding the cleaning mixture. The mixing of concentrated sulphuric acid and water engenders great heat, sufficient to crack stop-cocks, and such damage is invariably due to carelessness.

Drain off the cleaning mixture into its special bottle, and when drainage is complete (note the above remark about heat, water, and sulphuric acid) rinse with tap-water and finally with distilled water. After all water has drained away, if any drops remain on the surface cleaning has been insufficient, and the process must be repeated.

Unless the apparatus is to be used immediately, cork, or

cover inlets with filter paper, and lay aside carefully until it is wanted.

The apparatus, once thoroughly cleaned, should be rinsed out with distilled water each time before use, but need not be again cleaned with the cleaning mixture until when, after rinsing, residual drops of water remain clinging to the sides, indicating that dirt has again accumulated.

Standardisation of Apparatus

All graduated glass apparatus, unless purchased with a certificate of accuracy from some bureau of standards, should be standardised before it is used for accurate work. Because a piece of apparatus has had certain marks engraved upon it by the manufacturers, it by no means follows that these truly indicate what they are supposed to.[1]

Standardisation is carried out by measuring volumes of liquid in the apparatus that is to be standardised, noting the temperature, and weighing the measured volume.

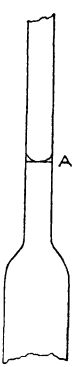


FIG. 9.

Standardisation of Pipettes. Weigh a small beaker containing some petrolatum or other paraffin oil. Fill the pipette with distilled water so that the lower curve of the meniscus coincides with the mark on the pipette, as at A, Fig. 9. Then drain off (without blowing through) into the beaker, being careful that the tip of the pipette is in contact with the side of the beaker just *above* the surface of the oil. After all the water that can has drained off, blow through once. Weigh the beaker again. Note the temperature. The difference in weight is the weight of the water from the pipette. From the table at the end of this Exercise calculate the volume to which this weight corresponds at the temperature noted. The volume found is the true volume of the pipette.

Note. Various methods can be employed when draining

[1] Recently it has become fashionable to graduate apparatus in terms of *millilitres* rather than cubic centimetres, since 1 litre (defined as the volume of 1,000 gm. of water at maximum density) is not exactly equal to the volume of a cube of 10 cm. side (1,000 c.c.), but equals 1000·027 c.c. It is obvious that the difference between one *ml.* and one c.c. is negligible for all ordinary chemical (and biochemical) measurements.

the pipette. It can be blown through not at all, or once, or twice. Each method will remove a different amount of water. **Whichever method is employed for standardisation, that method must be used whenever the pipette is subsequently employed for quantitative work.**

Example. The 5 c.c. pipette is taken and treated as described. The temperature noted is 17° C. The weight of the beaker with oil is initially 10·847, and after adding water from the pipette 15·825 gm., so that the difference is 4·978 gm. At 17° the volume of 1 gm. of water is 1·0023. Hence multiply 4·978 by 1·0023. The result is 4·99 c.c., which is the true value of that pipette under the conditions used for standardising. Each time the pipette is used 4·99 c.c., and not 5·00 c.c., will be taken.

Alternative Method of standardising Pipettes. Partially fill the burette with distilled water. Read accurately to the hundredth of a cubic centimetre (see p. 112, in the paragraph entitled " Reading the Burette "). Fill the pipette to be standardised with water to the meniscus, as described on p. 110. Drain into the burette and blow through once. Take a second reading. The difference gives the volume of the pipette provided the burette reads accurately. If the burette has a correction factor, then the figure found must be multiplied by this factor.

Example. The burette reading (from above downwards) is 43·54 c.c. Water is run in from a 10 c.c. pipette, and then the reading is 33·62, so that the volume of the pipette *in terms of the burette* is 9·92. But it has been ascertained that 50 c.c. in this particular burette has a true volume of 51·17 c.c., so that its factor is 1·023. Multiply 9·92 by this factor, and the true volume of the pipette, 10·15 c.c., is found.

Standardisation of a Burette. Weigh a beaker containing some oil. Fill the burette with distilled water to the 50 c.c. mark (see next section), taking care that there are no bubbles of air below the stopcock. Run off 50 c.c. into the beaker rapidly, and close the stopcock. The water left on the sides of the burette slowly drains down. After two minutes run off exactly to the 50·00 mark. Weigh the beaker again, noting the room temperature (or the temperature of the water, if water colder than room temperature is used). From the difference in weight calculate the volume of water. This is the true volume of the burette. If different from

50·00 c.c., the necessary correction must be made whenever the burette is used.

Note. Burettes are very seldom of equal internal diameter throughout, since they are made from drawn tubing. If time permits, a burette should be standardised for each 5 c.c. throughout its graduated length, or, still more preferably, for each 2 c.c.

Example. The beaker and oil are found to weigh 32·47 gm. After addition of water from the burette the weight is 82·47 gm. The bench temperature is 15° C. The correct burette volume is therefore 50·00 multiplied by 1·0019, or 50·10 c.c. The burette factor is 1·002, and volumes measured by that burette must be multiplied by this factor.

Reading the Burette. The lower surface of the meniscus of liquid in the burette will usually lie between two of the 0·1 c.c. graduation marks. Estimate with the eye to the nearest 0·01 c.c. when making a reading (see Fig. 10, in which the meniscus reads at 26·36). When the meniscus is coincident with a mark, this coincidence must be indicated in the reading. *E.g.*, 27·1 is incorrect for such a reading, which should be written 27·10 c.c.

Standardisation of a Graduated Flask. Clean and dry the flask. (Draining for some hours will give a sufficient approximation to dryness.) Run in water from the burette until the meniscus coincides with the mark on the flask, remembering that time must be allowed for drainage from the walls of the burette. The measured amount of water (corrected for burette error if necessary) gives the true volume of the flask.

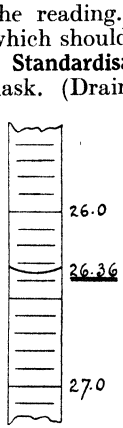


FIG. 10.

Example. To fill the 100 c.c. flask to the mark the burette had to be used three times, the total volume supplied being the sum of 50·00, 50·00 and 1·12 c.c., *i.e.*, 101·12 c.c. The burette had a factor of 0·997. Multiplying by this factor, the true volume of the flask is found to be 100·82 c.c.

Change of Volume of Water with Temperature. This is shown in the following table, which covers the usual range of room temperatures, and corrects for the differences of air-displacement of the water and the brass weights employed :—

Temperature ° C.	Volume of 1 gm. of water in c.c.	Temperature ° C.	Volume of 1 gm. of water in c.c.
10	1·0013	20	1·0028
11	1·0014	21	1·0030
12	1·0015	22	1 0033
13	1·0017	23	1·0035
14	1 0018	24	1·0037
15	1 0019	25	1·0040
16	1·0021	26	1·0042
17	1·0023	27	1·0045
18	1·0024	28	1·0048
19	1 0026	29	1·0051

Note. If, subsequent to standardisation, the tip of the burette or of any pipette is broken, the damaged apparatus cannot thereafter be used for quantitative work.

Note on the Required Degree of Accuracy in Quantitative Work

Most of the volumetric procedures which will be outlined in this course permit an accuracy of within 1 per cent. If, *e.g.*, the glucose present in a diabetic urine is 2·04 per cent., the result found should be either 2·02, 2·03, 2·04, 2·05 or 2·06 per cent., but should not exceed the limits of these figures. The colorimetric procedures are perhaps not quite so accurate, but at any rate permit an accuracy of within 2 per cent.

The extent to which corrections must be applied for apparatus, factors for standard solutions, etc., in order to obtain this degree of accuracy, is a matter of acquired judgment. A pipette which delivers 9·99 c.c. instead of 10·00 c.c. introduces an error of 0·1 per cent., which is generally negligible, so that a correction is unnecessary. A pipette which delivers 4·95 c.c. instead of 5·00 c.c. introduces a 1 per cent. error, obviously not to be disregarded.

Results should not be expressed in terms greater than the possible accuracy warrants. An estimation of glucose with a possible accuracy of 1 per cent. should not be stated, *e.g.*, as 2·0357 per cent., but at most as 2·036 per cent., while for most purposes 2·04 per cent. is adequate. The examples given in the following exercises afford further illustrations.

EXERCISE XVIII

THE ESTIMATION OF NITROGEN BY KJELDAHL'S METHOD [1]

(Two Three-hour Periods)

THIS method is accurate for biological fluids including urine, although it does not give accurate results with all organic compounds containing nitrogen. Preliminary heating with concentrated sulphuric acid converts nitrogen to ammonia, while the subsequent boiling with strong alkali liberates the ammonia, which, distilled off, is collected in a known volume of standard acid, and finally the amount of free acid left is determined by titration against alkali.

Experiment 1. Make up accurately a 2 per cent. solution of pure urea. Pipette accurately 5 c.c. of this solution (or whatever the 5 c.c. pipette actually delivers) into a 500 c.c. long-necked Kjeldahl flask.

Add 5 c.c. of concentrated sulphuric acid and a small pinch of powdered crystallised copper sulphate. Heat in the fume-chamber above a small Bunsen flame for forty minutes.

Note. Most biological fluids will char on adding the concentrated acid, and are then heated until clear. The urea solution does not char. Finally all solutions subjected to this treatment should be coloured a faint blue, due to the copper, or will be colourless.

After the forty minutes' heating, cool, add 200 c.c. of water carefully (water plus concentrated acid gives heat), then a little pumice or talc, and again cool if necessary. Then add carefully 80 c.c. of 40 per cent. sodium hydroxide. When nearly all the alkali has been added a faint precipitate (of

[1] Kjeldahl described his method in the *Zeitschr. anal. Chem.* in 1883. It has been modified in various ways, but the principle has remained unaltered.

copper hydroxide) should be seen. This may dissolve in excess of alkali to a blue solution.

Note. **Before** the alkali is added the flask should be connected with a long, bent tube (or a tube leading into a condenser) by a single-holed cork. The cork should be removed during addition of alkali, and **immediately** replaced as otherwise loss of ammonia may occur. A special trap is

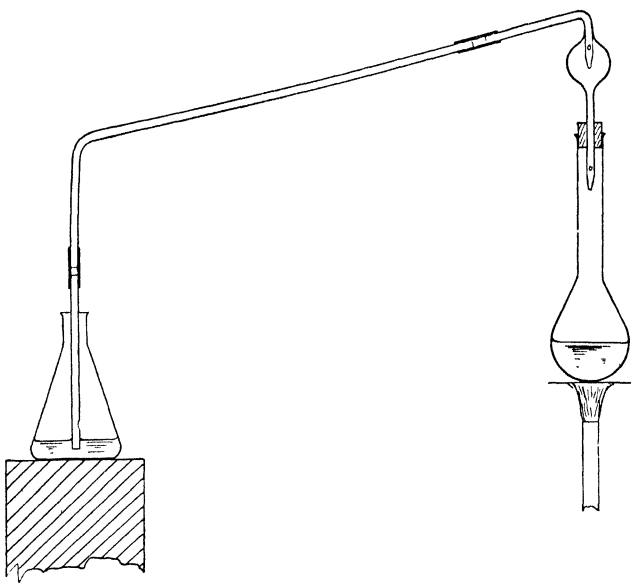


FIG. 11. Kjeldahl distillation apparatus for nitrogen determinations.

inserted in the distilling apparatus, to prevent spray passing over. A glass tube is joined by rubber tubing to the end of the delivery tube (or to the end of the condenser) and dips below the surface of 50 c.c. of N/10 sulphuric acid in a 250 c.c. Erlenmeyer flask. This acid is coloured with 3 drops of a solution of methyl red (0·2 per cent. in 95 per cent. ethyl alcohol). The arrangement is shown (without the condenser) in Fig. 11.

Heat the flask with a medium-sized flame and distil for

thirty to forty minutes, until about half the contents of the flask has distilled over. Disconnect. Wash the delivery tube with distilled water, allowing the washings to fall into the collecting flask. Then titrate the excess of N/10 acid against N/10 sodium hydroxide.

Note. When determinations are being made on unknown solutions it may happen that insufficient acid is taken for the distillation. In that case the methyl red will change colour during the distillation (to yellow). This should be watched for, and at the first sign of colour change another 25 c.c. of acid should be added to the Erlenmeyer flask, otherwise the determination will be ruined.

Note on Standard Acid and Alkali.[1] It is possible to obtain by manipulation exactly tenth-normal acid and alkali. This procedure is time-wasting and unnecessary except where many routine analyses have to be performed. A more scientific procedure is to make up approximately tenth-normal solutions, then standardise the alkali against pure oxalic acid (weighing out an accurate amount of pure oxalic acid crystals), using the same methyl-red indicator, then standardise the acid against the standardised alkali. In each case a factor will be obtained the use of which converts the actual titrimetric reading into its equivalent for true N/10 solutions.[2]

Example. Factor for N/10 acid used, 0·987; for N/10 alkali, 1·114; 50 c.c. of acid taken and 14·87 c.c. of alkali required. The amount of acid taken is equivalent to 49·35 c.c. of N/10 acid; the amount of alkali taken is equivalent to 16·57 c.c. of N/10 alkali. Hence 32·78 c.c. of N/10 acid was used up in neutralising the ammonia, and this acid is equivalent to 32·78 c.c. N/10 ammonia, which is equivalent to 32·78 c.c. of N/10 nitrogen. An N/10 solution of nitrogen

[1] A *molar* solution contains the molecular weight in grams of the dissolved reagent in 1 litre of solution. A *normal* solution contains 1 *gram-equivalent* of the reagent in 1 litre of solution, a gram-equivalent being the amount of the reagent capable of reacting with or being substituted for 1 gram-atom (1·008 gm.) of hydrogen. Hence equal volumes of solutions of the same degree of normality are equivalent to each other. *E.g.*, 5·0 c.c. of N/10 hydrochloric acid is equivalent to 5·0 c.c. of N/10 sodium hydroxide (and is exactly neutralised by it).

[2] A solution in which has been dissolved exactly 6·3 gm. of pure crystallised oxalic acid in 1 litre is exactly tenth-normal. Approximately N/10 solutions of acid and alkali are obtained by dissolving, each in a litre of water, 2·8 c.c. of concentrated sulphuric acid, and 4 gm. of pure stick sodium hydroxide.

theoretically should contain 14/10 gm. per litre, *i.e.*, 0·0014 gm. per cubic centimetre.

Hence $32{\cdot}78 \times 0{\cdot}0014 = 0{\cdot}0459$ gm. of nitrogen distilled over as ammonia, and this 0·0459 gm. of nitrogen was present in 5 c.c. of the urea solution, whence 100 c.c. of the urea solution contained 0·918 gm.

The theoretical amount in 100 c.c. of 2 per cent. solution is $2 \times 28/60$ gm. (since the molecular weight of urea is 60, and the weight of the nitrogen in it is 28), which is 0·933 gm., and the error is therefore

$$-\frac{15}{933} \times 100 \text{ per cent.} = -1{\cdot}6 \text{ per cent.},$$

just a little too great for an accurate determination.

Note especially that in the above estimation, unless careful watch is kept during distillation, if the flame of the burner fluctuates there is strong risk of acid being sucked back from the Erlenmeyer flask into the Kjeldahl flask, in which case, of course, the determination is spoiled and repetition is necessary.

Experiment 2. To be carried out during the subsequent examination of a twenty-four hours' specimen of urine (see Exercise XXXIV.).

Repeat as in Experiment 1, taking 5 c.c. of urine and 10 c.c. of concentrated sulphuric acid, and after the necessary heating, cooling and dilution adding 60 c.c. of 40 per cent. sodium hydroxide. The results can be expressed either per 100 c.c. of urine or preferably, when a twenty-four hours' sample is analysed, in terms of the total twenty-four hours' output.

Experiment 3. Determination of Nitrogen in Milk. (To be carried out if and when time permits.)

Take 5 c.c. of milk, 20 c.c. of concentrated sulphuric acid, and subsequently 120 c.c. of 40 per cent. sodium hydroxide, proceeding otherwise as in Experiment 1.

MICRO-KJELDAHL DETERMINATION OF NITROGEN

When only small amounts of material are available for nitrogen analysis, it is usually carried out with micro-technique. The following Experiment exemplifies usefully the relation of such technique to that on a larger scale, and illustrates a method that is now frequently used in bio-chemical work.[1]

[1] *Cf.* Pregl-Fyleman, "Quantitative Organic Microanalysis," p. 94 (London, J. and A. Churchill, 1924).

Experiment 4. Prepare a 0·2 per cent. solution of urea by pipetting 10 c.c. of the 2 per cent. solution (*cf.* Experiment 1) into a 100 c.c. volumetric flask, and filling to the mark with distilled water. Transfer accurately 1 c.c. of this dilute solution to a pyrex micro-Kjeldahl distillation flask (A in Fig. 11A), add 1 c.c. of concentrated sulphuric acid, a pinch of copper sulphate, and a crystal of potassium sulphate, and set up in a fume chamber over a small burner. After the addition of a small glass bead (to prevent bumping), apply heat, gently at first until the water has been driven off, and

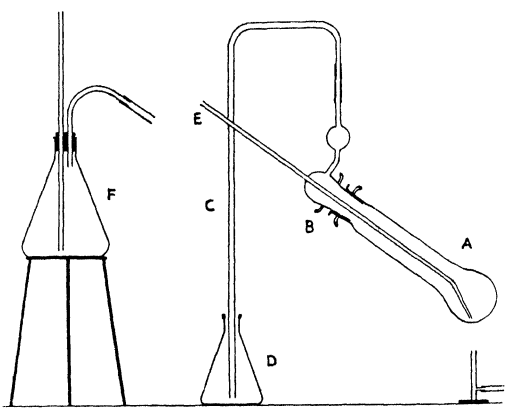


FIG. 11A. Diagrammatic sketch of micro-Kjeldahl apparatus for nitrogen determinations.

then adjust the heating so that the liquid boils steadily but not too vigorously for about ten minutes. Decomposition is then generally complete.

Note. With substances relatively poor in carbon after the solution has become clear it is often advantageous to add a few drops of alcohol or a small pinch of sucrose, and then to continue the heating until the solution is clear again.

Allow the flask to cool, add 5 c.c. of distilled water, and connect up as shown in Fig. 11A. The head-piece, B, which fits into the mouth of the flask A by means of a ground-glass connection, if connected to a vertical air condenser *C*, the end of which dips under the surface of 10 c.c. of N/70 sulphuric acid in the Erlenmeyer flask receiver D.

Note. All parts of the apparatus (except the very short rubber connections) should be made of pyrex glass. If a pyrex tube is not available as air condenser, a small water-jacketed condenser should be used.

Introduce into the distillation flask **A** 6 c.c. of 40 per cent. sodium hydroxide by means of a small filter funnel connected to tube **E** by a short length of rubber tubing. Remove the filter funnel and connect **E** immediately to a previously-heated steam generator **F**, and at the same time start heating the contents of the distilling flask with a micro-burner. This heating must be carried out throughout the distillation, since steam distillation alone is insufficient to drive all the ammonia over into the receiving flask. Continue distillation until deposition of salts causes bumping, then lower the receiving flask until the end of the condenser tube is about 1 cm. above the level of the sulphuric acid in the flask, and allow the steam distillation to proceed for another minute. Then disconnect the receiving flask and titrate the excess of sulphuric acid with N/70 sodium hydroxide from a 10 c.c. micro-burette graduated to one-hundredths of a cubic-centimetre, using methyl red as indicator. Interrupt the titration when almost at the end point, boil the contents of the flask to remove carbon dioxide, cool, and complete the titration.

Note. The N/70 acid and alkali can be conveniently prepared by appropriate dilution of the N/10 solutions used in Experiment 1.

Calculation. The titration figure subtracted from 10 c.c. (the amount of acid taken) gives the amount of acid used up in combining with the ammonia distilled over. Since 1 c.c. of N/70 acid is equivalent to 0·2 mg. of nitrogen, the amount of acid used up multiplied by 0·2 gives the number of milligrammes of nitrogen.

Note. A blank determination should be carried out, employing all the reagents required by the method, and the blank value subtracted, before calculating the nitrogen content of the sample.

Example. The titration required 5·345 c.c. The acid used was therefore 10·000 − 5·345 equal to 4·655 c.c., representing 4·655 × 0·2, equal to 0·931 mg. nitrogen.

The corresponding figures with the blank determination gave the value 0·015 mg. nitrogen.

Hence the true amount of nitrogen present in the material

taken for analysis was 0·931 − 0·015 = 0·916 mg. This was present in 1 c.c. of solution, so that 100 c.c. contained 91·6 mg.

The theoretical value is 93·3 mg., whence the error is − 1·8 per cent.

Note. In carrying out this determination with urine, it should be diluted 1 to 10, and 1 c.c. of the diluted urine used.

EXERCISE XIX

THE COLORIMETER AND THE ESTIMATION OF CREATININE AND CREATINE IN URINE

(Two Three-hour Periods)

The Colorimeter

THIS, as its name implies, is a colour measurer. The colour is not measured absolutely, but matched. Two solutions of exactly the same shade, but of different depths of colour, are matched by adjusting the lengths of the columns of liquid through which the light is passing until the depth of colour is the same in the two. This adjustment is brought about by raising cups containing the solutions so that clear glass " plungers " are immersed to desirable depths. Light passes from a mirror up through the cups, through the liquid, becoming thereby coloured, then through the plungers and through prisms to the eye. The eye sees a circular field, one half of which has come through one solution, the other half through the second solution.

The essence of a colorimetric determination consists therefore in converting the substance under examination into a coloured compound (or causing it to take part in some chemical reaction by which a coloured compound is produced) and comparing it with the same coloured compound made up from a standard solution of the same substance. Since the colour seen by the eye at any point in the field of vision is due to a linear column of coloured molecules corresponding to that point, then if the two halves of the field match, but the lengths of the columns of liquid through which the light has passed differ, in these lengths of liquid there must be the same number of coloured molecules, so that it follows that *the concentrations of coloured molecules in the*

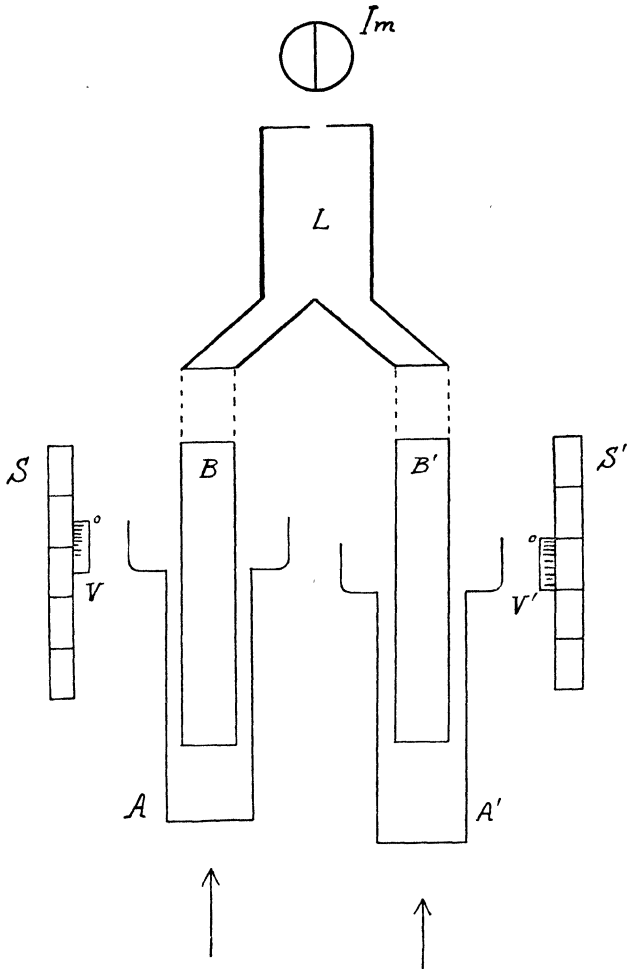


FIG. 12. Schematic diagram of a colorimeter. A, A′, movable cups ; B, B′, fixed plungers ; S, S′, fixed millimetre scales ; V, V′, verniers moving synchronously with A and A′ respectively ; L, system of lenses ; Im, field of vision as seen.

The zero of the vernier gives a reading on its fixed scale of the distance between the bottoms of the corresponding plunger and cup.

solutions will be inversely proportional to these lengths. A skeletal arrangement of the essential parts of the colorimeter is shown in Fig. 12.[1] [2]

Estimation of Creatinine in Urine

The method employed is due to Folin,[3] who applied the Jaffé reaction (see p. 102),[4] and through this procedure was the first to make colorimetric methods easily and accurately applicable to metabolic investigations.

The method should first be demonstrated to groups of students, and used at the same time to explain the principle of the colorimeter. Students should then carry out a determination individually or in pairs.

Experiment 1. Pipette out accurately 1 c.c. (or whatever the 1 c.c. pipette really measures) of urine into a 100 c.c. graduated flask, add 10 c.c. (measured approximately in a graduated cylinder) of a saturated solution of picric acid,[5]

[1] There are now many good colorimeters on the market, and to distinguish between them is somewhat invidious. In this laboratory we have found Klett's biocolorimeter, the Bausch and Lomb "Biological" and "Micro-" and the Spencer colorimeters, all capable of giving good results. Cheap colorimeters are to be avoided. Cups of "all-fused" glass are preferable.

[2] Kober and Bloor have extended the use of the colorimeter to measure the concentration of a substance present in particulate form (as a fine and uniform suspension). In the adapted colorimeter, used as a *nephelometer* (cloud-measurer), light is not transmitted through the liquid and suspension, but is directed on to them at right angles to the line of vision, and reflected upwards by the particles. The principle has been adapted for numerous determinations, *e.g.*, that of fats in milk and blood (Bloor, *J. Biol. Chem.*, 1914, XVII., 377 ; *J. Am. Chem. Soc.*, 1914, XXXVI., 1300).

[3] *J. Biol. Chem.*, 1914, XVII., 469.

[4] The chemistry of the reaction has been elucidated by Greenwald, *J. Am. Chem. Soc.*, 1925, LXVII., 1443 ; *J. Biol. Chem.*, 1928, LXXX., 103.

[5] Creatinine determinations require the use of pure picric acid. S. R. Benedict (*J. Biol. Chem.*, 1922, LIV., 239 ; 1929, LXXXII., 1) has devised several simple methods of purifying the moist commercial preparations. We have found the older method of Halverson and Bergeim to give excellent results. Add 50 gm. of the crude acid to 700 c.c. of water and dissolve with aid of heat. Add 10 c.c. of concentrated hydrochloric acid to the boiling liquid. Cool, decant from the crystals, wash by decantation with 100 c.c. of distilled water, and then repeat the recrystallisation. Filter finally through a Buchner funnel, and wash with 150 c.c. of distilled water. Dry between filter paper.

and 1 c.c. (measured in an accurate pipette) of a 10 per cent. solution of sodium hydroxide. (The ordinary bench reagent is 10 per cent.) Shake up and allow to stand for ten minutes in order to allow the maximum colour to develop. Then add distilled water to the 100 c.c. mark, stopper, and shake up thoroughly, and the solution is ready for comparison.

Simultaneously prepare the standard by taking 1 c.c. of a standard solution of creatinine (containing 1 gm. of pure creatinine in 1 litre of N/10 hydrochloric acid, and therefore 1 mg. in each cubic centimetre),[1] transferring this to a 100 c.c. flask, adding picric acid and sodium hydroxide as before, waiting ten minutes, and then diluting to the mark and shaking.

In this way two orange-red solutions are obtained, usually somewhat differing in intensity, but of precisely the same shade of colour.

Fill each one of the colorimeter cups with one of the two solutions. In filling rinse out first with the solution that is to be placed in the cup (unless the cup was perfectly dry and clean, which it usually is not). Fill only to where the cup flanges outwards (or only half fill where there is no such flange), so that when the plunger is deep in the cup liquid is not forced out and spilled.

It is usually preferable to place the standard solution always either at the right or at the left, to avoid alternation and confusion. Since some colorimeters have a rack and pinion adjustment for the right cup only, make a rule of using the left one for the standard.

The plunger should be clean and dry. If it is not, clean it by immersing in distilled water and drying with a towel or with filter paper, or preferably, when aqueous solutions are being used, when rinsing out the cup immerse the plunger in the solution to be tested also, so that it is wet with that solution.

Set the standard at such a convenient level that there is a reasonable depth of colour. Note that *the scale corresponds to the* (vertical) *distance from the bottom of the plunger to the bottom of the cup, i.e., to the length of liquid through which the light passes on its way to the eye*, also that the zero of the vernier scale (which moves upwards as the cup moves

[1] Creatinine zinc chloride is easily purified by recrystallisation from hot water; 1·603 gm. per litre is equivalent to 1 mg. creatinine per cubic centimetre.

upwards) gives on the fixed scale the accurate reading. (If the student is unaccustomed to reading with a vernier, the principle should be thoroughly explained to him.)

For creatinine solutions a convenient depth is 15 mm., but 20 mm. can be taken if desired.

Raise or lower the cup containing the unknown solution, using the screw adjustment until the colour matches. Take a reading. Then move this cup and again adjust it to equal colour and take a second reading. Repeat this four times and take the mean. (This is necessary, since the eye tires rapidly, especially with certain colours, and the error of reading is therefore materially reduced by taking such an average.)

The nature of the calculation can best be seen from an example :—

Example. Standard set at 15 mm. Readings of urine unknown, 13·2, 13·0, 13·3, 13·2. Average reading, 13·2 mm.

$$\text{Calculation} = \frac{\text{Concn. of unknown}}{\text{Concn. of standard}} = \frac{15{\cdot}0}{13{\cdot}2}.$$

But concn. of standard is 1 mg. per cubic centimetre. Hence concn. of unknown is,

$$1 \times 15{\cdot}0/13{\cdot}2,$$

i.e., 1·14 mg. per cubic centimetre, or 0·114 gm. per 100 c.c., or 1·14 gm. creatinine per litre.

Note. This calculation is rendered easier because in each case 1 c.c. was taken and diluted to 100 c.c., so that the solutions were quite comparable.

Generalisation. It should easily be seen that the result is given in this estimation by—

$$\frac{\text{Concn. of standard (grams per litre)} \times \text{Reading of standard}}{\text{Reading of unknown}}$$

in grams per litre.

Checking the Colorimeter. The general principles of the colorimeter having been mastered, attention should be drawn to one or two points of detail that must be observed for accurate results.

The correct adjustment between scale and cups should be checked by moving up the cups carefully till the bottom of the plunger is *just* in contact with the bottom of the cup on each side. Both scales should then read zero accurately.

If this is not the case, then the scale should be readjusted, if that is possible with the make of instrument, or else a scale correction must be applied to each reading. (Such a check is especially necessary when cups have been broken and replaced by new ones of possibly slightly different bottom thickness.)

The use of a prism to bring light from both cups to the same eye piece may slightly alter the colour or modify the intensity, and this should be controlled by taking an initial reading with the standard solution *in both cups*. (The average of four readings should be taken as usual.) If there is not complete agreement, then the reading in the cup to be subsequently used for the unknown is to be taken as the true reading of the standard. Thus in the above example, if this check had been made and it had been found that the reading was (average) 15·1 mm., in the calculation 15·1, and not 15·0, should be used.

It will be assumed that this check is made in subsequent work.

Estimation of Creatine in Urine [1]

This is determined by measuring the " pre-formed " creatinine in a portion of the urine and then converting the creatinine in another portion into creatinine and measuring the " total creatinine " in this. The difference gives the creatine content in terms of creatinine.

A sample of mixed urine from young children should be used, since this always contains creatine.

Experiment 2. Take a sample of mixed children's urine. On 1 c.c. of it determine the creatinine as in Experiment 1.

To determine the creatine content, pipette 1 c.c. into a small Erlenmeyer flask, and then add 10 c.c. of a saturated solution of picric acid. Place the flask in an autoclave and heat at 117° to 120° C. for twenty minutes. (Care must be taken to ensure that the autoclave contains sufficient water to last this period.) Cool, remove from the autoclave and add accurately 1 c.c. of 10 per cent. sodium hydroxide. Allow to stand ten minutes, transfer to a 100 c.c. graduated flask, wash the small flask twice with a small amount of distilled water and add the washings to the graduated flask,

[1] After Folin, *J. Biol. Chem.*, 1914, XVII., 469.

and then make up to the 100 c.c. mark with distilled water. Shake and read against the standard (made up as in Experiment 1) in the colorimeter. Calculate as before.

Example. A child's urine is estimated for creatinine, using a creatinine standard. The standard is set at 15 mm. The readings of the unknown are 21·3, 21·5, 21·6, 21·3, giving a mean of 21·4 mm.

Hence the creatinine content is

$$\frac{1 \times 15}{21{\cdot}4}, \textit{ i.e.}, 0{\cdot}70 \text{ gm. per litre.}$$

The creatinine is then converted into creatine and the "total creatinine" measured as in Experiment 2. The readings are found to be 16·8, 16·9, 17·1 and 16·9, giving an average of 16·9 mm.

Hence the total creatinine is

$$\frac{1 \times 15}{16{\cdot}9}, \textit{ i.e.}, 0{\cdot}89 \text{ gm. per litre.}$$

Hence, since preformed creatinine is 0·70 gm. per litre,

Creatine-creatinine is 0·89 — 0·70, *i.e.*, 0·19 gm. per litre.

Convert this into the figure for *creatine* by multiplying by the ratio of the molecular weights, 131/113, or 1·16. This gives the value

0·22 gm. creatine per litre.

Generalisation. If preformed creatinine is x gm. per litre, and total creatinine is y gm. per litre, then creatine is

$$(y - x) \times 1{\cdot}16 \text{ gm. per litre.}$$

EXERCISE XX

ESTIMATION OF URINE ACIDITY BY TITRATION AND BY *pH* DETERMINATION ESTIMATION OF AMMONIA IN URINE

(*One or two Three-hour Periods*)

Urine Acidity

WHEN this is estimated by titration against N/10 sodium hydroxide, using phenolphthalein as indicator, sodium hydroxide must be added to neutralise the various buffers present in the urine (phosphates, carbonates, etc.) and bring the *pH* value to 9 (the value at which the indicator changes colour).

When it is estimated by determining the *pH* value colorimetrically, indicators are used which give the hydrogen-ion concentration. The latter is the preferable method. There is no direct relationship between the two results, on account of the varying amounts of the different buffers present in different urines.

Titrimetric Method

Experiment 1. Place 25 c.c. of urine in a medium-sized Erlenmeyer flask (or in a large evaporating basin). Add about 15 gm. of powdered potassium oxalate. (This precipitates the calcium present, which would otherwise interfere with the titration by throwing down calcium phosphate on neutralisation, and so giving a different end-point.) Add 2 drops of a 1 per cent. alcoholic solution of phenolphthalein. Shake or stir up.

Rinse out the burette with standardised N/10 sodium hydroxide. Then fill to a convenient mark. *It is by no means necessary to fill to the* 0·00 *mark every time.* Read accurately. (This should be done before the potassium oxalate is added to the urine. Be careful that no air bubbles remain below the tap.)

Immediately the urine has been shaken or stirred up with the potassium oxalate run in the sodium hydroxide a little at a time with continued shaking or stirring, and finally drop by drop, until a permanent pink colour is seen. The faintest pink tinge should be taken as end-point. Read the burette again.

Calculation. Express the result in terms of N/10 acid. To do this, multiply the burette reading (the difference) by the factor for the N/10 sodium hydroxide, divide by 25 (the number of cubic centimetres of urine used) and multiply the result by 100 to get the figure per 100 c.c. of urine, remembering that N/10 acid and N/10 alkali when accurate are exactly equivalent.

Example. The first burette reading was 2·34, the second 15·47 c.c., whence the amount of alkali used was 13·13. The sodium hydroxide factor was 0·964.

Hence 13·13 × 0·964 gives 12·66 c.c. of N/10 alkali as the true value, and 12·66 × 100/25 gives the result:

Acidity per 100 c.c. of urine is equivalent to 50·64 c.c. N/10 acid.

Note. The urine after this titration can be used immediately for the determination of ammonia and amino-acids by Malfatti's method (see Experiment 4, p. 134).

Note. **In this, and in most estimations of this nature, the student should carry out duplicate determinations, and the results should show reasonable agreement.**

ESTIMATION OF THE *pH* OF URINE

The determination of hydrogen-ion concentration colorimetrically depends primarily upon a comparison between solutions of definite compositions and known *pH* (determined electrometrically) and unknown solutions, using an indicator which gradually changes colour as the *pH* changes, and so permits a close colour comparison.[1] *pH*, the logarithm of 1/ (hydrogen-ion concentration), is a convenient shorthand form of expressing relationships of this concentration.

It is essential in preparing solutions of exact composition, so that their *pH* values may be exactly known, that great attention be paid to purity of all reagents.

[1] The student should refer to a theoretical text-book for the theory of hydrogen-ion concentration.

The procedures are usually thereby rendered somewhat laborious.

Perhaps one of the simplest sets of " buffer mixtures " to prepare is that of Kolthoff.[1] Three solutions only are required, of succinic acid, of potassium dihydrogen phosphate, and of borax, all of which can easily be obtained pure or easily purified. Redistilled water is preferable in making up the solutions.

The three *standard solutions* are :—succinic acid, 0·05 molar. Dissolve 5·90 gm. of pure succinic acid in water and make up exactly to 1 litre. (A trace of thymol should be added to this solution as preservative.)

Potassium dihydrogen phosphate, 0·1 molar. Dissolve 13·61 gm. of KH_2PO_4 in water and make up to 1 litre.

Borax, $Na_2B_4O_7$, 10 H_2O, 0·05 molar. Dissolve 19·10 gm. in 1 litre of water.

Standard Buffer Mixtures. The following mixtures (each totalling 10 c.c. of solution) have the stated *pH* value as determined electrometrically by Kolthoff :—

Succinic acid.	Borax.	*pH.*	KH_2PO_4.	Borax.	*pH.*
c.c.	c.c.		c.c.	c.c.	
8·22	1·78	4·0	9·21	0·79	5·8
7·78	2 22	4·2	8·77	1·23	6·0
7·38	2·62	4·4	8·30	1·70	6 2
7·00	3·00	4·6	7·78	2·22	6·4
6·65	3·35	4·8	7·22	2·78	6·6
6·32	3·68	5·0	6·67	3·33	6·8
6·05	3·95	5·2	6·23	3·77	7·0
5·79	4 21	5·4	5·81	4·19	7·2
5·57	4·43	5·6	5·50	4·50	7·4
5·40	4·60	5·8	5·17	4·83	7·6
—	—	—	4·92	5·08	7·8
—	—	—	4·65	5·35	8·0
—	—	—	4·30	5·70	8·2
—	—	—	3·87	6·13	8·4
—	—	—	3·40	6·60	8·6
—	—	—	2·76	7·24	8·8
—	—	—	1·75	8·25	9·0

[1] *J. Biol. Chem.*, 1925, LXIII., 135.

Indicators. The following indicators of the Clark-Lub series should be prepared by grinding up 0·1 gm. of the dry powder with the quantities of N/100 sodium hydroxide given in the table and then, after solution is effected, transferring to a 250 c.c. volumetric flask and adding water to the mark to give a 0·04 per cent. solution :—

Common name.	Compound.	*pH* colour range.	N/100 NaOH required.
			c.c.
Brom cresol green	tetrabromo - m- cresol-sulphonphthalein .	3 8 (yellow) to 5·4 (blue)	14·3
Chlor phenol red	dichloro - phenol - sulphonphthalein . .	4·8 (yellow) to 6·4 (red)	23·6
Brom phenol red	dibromo - phenol - sulphonphthalein . .	5·2 (yellow) to 6 8 (red)	19·5
Brom cresol purple	dibromo - o - cresol - sulphonphthalein .	5 2 (yellow) to 6 8 (purple)	18·5
Brom thymol blue	dibromo - thymol - sulphonphthalein . .	6 0 (yellow) to 7 6 (blue)	16·0
Phenol red	phenol-sulphonphthalein	6 8 (yellow) to 8 4 (red)	28·2
Cresol red	o-cresol-sulphonphthalein	7·2 (yellow) to 8·8 (red)	26·2

DEMONSTRATION

Experiment 2. Take nine clean, dry test-tubes of approximately 16 mm. internal diameter, and from burettes run in the requisite volumes of potassium dihydrogen phosphate and borax solutions to give 10 c.c. of mixture with respective *pH* values from 6·0 to 7·6. Add to each tube 5 drops of the diluter solution of brom thymol blue. Invert. Note the series of colours ranging from yellow-olive through olive-green to blue.

Pipette accurately 10 c.c. of the urine under examination into a similar clean test-tube and add 5 drops of the same indicator. Invert. If the colour is within the range of colours shown by the nine tubes match as closely as possible. It should be possible to read to 0·1.

If, on the other hand, the colour is as blue as that of the tube of *pH* 7·4, make up a set of seven tubes with the requisite mixtures to give *pH* values from 7·0 to 8·2 and add to each 5 drops of the diluter solution of phenol red. Compare with a tube of urine to which the phenol red has also been

added. If still outside this range, repeat similarly with the cresol red indicator.

Similarly, if at first the result is yellow, repeat with a set of tubes to which brom cresol purple is added, and if the urine does not fall within this range of colours, repeat with a further set and methyl red.

Note. Near the ends of the ranges of each indicator the colour match is less definite. This is controlled by that overlapping of *pH* values shown for these indicators in the table on p. 131.

A more rapid, though less accurate, procedure is to use the " Colour Chart of Indicators " prepared by W. M. Clark (Williams and Wilkins Co., Baltimore, U.S.A.), in which the actual colours developed by the whole Clark-Lub series of indicators for their complete *pH* ranges, under the stated conditions of test-tubes of 16 mm. internal diameter, 10 c.c. of solution, and 5 drops of indicator, have been reproduced as accurately as possible on paper. With this chart the *pH* of a urine can be determined with moderate accuracy in two or three minutes.

Experiment 3. Take 10 c.c. of urine in a clean test-tube, add 5 drops of brom thymol blue, invert, and compare with the " Chart of Indicators." If blue, repeat with fresh urine and phenol red, and if necessary with fresh urine and cresol red. If, however, olive-yellow, repeat with brom cresol purple, and if necessary with methyl red. Continue till a good colour match is obtained.

Note. In these colorimetric tests for *pH* determination, if the urine is cloudy it must be filtered, but if possible fresh urine should always be used. If the urine is markedly coloured, take 1 c.c. and add 9 c.c. of distilled water. The error from this dilution may vary from 0·1 to 0·4.

Note. The indicators employed permit determination of *pH* within the ranges 4·4 and 8·8. Only very occasionally do pathological urines freshly voided give higher values (which will react with phenolphthalein). The usual range for most urines is between 6·2 and 7·4, though normal urines may be found giving any value between 5 and 8.

Improperly preserved urines may give higher values, due to bacteriological decomposition and formation of ammonia. Such values are of course without significance.

Note. The above procedure can be used with any colourless or almost colourless biological fluid, but dilution is not permissible unless such a fluid is naturally heavily buffered.

Note. Presence of inorganic compounds in a solution frequently affects the indicator so that the colour change does not quite truly correspond to the *pH* as determined in absence of such "salts." This is referred to as the "salt error," but is not of sufficient importance in the present connection to call for more than mention.[1]

Estimation of Ammonia and Amino-acids in Urine (Malfatti's Method)

When ammonium salts react with formaldehyde, hexamethylene tetramine (urotropine) is formed, and acid is set free in corresponding amount :—

$$4\ NH_4Cl + 6\ HCHO = N_4(CH_2)_6 + 4\ HCl + 6H\ OH.$$

Hexamethylene tetramine

Titration of the free acid against sodium hydroxide therefore permits estimation of the ammonia originally present.

Amino-acids, behaving as substituted ammonias, also react to give methylene derivatives, in which an alkaline amino group is replaced by a neutral radical :—

$$R\ .\ CH(NH_2)\ .\ COOH + HCHO = R\ .\ CH\ .\ (N : CH_2).\ COOH + HOH.$$

As a result of this replacement, the practically neutral "amphoteric" compounds are replaced by organic acids capable of reacting with sodium hydroxide; there is no longer an amino group to inhibit this reaction.

The ammonium salts and the much smaller amounts of amino-acids in urine can therefore be estimated in terms of ammonia after appropriate treatment of the urine by formaldehyde. In order to carry out the determination, the urine must first be neutralised. Hence the determination can usefully be made after that of the acidity of urine by titration (as in Experiment 1).

[1] Myers and Muntwyler (*J. Biol. Chem.*, 1928, LXXVIII., 225) have elaborated a method for the accurate colorimetric determination of the *pH* of urine.

If it is desired to ascertain the amino-acid content, then a determination can be made as in Experiment 3, ammonia subsequently determined on a separate sample of urine by an aspiration method (see Exercise XXII.) and the amino-acid content determined by calculation from the two results.

Experiment 4. Take the neutralised urine that has been titrated with sodium hydroxide to determine acidity, and add to it formaldehyde.

Note. The formaldehyde is prepared as follows. Since commercial formalin usually contains a little free formic acid, take 10 c.c. of formalin in a graduated cylinder, add 20 c.c. of distilled water and 2 drops of phenolphthalein, shake, run in N/10 sodium hydroxide until just pink, and then add the whole to the urine. **Obviously the sodium hydroxide used for the formaldehyde is not considered in the following calculation.**

On addition of the pink formaldehyde to the pink urine the colour is discharged through the formation of free acid. Immediately titrate with more N/10 sodium hydroxide until the pink colour is just restored. Note the amount of sodium hydroxide added. The calculation is shown in the following example :—

Example. Twenty-five cubic centimetres of urine required 8·67 c.c. of sodium hydroxide to turn phenolphthalein pink, the factor to convert the alkali to N/10 being 1·173. Calculate the acidity.

1·70 c.c. of this sodium hydroxide were used to neutralise the diluted formaldehyde. This figure has no further significance.

For the subsequent titration the burette readings were—before, 12·17, and after, 17·48, the difference being therefore 5·31 c.c. This, multiplied by the factor, gives 6·23 c.c. This, multiplied by 0·0014, gives the result in terms of nitrogen (see calculation for the Kjeldahl determination), or by 0·0017 in terms of grams of ammonia, NH_3, in the amount of urine taken, in this case 0·0106 gm. NH_3 per 25 c.c. of urine, whence, multiplying by 40, the final result is

0·424 gm. " ammonia " per litre of urine,

remembering that " ammonia " in this estimation includes amino-acids, calculated as NH_3.

EXERCISE XXI

ESTIMATION OF GLUCOSE IN PURE SOLUTION AND IN URINE

(Two Three-hour Periods)

GLUCOSE is now usually estimated quantitatively by Benedict's procedure, using his special reagent.[1] Its determination is of especial importance, clinically, in the control of diabetics.

Benedict's Reagent for Quantitative Sugar Determinations

This is made up as follows :—

Dissolve 18·000 gm. of crystallised copper sulphate in about 100 c.c. of distilled water. The copper sulphate must be pure and weighed out accurately.

Weigh out to within 1 gm. 200 gm. of crystallised sodium carbonate (or 100 gm. of anhydrous sodium carbonate), 200 gm. of sodium or potassium citrate, and 125 gm. of potassium thiocyanate, and, with the application of slight heat, dissolve these in enough water to make 800 c.c. of solution. Transfer to a graduated litre flask, pour the copper sulphate solution into the other with constant shaking, rinse the flask containing that solution twice with distilled water and add the washings to the bulk, then add 5 c.c. of a 5 per cent. solution of potassium ferrocyanide, and finally make up the total volume (at room temperature) to 1,000 c.c.

Provided pure copper sulphate is taken and exactly 18 gm. of it weighed out, the solution is of such strength that 25 c.c. of it is reduced by 50 gm. of glucose. No accurate equation can be written for the reaction.

Experiment 1. An accurate 2 per cent. solution of pure glucose has been prepared in bulk. Take a sample of this in a clean, dry flask. If such a flask is not available rinse out a clean flask with a very little of the solution, and then

[1] S. R. Benedict, *J. Am. Med. Assoc.*, 1911, LVII., 1193.

transfer about 30 c.c. to the flask. Rinse out the burette with glucose solution from the flask, drain it carefully, and finally half fill it and take an accurate reading.

Pipette out accurately 25 c.c. of the reagent into a fairly large porcelain basin. (Remember that if the 25 c.c. pipette does not deliver 25 c.c. a correction must be applied as determined by the standardisation.) Add 15 gm. of crystalline sodium carbonate or 8 gm. of anhydrous sodium carbonate (weighed roughly) along with a little powdered pumice stone or talc powder. Stir up. Heat to boiling over a low flame. Continue stirring constantly to prevent spirting. Run in the sugar solution from the burette $\frac{1}{2}$ c.c. at a time, stirring for at least half a minute between each addition. When 2·5 or 3·0 c.c. of this pure 2 per cent. glucose have been added the colour will change rapidly to dead white.

Whenever this takes place before 5 c.c. have been added, the amount added is too small to give an accurate estimation, and the sugar solution must be diluted before the titration is repeated. The same holds true for a diabetic urine. If, however, 5 c.c. or more are required, then repeat, but more accurately, with the undiluted solution or urine, as described below.

Dilute the sugar solution by running in accurately from the burette 10 c.c. into the 100 c.c. standardised flask (remembering to make any necessary correction for its standardised figure in the final calculation), and shake up. Discard the rest of the sugar solution (or transfer to a flask for further preparation of the diluted solution if necessary), rinse out the burette with water, then with the diluted sugar solution, and fill up to the mark.

Repeat the determination exactly as before,[1] but *just* as *soon as any colour change is observed* in the contents of the basin commence to run in the solution only 2 or 3 drops at a time, and when the colour has almost gone, 1 drop at a time, waiting (stirring) twenty or thirty seconds between addition of each drop, until all colour has **just** disappeared (the usual error is to run in too much), and then read the burette.

Note. If the titration is too long delayed and cooling is permitted, some oxygen may be absorbed from the air, tending to recolour the mixture, and so too great a volume

[1] Whenever the solution is boiled down so far that spirting is difficult to avoid, a little water should be added. When this is necessary, the note concerning error from cooling should be carefully heeded.

will be run in. In proof of this it should be noticed that after the titration is completed, if the contents of the basin are allowed to cool some colour will return within a few minutes.

Carry out an exactly parallel second determination. If the two results show fairly good agreement, that will suffice. Otherwise a third determination must be made. The calculation is seen from the following example :—

Example :

Initial burette reading	0·00 c.c.		
After first titration .	24·96 c.c.	Difference .	24·96 c.c.
After second titration	49·81 c.c.	Difference .	24·85 c.c.
		Mean .	24·91 c.c.

Calculation :

Twenty-five cubic centimetres of Benedict's solution is reduced by 50 mg. of glucose. Hence 50 mg. of glucose is contained in 24·91 c.c. of solution. Hence 100 c.c. of solution contains $50/24{\cdot}91 \times 100$,

i.e., 201·1 mg., or 0·2011 gm.

Hence (neglecting the very slight change in specific gravity) the solution contains 0·2011 per cent. of glucose, and the original undiluted solution contains 2·011 per cent.

Error : $+ \frac{0{\cdot}011}{2} \times 100$ per cent., *i.e.*, + 0·55 per cent.

(satisfactory).

Note. After some practice with these quantities students should be able to get sufficiently accurate results taking only 10 c.c. of Benedict's solution, and proportionately less sodium carbonate.

Experiment 2. Repeat with an unknown solution of glucose.

Experiment 3. Repeat with a diabetic urine. The end-point is a little more difficult to determine, since instead of a dead white colour a dirty yellow colour (due to the urine) will remain. Note also that excess of either pure glucose or of diabetic urine leads to a yellowish colour.

EXERCISE XXII

ESTIMATION OF AMMONIA AND UREA IN URINE

(Two Three-hour Periods)

THE principles involved in this double determination are that addition of a strong alkali to a solution of an ammonium salt liberates ammonia, which can then be distilled off (as in the Kjeldahl procedure) or aspirated off by sucking air through the solution (as in the present procedure), and further that the enzyme urease decomposes urea to ammonia practically quantitatively, so that if ammonia be determined on one sample of urine, and this *pre-formed ammonia* plus *urea-ammonia* be determined on a second sample of the same urine, from the difference between the two results the amount of urea present can be calculated.

Experiment 1. Pipette accurately 10 c.c. of urine into a 100 c.c. volumetric flask, and add (ammonia-free) distilled water to the mark. Shake up. Pipette 5 c.c. of this diluted urine into a thick-walled test-tube of the Van Slyke-Cullen urea apparatus,[1] crush two urease tablets of a sample known to be active [2] and add the powder to the tube, and incubate at 40° for thirty minutes. Into a second similar tube pipette accurately 25 c.c. of N/50 sulphuric acid and add 2 drops of methyl-red indicator. At the expiry of the thirty minutes connect the two tubes together as indicated in Fig. 13, but **just before** making the connection add to the urine tube 20 drops of caprylic alcohol (to prevent foaming) and 7 c.c. of 20 per cent. potassium carbonate. After making the necessary connections place the urine tube in a water-bath

[1] Van Slyke and Cullen, *J. Biol. Chem.*, 1914, XIX., 211.

[2] The activity of commercial urease tablets should always be tested on any fresh batch by carrying out a test, similar to Experiment 1, with a urea solution of known strength.

or beaker containing water at 70° to 80° C. and aspirate with a water-pump **so that air is drawn through the urine tube**

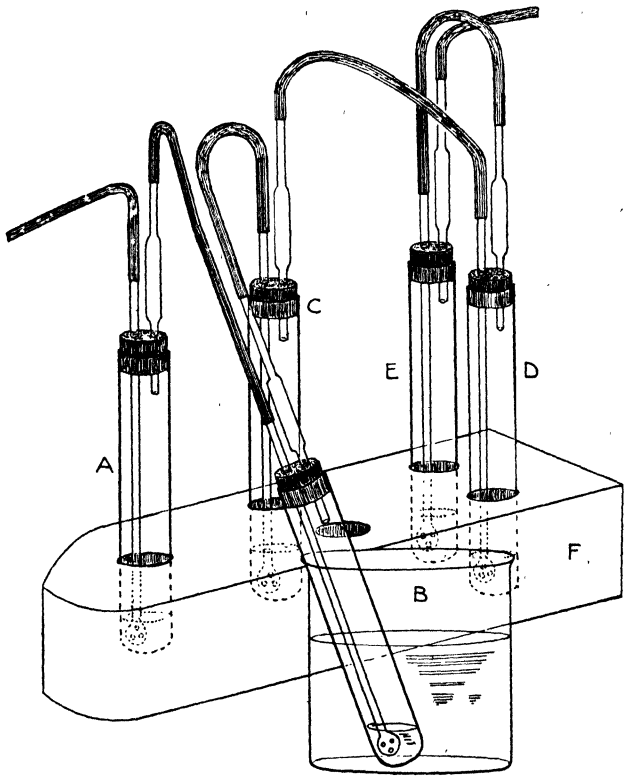


FIG. 13. Van Slyke and Cullen apparatus for estimating urea and ammonia.

A, tube containing concentrated sulphuric acid ; B, tube containing diluted urine, urease, and potassium carbonate, immersed in a beaker containing hot water ; C, tube containing N/50 sulphuric acid ; D, tube containing undiluted urine and potassium carbonate (for ammonia determination) ; E, tube containing N/50 sulphuric acid ; F, wooden block.

and ammonia then carried along with it to the acid tube for twenty minutes. In order to prevent any error from

presence of a trace of ammonia in the air of the laboratory, a tube containing concentrated sulphuric acid should be inserted in the series and air **first** drawn through it.

Stop aeration, disconnect the apparatus and titrate the N/50 acid against N/50 alkali.

Calculation. Convert the acid and alkali into their true N/50 values by use of the previously determined factors for each. Subtract these values. The difference is the amount of N/50 acid used to neutralise ammonia. It therefore is equivalent to the same number of cubic centimetres of N/50 NH_3, and therefore to the same number of cubic centimetres of N/50 " N " solution.

A normal solution of N theoretically contains 14 gm. of N per litre ; hence an N/50 solution should contain 0·28 gm. per litre, *i.e.*, 0·28 mg. per cubic centimetre. Hence multiply by 0·28 to obtain the amount of (urea- plus ammonia-) N in 5 c.c. of diluted, or 0·5 c.c. of undiluted, urine. Multiply the result by 200 to get the figure for 100 c.c. of urine.

Example. Twenty-five cubic centimetres of N/50 sulphuric acid with a factor 0·961 were taken, and 11·25 c.c. of N/50 alkali with a factor 1·048 were required. The true values are therefore 24·03 and 11·79 c.c. respectively, and the difference, 12·24 c.c., corresponds to 12·24 c.c. of N/50 nitrogen. This, multiplied by 0·28, gives 3·43 mg. of (urea + NH_3)-N present in 0·5 c.c. of undiluted urine, and finally (multiplying by 200)—

0·686 gm. (urea + NH_3)-N are present in 100 c.c. of the urine.

Simultaneous Experiment. To determine the " pre-formed ammonia " in the urine under examination pass the gas (air) from the N/50 sulphuric acid tube in the above determination through a third tube, into which has been accurately pipetted 5 c.c. of **undiluted** urine and 7 c.c. of 20 per cent. potassium carbonate, and then finally into a fourth tube, containing 25 c.c. of N/50 sulphuric acid (and 2 drops of methyl-red solution). After the twenty minutes' aspiration also titrate the fourth tube against N/50 alkali.

Calculation. Apply the factors to get the true N/50 values. The difference in the corrected volumes gives the figure for N/50 acid used up, and therefore the same figure for N/50 N. Multiply by 0·28 ; the result is the number of milligrams of NH_3-N in 5 c.c. of urine. Multiply by 20 for the figure per 100 c.c. urine.

Example (a continuation of that above). 9·10 c.c. of alkali were required. Corrected values are 24·03 c.c. acid and 9·54 c.c. alkali. The difference, 14·49 c.c. × 0·28 gives 4·06 mg. NH_3-N in 5 c.c. urine, *i.e.*, 0·081 gm. NH_3-N in 100 c.c. urine.

Hence, combining the two results,

1 litre of urine contained 0·81 gm. of NH_3-N,
6·05 gm. of urea-N.

To obtain the result in terms of urea and ammonia, multiply respectively by

2·14, the ratio given by $(CON_2H_4)/N_2$, and
1·21, the ratio given by $(NH_3)/N$.

EXERCISE XXIII

ESTIMATION OF CHLORIDES IN URINE

(One Two-hour Period)

FOLLOWING the Volhard-Arnold procedure, excess of silver nitrate is added to a measured volume of urine, and the amount of the excess is determined by titration with ammonium thiocyanate. In order to prevent the silver chloride from affecting the end-point, it is filtered off before titration. Ammonium ferric sulphate (ammonium iron alum) is used as indicator. When all silver is precipitated (but not before) formation of ferric thiocyanate—red—indicates the end-point. The first brownish-red tinge is taken.

Preparation of Standard Solutions

Silver Nitrate Solution. 29·061 gm. of pure silver nitrate are dissolved in distilled water, and more water added to exactly 1 litre. This solution is of such strength that 1 c.c. is equivalent to 0·01 gm. of sodium chloride or 0·006 gm. chlorine (as chloride).

Ammonium Thiocyanate Solution. While where large numbers of titrations have to be carried out as a routine procedure it is convenient to make up such a solution that 1 c.c. is equivalent to 1 c.c. of silver nitrate solution, yet for training purposes it is a useful exercise for the student to standardise his own thiocyanate solution. The solution provided should contain approximately 13 gm. of ammonium thiocyanate per litre.

Transfer to a small flask with the 10 c.c. pipette 20 c.c. of the standard silver nitrate solution ; add 5 c.c. of ferric alum (saturated solution), 4 c.c. of dilute nitric acid, and about 70 c.c. of distilled water. Shake. Run in ammonium thiocyanate solution from a burette until there is the

slightest **permanent** brown-red colour. Read the amount withdrawn from the burette. The factor for the thiocyanate is given by 20/(number of c.c. of thiocyanate used). After multiplying by this factor, 1 c.c. of thiocyanate is therefore equivalent to 1 c.c. of standard silver nitrate solution.

Experiment 1. Pipette accurately 10 c.c. of urine into a 100 c.c. volumetric flask ; add approximately 1 c.c. of concentrated nitric acid and 2 c.c. of a saturated solution of ferric alum. (If there is at this stage a marked red colour due to other urinary constituents, add 8 per cent. potassium permanganate drop by drop until the colour disappears. This is not usually necessary.) Accurately transfer 20 c.c. of the standard silver nitrate solution to the flask, shaking during the addition. Allow to stand ten minutes, then add distilled water to the 100 c.c. mark, mix thoroughly, and filter through a **dry** filter paper. Measure out accurately 50 c.c. of the filtrate and transfer it to a clean Erlenmeyer flask ; titrate with the thiocyanate as above, taking the end point as already indicated.

Calculation. Multiply the number of cubic centimetres of ammonium thiocyanate used by the factor, and multiply the result by 2. This gives the total amount of silver nitrate remaining in the 100 c.c. flask **before filtration.** Subtract this from 20 c.c. The difference, x c.c., corresponds to $x \times 0{\cdot}01$ gm. NaCl present in the 10 c.c. of urine. Multiply by 100 to get the figure per litre.

Example. In the thiocyanate titration 20 c.c. of silver nitrate required 20·51 c.c. of thiocyanate. Hence the thiocyanate factor is 20/20·51, or 0·975.

In the urine determination 7·18 c.c. of thiocyanate were required. Hence $7{\cdot}18 \times 0{\cdot}975$ gives 7·00, which, multiplied by 2, gives 14·00 c.c. silver nitrate not used up by chloride. Hence 6·00 c.c. were used up in that reaction, and these correspond to 0·060 gm. NaCl.

Therefore the urine contained 6·0 gm. sodium chloride per litre.

EXERCISE XXIV

ESTIMATION OF PHOSPHATES IN URINE

(One Two-hour Period)

In very slightly acid solution (a condition ensured by addition of sodium acetate and acetic acid) uranium salts precipitate phosphate as uranium phosphate. This reaction takes place in preference to the formation of brown uranium ferrocyanide. The latter reaction can therefore be used to determine the end-point.

Standard Uranium Acetate Solution

Dissolve 35 gm. (weighed accurately) of uranium acetate, $UO_2(C_2H_3O_2)_2 2H_2O$, in 1 litre of distilled water. This solution is accurate enough for ordinary determinations. If the purity of the preparation is doubtful (*e.g.*, the amount of water of crystallisation may be variable) weigh out 36 gm. to the litre, and check it against a solution of potassium dihydrogen phosphate, KH_2PO_4, made by dissolving 3·831 gm. in a litre. Of this solution 50 c.c. contain 0·1 gm. P_2O_5. If it requires exactly 20 c.c. of the uranium acetate solution then 1 c.c. of the latter corresponds to 0·005 gm. P_2O_5. Usually a little less than 20 c.c. will be required, in which case the bulk of the solution should be so diluted that the exact ratio holds.

Experiment 1. Pipette 50 c.c. of urine accurately into a 200 c.c. Erlenmeyer flask, or an evaporating basin of corresponding size. Add 5 c.c. of acid sodium acetate (made by dissolving 100 gm. of sodium acetate in 800 c.c. of distilled water, adding 30 c.c. of glacial acetic acid with shaking, and then diluting to the litre) and heat over a Bunsen flame just to the boiling point. **Do not continue boiling.** Run into the hot solution a few drops at a time from a burette containing the standard solution of uranium acetate (see above).

When the precipitate which forms apparently ceases to increase in bulk, run in from the burette drop by drop, shaking or stirring after the addition of each drop and then removing a drop from the beaker (or basin) and bringing it into contact with a drop of 10 per cent. potassium ferrocyanide solution on a porcelain tablet.

It is desirable to put a number of drops of the ferrocyanide solution on the tablet, and probably six or eight tests will have to be made before a brownish-red coloration is seen developing in the yellow solution. The first time this is suspected take a burette reading. Then add another drop and repeat. A deeper colour should be obtained, confirming the first reading, which should then be accepted as the accurate one.

Calculation. Multiply the number of cubic centimetres of uranium acetate solution as measured by the burette by 0·005. The result gives the number of grams of P_2O_5 present in the 50 c.c. of urine taken. Multiply by 20 to express the result per litre of urine.

Example. The initial burette reading was 10·37 c.c. The reading after the first tinge of brown-red colour with ferrocyanide was 18·67, and the difference therefore 8·30 c.c. The factor for the burette used was 1·011. Multiplying by this factor, the true value is 8·39 c.c. Hence, multiplying by 0·005, 50 c.c. of this urine contained 0·04195 gm. of P_2O_5, and the result is

0·839 gm. P_2O_5 per litre of urine.

Note. Fiske and Subarrow [1] have devised a simple colorimetric method which gives accurate results, and which depends on the formation of phosphomolybdic acid from phosphate and ammonium molybdate in acid solution, and then the reduction of the phosphomolybdic acid by 1-2-4 amino-naphthol-sulphonic acid with production of a deep blue colour.

[1] Fiske and Subarrow, *J. Biol. Chem.*, 1925, LXVI., 375.

EXERCISE XXV

EXAMINATION OF PATHOLOGICAL URINES

(One Three-hour Period)

URINES that are possibly pathological should be tested initially for reducing substances and albumin.

Reducing Substances. A positive Benedict test indicates reducing substance present.

A doubtful Benedict's test indicates that Fehling's test should also be made.

A doubtful Benedict and positive Fehling indicates reducing substance present.

If reducing substance is present, an osazone or fermentation test should next be carried out to find out if glucose, some other sugar, or something else, not a sugar, is present.

Acetone Bodies. If glucose is found present acetone bodies should be tested for, *i.e.*, acetone and acetoacetic acid.

For acetone use Legal's sodium nitroprusside test (Exercise XV., Experiment 31). If the test appears to be positive check by carrying out a parallel test with a normal urine.

For acetoacetic acid use Gerhardt's ferric chloride test (Exercise XV., Experiment 31). The Le Nobel test (same reference) is positive for both acetone and acetoacetic acid.

Rothera's sodium nitroprusside test can also usefully be applied. To 10 c.c. of urine add 5 gm. of ammonium sulphate crystals and shake up to saturation. Then add 3 drops of **freshly prepared** (about 5 per cent.) sodium nitroprusside solution and 2 c.c. of concentrated ammonium hydroxide. Gradual development of a deep permanganate colour indicates the presence of acetoacetic acid.[1] (This test is also given by acetone, but is more delicate for acetoacetic acid.)

Albumin. The heat and nitric acid tests should be employed. If in the first test a slight cloudiness appears

[1] A modification of the test permitting greater rapidity is to prepare a mixture of powdered ammonium sulphate and sodium nitroprusside crystals in the proportions of 100 : 1, and to add about 5 gm. of this to 10 c.c. of urine. The reaction proceeds as usual.

this may of course be due to phosphates, and a drop of dilute acetic acid should be added. Phosphates dissolve; albumin does not (*cf.* Exercise XV., Experiment 30).[1]

In rare instances unusual proteins are met with in urine. The commonest of these is the "Bence-Jones protein." With this when the heat test is applied a turbidity commences to be seen between 40° and 60° C. Addition of a drop of acetic acid and continued heating to the boiling point causes disappearance of the precipitate in whole or in part.

Osgood and Haskins [2] have modified the heat test for proteins to facilitate detection of Bence-Jones protein. To 5 c.c. of urine add 1 c.c. of 50 per cent. acetic acid. (A precipitate at this stage is due to urates, or other non-protein material, and should be filtered off and neglected.) Then add 3 c.c. of 30 per cent. sodium chloride. If a precipitate forms at room temperature it is due to Bence-Jones protein, or to globulin if that is present in amount greater than 0·4 per cent. Apply heat. Precipitated Bence-Jones protein increases in amount up to 40° and then slowly redissolves. The blood proteins of albuminuria, on the other hand, are steadily precipitated with increasing temperature. (Bence-Jones protein, if present, may also be accompanied by blood proteins.)

The *sulphosalicylic acid* test for proteins can also be used with advantage. To 1 c.c. of urine add 3 drops of 20 per cent. sulphosalicylic acid. Boil, and then allow to cool. Albumin, globulin, proteose, and Bence-Jones protein are all precipitated at room temperature. The two latter dissolve on boiling and reappear on cooling. This test has been made the basis of a fairly satisfactory method for the quantitative estimation of protein in urine.[3]

Blood. Where the presence of blood is suspected (tinge of colour) apply the benzidine or orthotolidin test (Exercise X., Experiments 11 and 12).

Bile. When bile is suspected shake the urine. A yellow

[1] When albumin is present an approximation to its amount is obtained by the use of Esbach's "albuminometer." Addition of the special "Esbach's reagent" to a definite volume of urine throws down a bulky precipitate. This is allowed to stand for twenty-four hours, when its volume, read on a special scale, gives the number of grams of protein per litre of urine.

[2] *J. Lab. Clin. Med.*, 1931, XVI., 575.

[3] *Cf.* Nicholson, "Laboratory Medicine," 2nd edit., p. 430 (Lea and Febiger, Philadelphia, 1934).

foam strongly suggests bile. Confirm by adding 5 c.c. of urine to 5 c.c. of concentrated nitric acid carefully in a test-tube, so that the two do not mix. At the interface green, blue, violet, red and reddish-yellow rings will be seen if bile is present. The sulphur test should also be positive if much bile is present, but should not be used if thymol has been added as a preservative.

Melanins. These are indicated when a urine, normal in

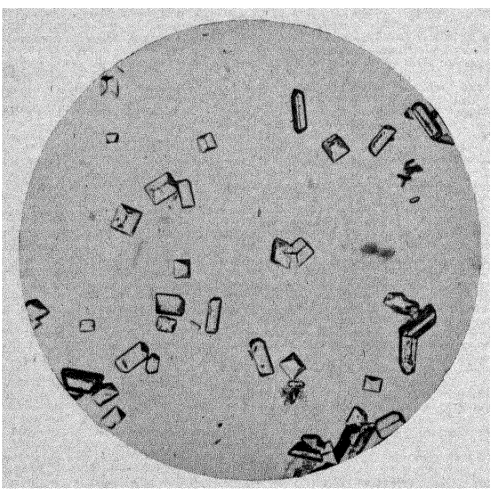


FIG. 14. Photo-micrograph of ammonium magnesium phosphate (triple phosphate) crystals from human urine, × 90.

colour when freshly voided, on exposure to the air deepens in colour to dark brown or black. Confusion with indigo can be avoided, since this is soluble in chloroform, while the brownish-black pigment is not. Urines containing homogentisic acid only darken on addition of alkali or ammonia. (Bacteriological decomposition may produce the necessary ammonia.)

Melanins can be tested for by the Von Jaksch-Pollak procedure. To 10 c.c. of urine add a few drops of ferric chloride solution. A **grey** precipitate indicates melanin.

On further addition of ferric chloride the precipitate should darken, and on still further addition it should redissolve.

Urorosein. In certain urines addition of concentrated hydrochloric acid and a drop of sodium nitrite solution gives a rose-red colour, due to production of a coloured compound from indole-acetic acid.

Examination of Urinary Residues. Examination of urinary sediments is of considerable importance for diagnostic purposes, but scarcely comes within the scope of this course, except in so far as these consist of chemical compounds and not merely tissue *débris*.

The urine should be centrifuged, a little of the sediment transferred to a glass slide, and the slide examined under the microscope.

Triple phosphate, calcium oxalate, and uric acid crystals are perhaps most frequently seen.

Ammonium magnesium phosphate (triple phosphate) occurs chiefly in prisms, often of the so-called " coffin-lid " shape. It is only formed during alkaline fermentation of urine, fermentation which may have occurred either before (pathologically) or after the urine has been voided. A photo-micrograph is shown in Fig. 14.

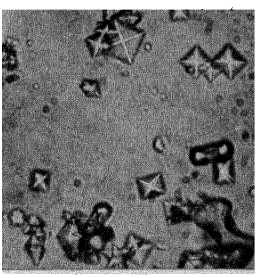


FIG. 15. Photo-micrograph of calcium oxalate crystals from human urine, × 270.

Calcium oxalate occurs as octahedra and in dumb-bell form (see Fig. 15).

Calcium (stellar) *phosphate* may occur in amorphous, granular or crystalline (wedge-shaped) formations or irregular rosettes.

Uric acid is common in acid urine, occurring in very varied forms: rhombic prisms, wedges, dumb-bells, whetstones, etc. These crystals are usually pigmented, yellow to reddish-brown. A photo-micrograph is shown in Fig. 16.

Urates of ammonium, calcium, magnesium, potassium and sodium, amorphous or crystalline, are of not infrequent occurrence.

Crystals of calcium sulphate are rare, and when found in markedly acid urines appear as long, thin, colourless prisms.

Hexagonal plates of cystine occur in the rare pathological

condition cystinuria. Leucine and tyrosine are found in the urine in cases of acute yellow atrophy of the liver and other cases involving liver degeneration. In liver conditions

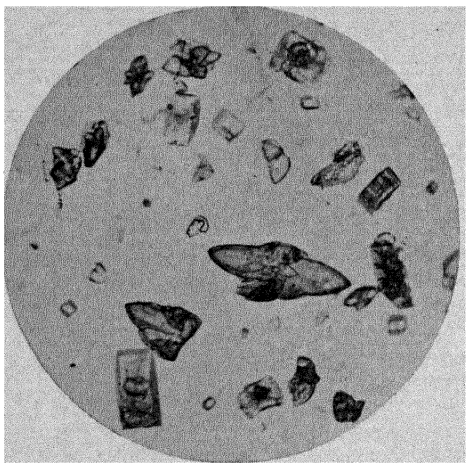


FIG. 16. Photo-micrograph of uric acid crystals from human urine, × 45.

crystals of haematoidin (which is perhaps identical with bilirubin) are found as small yellowish-red plates.

In (fermented) alkaline urines indigo crystals may occur as dark blue needles.

EXERCISE XXVI

GASTRIC ANALYSIS AND ANALYSIS OF GASTRIC CONTENTS

(One or two Three-hour Periods)

GASTRIC analysis is designed to ascertain whether the gastric juice contains " free " hydrochloric acid, and also whether gastric contents contain lactic acid, erepsin and trypsin. Peptic activity can also be measured.

Gastric analysis should be carried out on the fasting subject. One or two students should volunteer for the test, and should have taken no food for at least four or five hours previously, so that the stomach is practically empty.

In turn will be considered (i.) the Rehfuss stomach tube and its use, (ii.) the withdrawal of gastric contents, (iii.) the test-meal, and (iv.) the analysis of the gastric contents.[1]

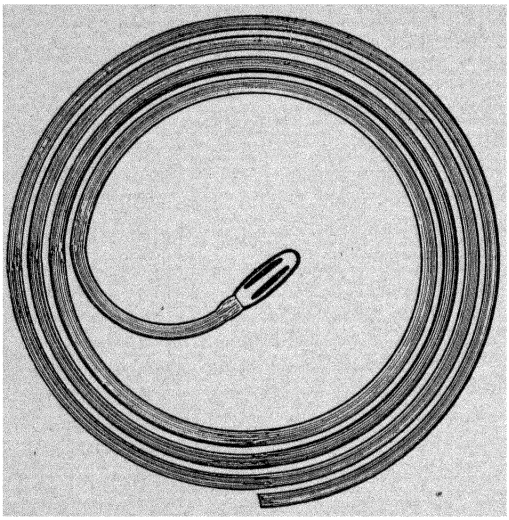
The Rehfuss tube [2] consists of a thin rubber tube attached at the lower end to a small, perforated metal " olive," and at the upper end to a glass syringe (Fig. 17). The whole apparatus should be boiled before use. The tube is usually swallowed without difficulty, and is so thin that it can remain in position throughout a test of two or three hours with no material discomfort to the subject. A sufficient length of tube should be swallowed to bring the " olive " near to the pyloric end of the stomach. The subject should remain still throughout the test, and preferably in a recumbent

[1] The development of the fractional test and the realisation of its importance are due to the work of Hawk and Rehfuss.

[2] *Cf.* Rehfuss, *J. Am. Med. Assoc.*, 1914, LXIV., 569. Ryle has devised a similar tube which would appear to possess certain advantages over the original Rehfuss model (Ryle, " Gastric Function in Health and Disease," Oxford University Press, 1926).

position. **Any change of position of the "olive" within the stomach may give rise to faulty results, since, of course, the acidity is not a constant value throughout the mass of stomach contents.**

Withdrawal of Contents. A considerable amount of



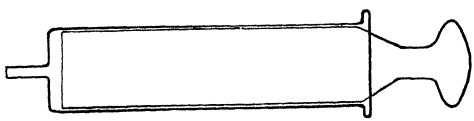


FIG. 17. Rehfuss tube and all-glass syringe, half natural size.

"residual contents" is present in the stomach, even when the test is commenced before breakfast. These "residual contents" are withdrawn through the tube and held for analysis. Since frequently filtration of the gastric contents proceeds very slowly on account of presence of mucus or other cause, these and subsequent specimens are poured

immediately on to a filter paper, the filtrates being collected in numbered test-tubes (to prevent confusion).

The test-meal is then administered. Counting from this as zero time, at thirty-minute intervals 10 to 15 c.c. of contents are withdrawn, until the stomach is empty. In withdrawing these samples suction should be used as little as possible. If possible, samples should be withdrawn until the end of three hours, but this frequently cannot be done, since the time of stomach-emptying is very variable in different individuals.

The Test-meal. Either an Ewald test-meal or a biscuit and water meal should be given. The first consists of two pieces of dry toast (theoretical weight, 35 gm.) and 8 oz. (250 c.c.) of tea without milk ; the second consists of three arrowroot biscuits and 250 to 300 c.c. of water.

One or other of these meals is taken by the subject with the tube in position, and immediately after withdrawal of the residual contents.

Removal of the Tube. The tube should be carefully withdrawn to the epiglottis and the student asked to swallow. It can then be withdrawn, and this procedure prevents any subsequent soreness of the throat.[1]

Analysis of the Gastric Contents

One of the most important points to be ascertained is the absence or presence of "**free**" hydrochloric acid. It is necessary to stress from the outset that when this test is used clinically so-called **achlorhydria**—absence of free hydrochloric acid from the gastric contents—in a patient **unaccustomed to the test** should only permit the conclusion that **the test ought to be repeated,** since many factors, and especially the unaccustomed presence of the tube in mouth and oesophagus, may inhibit gastric acid secretion.

" Free hydrochloric acid " is, of course, a very rough term. The gastric contents may contain hydrochloric acid, " combined " hydrochloric acid (or protein hydrochloride) and organic acids. The actual *pH* value of the mixture is due to the summation of the effects of these. For clinical purposes a sufficient approximation is obtained by assuming that

[1] Very occasionally, after a tube has been used many times, the olive becomes detached during a test ; in such an event it passes down the intestine without discomfort or untoward result.

the amount of alkali required to bring the fluid to a *pH* of a little less than 4 corresponds to free hydrochloric acid, while the further amount required to bring it to *pH* 9 corresponds to the sum of the combined and organic acids present, the total quantity of alkali used indicating the total acidity. Hence the following procedure is employed :—

Accurately pipette 5 c.c. of filtrate from each sample (or a lesser but definite amount if 5 c.c. are not available) into a series of clean test-tubes. Add to each 4 drops of dimethyl-aminoazobenzene (Töpfer's reagent), which colours the solution red, and titrate each in turn against N/10 alkali. In each case run in alkali until the colour is just turned to *salmon-pink* (*pH* less than 4). Note the amount of alkali added. Then immediately add to the solution 3 drops of 1 per cent. alcoholic phenolphthalein solution and continue titrating. The solution at first turns yellow, and at *pH* 9 changes to red. The first permanent pinkish tinge is taken. Note the final burette reading.

Calculation. If 5 c.c. are taken in each case, then multiply the two sets of figures by 20 to get the free hydrochloric acid and the total acidity per 100 c.c. of gastric contents. The resulting figures are in terms of N/10 acid. If less or more fluid was taken for titration make the corresponding correction. (If x c.c. are taken, multiply by $100/x$.)

Typical Examples. Six samples were obtained at 0, 0·5, 1·0, 1·5, 2·0 and 2·5 hours. Five cubic centimetres were used for each titration. The N/10 alkali had a factor 1·060. The initial burette reading was 0·00 c.c. The subsequent readings were :—

(i.) 0·45 and 0·55 ; differences, 0·45 and 0·55 ; corrected, 0·48 and 0·58 c.c.
(ii.) 0·59 and 0·65 ; differences, 0·04 and 0·10 ; corrected, 0·04 and 0·11.
(iii.) 1·80 and 1·95 ; differences, 1·15 and 1·30 ; corrected, 1·22 and 1·38.
(iv.) 3·56 and 3·84 ; differences, 1·61 and 1·89 ; corrected, 1·71 and 2·00.
(v.) 4·29 and 4·59 ; differences, 0·45 and 0·75 ; corrected, 0·48 and 0·80.
(vi.) 4·79 and 4·92 ; differences, 0·20 and 0·33 ; corrected, 0·21 and 0·35.

These figures, multiplied by 20, give :—

Time.	Free HCl.	Total acidity.
0 hour	9·6 c.c. N/10 acid	11·6 c.c. N/10 acid
0·5 ,,	0·8 ,, ,,	2·2 ,, ,,
1·0 ,,	24·4 ,, ,,	27·6 ,, ,,
1·5 ,,	34·2 ,, ,,	40·0 ,, ,,
2·0 ,,	9·6 ,, ,,	16·0 ,, ,,
2·5 ,,	4·2 ,, ,,	7·0 ,, ,,

Note. Methods of estimating gastric acidity based upon single analysis at a period of one hour after the test-meal are unsatisfactory.

Note. Gastric contents are stated to average, at the height of digestion, 0·2 per cent. hydrochloric acid. Since 100 c.c. of N/10 hydrochloric acid contain 0·365 gm. of this acid, such an average concentration corresponds to $100 \times 0{\cdot}2/0{\cdot}365$, equal to 55 c.c. of N/10 acid.

Note. The chief clinical significance of gastric analysis is the presence or absence of free hydrochloric acid. Patients showing complete absence of acid, through distaste of the test-meal, or other factors, frequently produce an acid gastric secretion following subcutaneous injection of a minute dose of histamine (no test-meal being necessary). Patients with pernicious anaemia give uniformly negative results.

Further Examination of Gastric Contents

The following tests can be carried out on the mixed samples left over from the above analyses, or on each sample separately, if sufficient of the filtrate has been obtained. **In any case the residual contents should be examined separately.**

Free Hydrochloric Acid. (Note remarks above concerning this term.)

Gunzberg's reagent is made by dissolving 2 gm. of phloroglucinol and 1 gm. of vanillin in 100 c.c. of 95 per cent. ethyl alcohol.

Place 2 drops of this reagent in a small porcelain basin and evaporate to dryness very carefully over a low flame. Moisten a clean glass rod with the filtrate that is to be tested and draw the moist end through the dried residue from

the reagent. Again warm very gently. A purplish-red colour indicates the presence of " free hydrochloric acid."

Lactic Acid. *Uffelmann's Reaction.* Add ferric chloride solution drop by drop to a 1 per cent. solution of phenol until an amethyst-blue colour has developed. To 5 c.c. of this solution add 5 c.c. of filtrate. A canary-yellow or greenish-yellow colour develops in presence of 0·01 per cent. lactic acid or more. Other organic acids also give this reaction, while presence of mineral acids such as hydrochloric acid weakens the response. The test therefore is only of value when positive.

Strauss Test (more satisfactory). Transfer 5 c.c. of gastric filtrate to a separating funnel, add 20 c.c. of ether, shake thoroughly and allow the liquids to separate. Drain off all liquid from the funnel except the last 5 c.c. To this ether extract add 20 c.c. of distilled water and 2 drops of 10 per cent. ferric chloride solution. Shake gently. A slight green colour is given if as much as 0·05 per cent. lactic acid was present in the original filtrate, while 0·1 per cent. gives an intense yellow-green.

Blood. Apply the benzidine or orthotolidin tests.

Bile. If bile is present in large amount the gastric contents will be coloured green. If the contents are colourless the presence of bile may be tested for as follows: Saturate 5 c.c. of filtrate with ammonium sulphate crystals, making certain the saturation is complete. Add 1 c.c. of acetone, and mix by inverting the test-tube five or six times (but not shaking). On standing the acetone rises to the surface. Allow a drop of **yellow** nitric acid to run down the side of the tube. If bile is present the acetone takes on a green colour.

EXERCISE XXVII

PREPARATION OF PROTEIN-FREE BLOOD FILTRATES AND THEIR ANALYSIS

(Three or four Three-hour Periods)

The Blood Sample

NUMEROUS methods have been devised for analysis of various blood constituents using only **drops** of blood. While in skilled hands such methods may lead to results of moderate accuracy, yet they tend to be needlessly inaccurate, and the procedures are not the most satisfactory for the training of students. It is just as easy, and no more painful to the subject (normal or patient), to obtain a reasonably large sample of blood from a vein, as to obtain drops of blood by pricking the ear or finger.

A piece of rubber tubing is twisted tightly about the arm just above the elbow. The veins of the inner flexure of the elbow usually stand out sharply. The median basilic vein is generally selected for puncture. The skin surface above it (or whatever vein is chosen) is washed with alcohol, and a sterilised hypodermic needle ($1\frac{1}{4}$ in., gauge 22, is a convenient size) attached to a syringe is inserted into the vein at an angle of about 50 degrees. Blood commences to flow into the syringe, and the piston is gradually withdrawn. About 10 c.c. is a convenient quantity to withdraw when a number of constituents are to be determined (3 to 4 c.c. are sufficient for determination of a single constituent). The blood is discharged immediately into a test-tube containing *one* drop of 20 per cent. potassium oxalate for *each* 5 c.c. of blood, and shaken up. The manipulation should be sufficiently rapid to prevent clotting. **Excess of oxalate should be avoided, as it frequently interferes with subsequent procedures.**

Utilisation of the Blood Sample. Blood constituents of clinical importance are determined on whole blood, blood plasma, blood serum, or filtrates freed from blood proteins, according to the relative distribution of such constituents in red cells and plasma, or the degree of interference of proteins or other substances with the necessary procedures.

Thus glucose, creatinine (and creatine), non-protein-N and uric acid are determined on protein-free filtrates from whole blood, urea and cholesterol in whole blood, chloride, phosphate and plasma proteins in blood plasma, phosphates in plasma or serum, and calcium in serum. Haemoglobin, present only in the cells, can be determined on whole blood, provided that at the same time the cell volume is also determined.

Preparation of Protein-free Filtrates from Blood

Various protein precipitants have been used, such as trichloracetic acid, tungstic acid (in the Folin-Wu procedure) and zinc hydroxide (Hagedorn-Jensen and Somogyi procedures). All of these have limitations to their uses. Thus the Folin-Wu filtrate contains besides glucose small amounts of other reducing substances (not fermentable by yeast), and so yields results for glucose which are too high by from 10 to 20 mg. per 100 c.c. It gives accurate results for creatinine, non-protein-N and uric acid. The filtrate for zinc hydroxide can be used to determine glucose and creatinine accurately, but gives too low values of non-protein-N (since most of the uric acid is precipitated by the zinc hydroxide). It is doubtful if a single precipitant for blood protein, suitable for all necessary determinations on the filtrate, is yet available.

Preparation of the Folin-Wu Filtrate.[1] The tungstic acid precipitant is obtained by mixture of accurately measured amounts of sodium tungstate and sulphuric acid. Accuracy is necessary to produce a mixture with a *pH* within well-defined limits.

[1] Folin and Wu, *J. Biol. Chem.*, 1919, XXXVIII., 81.

Volumes of water and of reagents bearing a strict ratio to whatever volume of blood is used must be employed, and consequently special pipettes have been devised (see Fig. 18, B).

Measure out accurately by means of a suitable pipette the volume of blood that is to be treated, and transfer it to a 200 c.c. Erlenmeyer flask (which should be clean and dry). The amount taken is considered as **one volume.** Add seven volumes of distilled water and shake up gently to lake the blood. Then add one volume of 10 per cent sodium tungstate and mix. Finally add very slowly drop by drop one volume of two-thirds normal sulphuric acid, with constant shaking. Close the flask by a rubber cork and continue shaking. Scarcely any air bubbles should form. After standing for five minutes the coagulated mass should have changed from red to chocolate-brown in colour.

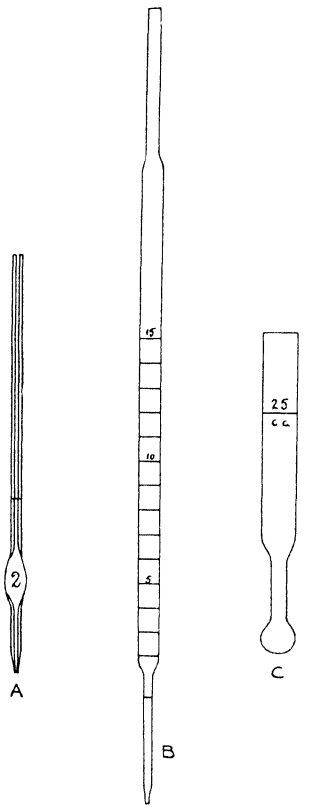


FIG. 18. A, Ostwald-Folin pipette; B, Folin-Wu dilution pipette; C, Folin-Wu sugar tube. One-quarter natural size.

Note. The sulphuric acid used should be standardised, although 35 gm. (19 c.c.) of concentrated acid made up to a litre with water give the approximate strength. Only sufficient sulphuric acid must be added to transform the tungstate to tungstic acid (leaving but a trace of free sulphuric acid over). In other words, the *pH* value

resulting must be within well-defined limits; too great an acidity must be avoided.

Note. Through various causes, and especially through addition of too much oxalate, the change of colour to dark chocolate-brown may be incomplete. Filtration at this stage gives a cloudy filtrate, the use of which leads to inaccurate results. Hence, if such incomplete colour change occurs add 10 per cent. sulphuric acid **drop by drop,** shaking vigorously for three or four minutes after each drop, and continue until there is no foaming and the colour is definitely dark brown. Since only a few drops are usually required, the volume change can be neglected.

Fit a filter paper to a funnel accurately, selecting a size of paper and funnel that will hold all the mixture of precipitate and filtrate. Both paper and funnel must be dry. Add a few drops of the mixture, pouring it down the double thickness side of the paper, and then wait until the whole of the paper has become moistened. Then pour all the mixture on to the paper, and cover with a large watch-glass. If the proportions taken have been accurate, the first drops of filtrate (as well as the remainder) should be perfectly clear.

From 10 c.c. of blood taken and so treated 60 or 70 c.c. of filtrate should easily be obtained.

Preparation of the Zinc Hydroxide Filtrate (after Somogyi).[1] To one volume of blood add seven volumes of distilled water, and shake up gently. Then add one volume of 10 per cent. sulphate ($ZnSO_4$, $7H_2O$) and one volume of 0·5 *N* sodium hydroxide.[2] Shake up, and filter. A clear solution should be obtained.[3]

[1] Somogyi, *J. Biol. Chem.*, 1930, LXXXVI., 655; LXXXVII., 339.

[2] The two solutions must be so related that when 10 c.c. of the zinc sulphate solution is diluted with 50 to 70 c.c. of water before titration, and titrated against the 0·5 *N* sodium hydroxide, added slowly, with continuous shaking, 10·8 to 11·2 c.c. of the latter are required to produce a permanent pink colour with phenolphthalein.

[3] Letonoff (*J. Biol. Chem.*, 1934, CVI., 693) has modified Somogyi's procedure by using as a precipitant dry zinc hydroxide. He adds 1 gm. of the dry powder to 1 c.c. of blood diluted with 9 c.c. of water, shakes up thoroughly, and filters. He claims that the clear filtrate gives true values for glucose when employed with the original Folin-Wu reagents for glucose determination.

Estimation of Blood Glucose on Folin-Wu Filtrate.[1]

Special Solutions

(1) THE STANDARD GLUCOSE SOLUTIONS. A stock solution is made up by dissolving 1 gm. of purest glucose accurately weighed out in 0·25 per cent. benzoic acid, and adding more of the benzoic acid solution (used as a preservative) to make 100 c.c. Two dilutions are prepared from this. The first, made by diluting 10 c.c. to 1 litre (again using the 0·25 per cent. benzoic acid solution), contains 0·1 mg. per cubic centimetre, and is used for all normal bloods; the second is made by diluting 20 c.c. of the stock solution to 1 litre, and therefore contains 0·2 mg. per cubic centimetre, and should be used to check readings made with the first that indicate a blood sugar value of 0·2 per cent. and over.

(2) THE FOLIN-WU ALKALINE COPPER SOLUTION. Dissolve 40 gm. of pure anhydrous sodium carbonate in about 400 c.c. of water in a litre flask, add 7·5 gm. of tartaric acid, shake until dissolved, and then add 4·5 gm. of pure crystallised copper sulphate. After complete solution make up with distilled water to 1 litre.

(3) PHOSPHOMOLYBDIC ACID SOLUTION. Add to a flask 35 gm. of molybdic acid, 5 gm. of sodium tungstate, 200 c.c. of 10 per cent. sodium hydroxide and 200 c.c. of water. Boil vigorously for thirty minutes, until most of the ammonia (present as impurity in the molybdic acid) has been boiled off. Cool, dilute to 350 c.c., add 125 c.c. of syrupy (85 per cent.) phosphoric acid, and finally dilute to 500 c.c.

Experiment 1. A special tube is taken—the Folin-Wu sugar tube—so constructed (Fig. 18, C) that 4 c.c. of liquid a little more than fills the lower bulb, reaching half-way into the constricted portion. Thereby only a minimal amount of surface is exposed to air and so possibly to oxidation. (Tubes that do not conform to this specification should be rejected.)

Pipette accurately, using an Ostwald-Folin pipette (Fig. 18, A), 2 c.c. of the Folin-Wu filtrate into this tube and into a second similar tube 2 c.c. of the standard glucose solution containing 0·1 mg. per cubic centimetre, and into a third 2 c.c. of the standard containing 0·2 mg. per cubic centimetre. To each add 2 c.c. of the alkaline copper

[1] Folin and Wu, *J. Biol. Chem.*, 1919, XXXVIII., 81; 1920, XLI., 367.

solution. Place in a large beaker containing boiling water and continue boiling the water for six minutes. Then remove to a rack and add immediately to each tube 2 c.c. of the special phosphomolybdic acid solution. *During the heating some of the cupric salt has been reduced to reddish-yellow cuprous oxide* (hence the necessity of preventing atmospheric oxidation). *This dissolves in the phosphomolybdic acid, reducing it to an intensely blue molybdenum salt, the intensity of the blue colour being proportional to the degree of reduction, and therefore to the amount of glucose originally present.*

As soon as the reduced copper has redissolved immerse the tubes in a beaker of cold water. When cool add distilled water to the 25 c.c. mark, cork with a clean rubber cork or close firmly with the thumb and invert several times until the contents are thoroughly mixed.

Transfer sufficient quantities of the unknown and the weaker standard to the cups of a colorimeter, set the standard solution at 20 mm., and read the unknown. (Repeat with the stronger standard if necessary.)

Calculation. Since both tubes contained 25 c.c., they are directly comparable. The weaker standard contained 2 c.c. of a sugar solution containing 0·1 mg. per cubic centimetre, and hence 0·2 mg. in all; in the other tube 2 c.c. of blood filtrate were used corresponding to 0·2 c.c. of the original blood sample. Hence the amount of glucose present in 0·2 c.c. of blood is

$$0{\cdot}2 \times \frac{20}{\text{reading of unknown}} \text{ mg.}$$

Multiply by 500 (*i.e.*, 100/0·2) to get the corresponding value per 100 c.c. of blood in milligrams, or by 500/1,000, or 0·5, to get the figure in grams. The calculation with the stronger standard should be obvious.

Example. The reading of the unknown with the weaker standard was 16·3. Hence 0·2 c.c. of blood contained

$$\frac{0{\cdot}2 \times 20}{16{\cdot}3}, \text{ or } 0{\cdot}245, \text{ mg.}$$

Hence 100 c.c. of blood contained 123 mg., or 0·123 gm., of glucose.

Note 1. Since the exact amount of reduction depends upon the degree of heating, time of heating, etc., which may vary slightly in every determination, two standard tubes

should always be run with each unknown, and not more than two or three unknowns be run at one time.

Note 2. In this and many other colorimetric determinations the colour developed is not rigidly proportional to the concentration when markedly different concentrations are compared. Hence the necessity of more than one standard to ensure accurate results.

Note 3. The above estimation should first of all be

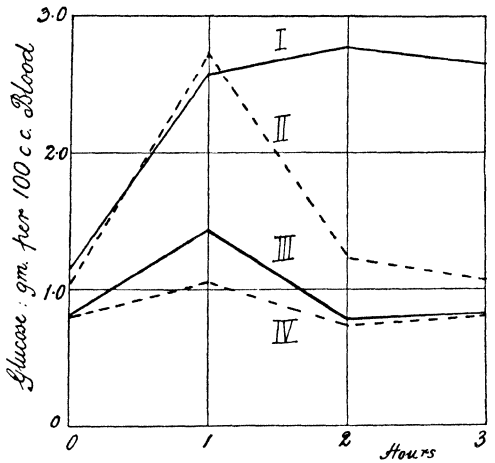


FIG. 19. Typical sugar tolerance curves.
I, Diabetes mellitus ; II, hyperthyroidism with glycosuria ; III, normal curve ; IV, renal glycosuria.

demonstrated to a group of students before they are allowed to carry out the determination themselves.

Estimation of Blood Glucose on Somogyi Filtrate

The estimation is carried out exactly as for the Folin-Wu filtrate, except that a different copper reagent is employed.

Somogyi's Modified Shaffer-Hartmann Copper Reagent.[1] Dissolve successively in 300 or 400 c.c. of water 7 gm. $CuSO_4$, $5H_2O$, 20 gm. Na_2CO_3, 25 gm. $NaHCO_3$, and 25 gm. Rochelle

[1] Somogyi, *J. Biol. Chem.*, 1928, LXXX., 733.

salt. Transfer to a graduated litre flask, and add distilled water to the mark.

DETERMINATION OF SUGAR TOLERANCE

A glucose tolerance test is frequently carried out in order to differentiate between the conditions of mild diabetes, hyperthyroidism with glycosuria and renal glycosuria.

Experiment 2. The subject should preferably have taken no breakfast, and in any case should have been several hours without food.

A sample of blood is taken, and the subject empties his bladder; the urine is preserved for test.

A solution containing 50 gm. of glucose [1] (fairly pure powder, not syrup) is made up to 200 or 300 c.c. with water and flavoured with the juice of a lemon. This is swallowed by the patient, and the time of swallowing it is considered as zero time. Then after one, two or three hours blood samples are taken and the bladder emptied.

All urine samples are tested for reducing sugar, and the glucose content is determined on each sample of blood. Typical sugar tolerance curves are shown in Fig. 19. In the abnormal conditions represented in three of these curves sugar is usually found in the urine during the greater part of the test.

Further Notes on Methods of determining Blood Sugar

Modifications of the Folin-Wu Procedure. As already stated, it is now recognised that sugar values obtained by this procedure are 10 to 20 mg. per 100 c.c. too high. Both Benedict [2] and Folin [3] proposed several modifications of the original copper reagent designed to yield values which approximate more closely to the true glucose content. They possess no advantage over the Somogyi procedure.

Titrimetric Procedures. The methods most commonly used

[1] There is little advantage in giving more than 50 gm. of glucose. Smaller amounts give a depressed tolerance curve. (*Cf.* Maclean and de Wesselow, *Quart. J. Med.*, 1920–21, XIV., 103.)

[2] Benedict, *J. Biol. Chem.*, 1925, LXIV., 207; 1926, LXVIII., 759; 1928, LXXVI., 457; 1931, XCII., 141.

[3] Folin, *J. Biol. Chem.*, 1926, LXVII., 357; 1929, LXXXII., 83; Folin and Svedberg, *J. Biol. Chem.*, 1926, LXX., 405; Folin, 1929, LXXXII., 83.

are those of Maclean,[1] Shaffer and Hartmann,[2] and Hagedorn and Jensen.[3] These methods all depend on the titration of iodine against standard thiosulphate solutions.

In Maclean's method the blood proteins are precipitated by dialysed iron, and an aliquot part of the filtrate is boiled with an alkaline copper solution containing potassium iodate and iodide. On the addition of hydrochloric acid free iodine liberated from the iodate combines with cuprous chloride, and the excess iodine is titrated with thiosulphate. This value, subtracted from that of a blank estimation, gives the amount of cuprous salt formed, whence the glucose content can be calculated. Although this method has had considerable clinical application, it is not much more accurate than the colorimetric procedure, involves more manipulation and requires much more time.

The principle of the Shaffer-Hartmann method is substantially the same as Maclean's, and similar results are obtained; used with Somogyi's zinc hydroxide precipitant it is said to be accurate, and more convenient.

In the Hagedorn-Jensen procedure the blood proteins are precipitated by zinc hydroxide, the filtrate heated with potassium ferricyanide solution, and iodide solution added, and the amount of iodine freed (equivalent to the ferricyanide reduced) is titrated against thiosulphate solution. In experienced hands this is probably the most accurate method, but it is not so suitable as the colorimetric method for clinical use.

Estimations on Small Amounts of Blood. Several of the micro-methods which have been used for the estimation of blood sugar in cases where only very small amounts of blood are obtained are modelled on existing macro-methods. With the original method of protein precipitation Byrd's modification of the Folin-Wu procedure [4] employs 0·1 c.c. of blood and one-fourth the quantities of reagents called for in the regular procedure. It is reasonably accurate, but, as with the macro-method, gives results which are too high.

Other methods which may be mentioned are those of

[1] Maclean, *J. Physiol.*, 1916, L., 168; *Biochem. J.*, 1919, XIII., 135.
[2] Shaffer and Hartmann, *J. Biol. Chem.*, 1921, XLV., 365.
[3] Hagedorn and Jensen, *Biochem. Zeitschr.*, 1923, CXXXV., 46; CXXXVII., 92.
[4] Byrd, J. A., *J. Am. Med. Assoc.*, 1924, LXXXIII., 508.

Bang,[1] Gardner and Maclean's modification [2] of Bang's, and Folin's new ferricyanide method.[3] Bang's method is time-consuming, and requires an experienced technician to obtain results which are of any value. In Folin's method the sugar is oxidised by alkaline potassium ferricyanide and the ferrocyanide so produced is estimated colorimetrically as Prussian blue. Benedict adapted his tungsto-molybdic method to micro-procedures and claimed that it is accurate and highly specific.[4]

As far as students' work is concerned, it does not seem advisable to teach these micro-methods of blood sugar analysis, since little is gained from the point of view of training in procedures, and the inaccuracy of result is bound to be considerable where the worker is inexperienced. From the clinical standpoint in almost all cases sufficient blood can be obtained to carry out sugar estimations by the usual procedure. Young children, with the fontanelle closed, and patients with small and difficultly accessible veins (especially when several determinations are necessary), may be considered exceptions. In such cases Byrd's modification seems suitable to be used by the average technician.

Estimation of Blood Creatinine [5]

The determination is carried out in a manner similar to that employed for urine, but modified on account of the much smaller amount of creatinine present. The colour is an intense orange instead of orange-red, and is correspondingly more difficult to read accurately.

The importance of a creatinine determination in disease lies in ascertaining whether the value is normal or much above normal. According to Gaebler [6] the creatinine which can be isolated from blood is present there in the form of some unknown precursor. Whether or not this be true, clinical conclusions based upon values for so-called blood creatinine are still valid.

[1] Bang, *Biochem. Zeitschr.*, 1913, XLIX., 1 ; 1914, LVII., 300.
[2] Gardner and Maclean, *Biochem. J.*, 1914, VIII., 300.
[3] Folin, *J. Biol. Chem.*, 1928, LXXVII., 421 ; Folin and Malmyos, *ibid.*, 1929, LXXXIII., 115 ; Folin and Svedberg, *ibid.*, 1930, LXXXVIII., 85.
[4] Benedict, *J. Biol. Chem.*, 1931, XCII., 141.
[5] Folin, *Zeitschr. f. physiol. Chem.*, 1934, CXXVIII., 268.
[6] Gaebler, *J. Biol. Chem.*, 1930, LXXXIX., 451.

Creatinine Standard. Pipette accurately 1 c.c. of the (urine) standard creatinine solution (1 gm. per litre; *cf.* Exercise XIX., Experiment 1) into a 500 c.c. volumetric flask, and then add distilled water to the 500 c.c. mark. Mix thoroughly. Five cubic centimetres of this solution contain 0·010 mg. creatinine.

Additional Reagents required are a 2 per cent. solution of sodium hydroxide and a 1 per cent. solution of pure sodium picrate.[1]

Experiment 3. Take three small, dry Erlenmeyer flasks. Into the first pipette 10 c.c. of the Folin-Wu or Somogyi blood filtrate, into the second 5 c.c. of the standard and 5 c.c. of distilled water, and into the third 10 c.c. of the standard. To each add 1 c.c. of 1 per cent. sodium picrate solution and 1 c.c. of 2 per cent. sodium hydroxide. Shake, allow to stand thirty minutes, and compare in the colorimeter the unknown with that standard solution nearest its intensity in colour. If the unknown appears to be much darker than the stronger standard, before comparison in the colorimeter dilute the unknown by the addition of 10 c.c. water, 1 c.c. picrate solution, and 1 c.c. sodium hydroxide.

Note. In this estimation it is particularly important to check the colorimeter by measuring the standard in both cups (*cf.* Exercise XIX., "Checking the Colorimeter").

Calculation. Since all solutions contain the same proportions of the reagents, they are directly comparable.

[1] *Preparation of pure sodium picrate* (Folin's method). Add 100 c.c. of acetone to 100 gm. of moist picric acid in a 500 c.c. flask, immerse the flask in warm water, and shake until all the picric acid has dissolved. Add about 4 gm. of norite, shake up, and filter, taking care to prevent evaporation of the solvent.

Dissolve 50 gm. of anhydrous sodium carbonate and 20 gm. of sodium chloride in 500 c.c. of warm water in a 1 litre beaker. Then add slowly, with stirring, the acetone solution of picric acid. When there is no further evolution of carbon dioxide, place the beaker in cold water for about thirty minutes, then filter with suction through a large Buchner funnel, and wash the precipitate with about 500 c.c. of 7 per cent. sodium chloride solution. Suck as dry as possible.

Recrystallise the precipitate of sodium picrate by transferring it to a beaker, adding about 500 c.c. of boiling water, and 4 gm. of anhydrous sodium carbonate. Then add slowly, with stirring, 30 gm. of sodium chloride. After solution is complete, cool, and filter off the sodium picrate. Wash with 7 per cent. and then with 2 per cent. sodium chloride, and finally with a little methyl alcohol. Dry at room temperature.

If the weaker standard is employed, then the amount of creatinine in 10 c.c. of the blood filtrate, **or in 1 c.c. of the original blood,** is

$$0{\cdot}01 \times \frac{\text{Reading of standard}}{\text{Reading of unknown}} \text{ in milligrams.}$$

This, multiplied by 100, gives the value per 100 c.c. of blood.

If the stronger standard is used, the value is

$$0{\cdot}02 \times \frac{\text{Reading of standard}}{\text{Reading of unknown}} \text{ in milligrams.}$$

Example. The weaker standard is used and set at 30 mm. The four readings of the unknown average 22·1 mm. Hence the creatinine in 1 c.c. of blood is

$$0{\cdot}01 \times 30/22{\cdot}1 \text{ mg., equal to } 0{\cdot}0136 \text{ mg.,}$$

and the creatinine per 100 c.c. of blood is 1·4 mg.

Estimation of Uric Acid in Blood

This estimation has little clinical application of value, except in cases of gout, and introduces no new type of procedure. One of the best methods available is that of Folin,[1] which depends on the precipitation of uric acid as silver urate and its subsequent reaction with a special phosphotungstic acid reagent (free from molybdate) to produce a blue coloured compound. The original papers should be consulted.

Estimation of Non-Protein-N

This can be more conveniently dealt with after the estimation of urea has been described (see next Exercise).

[1] Folin, *J. Biol. Chem.*, 1933, CI., 111 ; 1934, CVI., 311.

EXERCISE XXVIII

ESTIMATIONS ON WHOLE BLOOD NOT INVOLVING PROTEIN PRECIPITATION AND OF NON-PROTEIN NITROGEN

(*Two or three Three-hour Periods*)

UREA and cholesterol are generally estimated on whole blood by special procedures. These should be demonstrated to the student, who can thereafter carry out one or both of them.

Estimation of Urea

The principle is the same as that of the estimation of urea in urine. Since blood freshly drawn contains but a negligible trace of ammonia, no simultaneous determination of ammonia is required (*cf.* Exercise XXII.).

Experiment 1. Pipette accurately 3 c.c. of oxalated blood into a thick-walled test-tube of the Van Slyke-Cullen apparatus, crush up a urease tablet (of a sample known to be active) and add the powder to the tube, and incubate at 37° to 40° C. for thirty minutes. Then connect up with a second tube containing 10 c.c. of N/50 sulphuric acid and 2 drops of methyl red indicator, and proceed exactly as in the urea determination of Exercise XXII.

Note. With pathological blood it not infrequently happens that the amount of ammonia formed is sufficient to more than neutralise the amount of acid taken. Hence at the first sign of colour change in the acid tube compress the rubber tubing connecting the two tubes, and rapidly remove the cork from the acid tube and introduce a further 10 c.c. of N/50 acid, immediately reclosing the tube and reconnecting.

Calculation. Convert the acid and alkali figures into their true N/50 values by using the previously determined factors for each. Subtract these values. The difference is the

amount of N/50 acid used to neutralise the ammonia that has been formed from urea. It is therefore equivalent to the same number of cubic centimetres of N/50 ammonia, and therefore of N/50 solution. Multiply by 0·28 (*cf.* calculation of Exercise XXII.). The figure obtained is the number of milligrams of urea-N in 3 c.c. of blood. Multiply by 100/3 to get the value for 100 c.c. of blood. To obtain the figure for **Urea** multiply by 2·14 (*cf.* Exercise XXII.).

Example. Ten cubic centimetres of N/50 sulphuric acid with a factor 0·961 were taken, and 7·64 c.c. of N/50 hydroxide with a factor 1·023 were required. The corrected values are respectively 9·61 and 7·82 c.c., and the difference is 1·79 c.c. This, multiplied by 0·28 × 100/3, gives

16·7 mg. urea-N per 100 c.c. blood.

This figure, multiplied by 2·14, gives

35·7 mg. **Urea** per 100 c.c. blood.

Note. Figures for urea-N are generally used clinically. Normal limits are :—

12 to 15 mg. urea-N per 100 c.c. blood ;
25 to 32 mg. **Urea** per 100 c.c. blood.

Higher values are found, especially in cases of nephritis, and increase with increasing severity of the kidney condition.

Note. Accuracy of technique in using this procedure can be checked by running a control in which, instead of blood, 3 c.c. of an exactly 0·03 per cent. solution of pure urea are taken, corresponding in urea content to an average normal blood. The result 14 mg. urea-N per 100 c.c. should be obtained.

Various methods have been devised involving nesslerisation of the ammonia and colorimetric measurement. That of Karr [1] is one of the most expeditious. Such types of procedure are also employed in determining non-protein nitrogen, after the initial digestion. In our experience reasonably accurate results are obtained somewhat more easily by the titrimetric procedures described in the test.

[1] Karr, *J. Lab. Clin. Med.*, 1924, IX., 3.

Estimation of Non-Protein Nitrogen [1]

A combination of micro-Kjeldhal and aspiration methods is used.

Special Acid Mixture. Add 30 c.c. of 85 per cent. (syrupy) phosphoric acid to 5 c.c. of 5 per cent. copper sulphate. After mixing add 10 c.c. of pure concentrated sulphuric acid and mix. Dilute with an equal volume of water before using. (Keep stoppered and away from all possible contact with ammonia fumes.)

Experiment 2. Transfer 5 c.c. of the *Folin-Wu filtrate* to a large, dry Pyrex test-tube. Add 1 c.c. of the diluted acid mixture and a glass bead. Boil vigorously over a small flame (preferably of a micro-burner) until dense fumes fill the whole of the tube (three to seven minutes). Insert a funnel into the mouth of the tube, and continue heating **gently** until the solution is almost colourless (two or three minutes). Allow to cool. Transfer to a urea tube of the Van Slyke-Cullen apparatus, connect up as described in the previous experiment with a tube containing N/50 sulphuric acid, add potassium carbonate and aspirate and titrate as described in Experiment 1.

The calculation is similar. The amount of Folin-Wu filtrate taken corresponds to 0·5 c.c. of blood, instead of the 3 c.c. used for the urea determination. Hence an error of 1 drop in the final titration is of much greater significance.

Estimation of Cholesterol

The principle of the determination is based upon the blue-green colour developed by cholesterol under the action of acetic anhydride (*cf.* Exercise VII., Experiment 9). The difficulties of the determination are two; first, the exclusion of water; and secondly the development of a brownish tinge in the blood extract, which renders exact comparison with the standard difficult. The nature of the brown impurity is still undetermined. The method outlined is that of Cornell [2] and removes

[1] Although this determination is carried out on the Folin-Wu filtrate, it is more conveniently described at this point, since it involves much of the procedure described in the previous experiment.

[2] Cornell, *J. Lab. Clin. Med.*, 1928, XIV., 251.

these difficulties fairly satisfactorily. It is a modification of Bloor's original method.[1]

STANDARD SOLUTION OF CHOLESTEROL. Dissolve 1 gm., accurately weighed, of pure recrystallised cholesterol in **pure, dry** chloroform in a 100 c.c. graduated volumetric flask. Fill up to the mark with the chloroform. One cubic centimetre of this solution contains 10 mg. of cholesterol. Pipette accurately 1 c.c. of this into a second similar 100 c.c. flask, and add chloroform to the mark. *One cubic centimetre of this diluter solution contains* 0·1 *mg. of cholesterol.*

Experiment 3. Prepare an alcohol-ether mixture containing two parts of absolute alcohol (99·8 per cent.) and one part of **redistilled** ether.

Pipette accurately 3 c.c. of (oxalated) blood into 75 c.c. of the alcohol-ether mixture, allowing the blood to run in slowly with shaking or stirring. Use a previously *dried* 150 c.c. beaker. Shake carefully. Filter into a **dry** 250 c.c. Erlenmeyer flask. Wash the beaker and the residue on the filter paper with an additional 50 c.c. of the alcohol-ether mixture, using small amounts at a time. Reduce the amounts of the combined filtrates to 80 to 90 c.c. by boiling carefully on a water-bath containing boiling water. Cool, transfer to a **dry** 100 c.c. graduated volumetric flask, rinse the Erlenmeyer flask with a little of the alcohol-ether mixture, add the rinsings, and make up with more of the mixture exactly to the 100 c.c. mark.

Pipette accurately 50 c.c. of this 100 c.c. into a **dry** 150 c.c. Erlenmeyer flask, evaporate to dryness on the *water-bath* at 80° (not an electric plate, and temperatures above 80° must be avoided) and carefully apply a partial vacuum (by connecting with a suction pump) to the last drop or two while the flask is **still hot.** (During the heating on the water-bath insert a small funnel in the mouth of the flask to avoid splashing.)

Treat the residue three or four times with 5 or 6 c.c. of pure **dry** (water-free) chloroform, transferring through a funnel to a 25 c.c. graduated flask. Make up finally with the chloroform to the 25 c.c. mark. *This 25 c.c. of chloro-*

[1] Bloor and Knudson, *J. Biol. Chem.*, 1916, XXVII., 463; Bloor, *ibid.*, 1917, XXIX., 437.

form solution contains all the cholesterol originally present in 1·5 c.c. of blood.

Pipette accurately 5 c.c. of this solution into a graduated 10 c.c. cylinder ; add 2 c.c. of pure acetic anhydride and 0·1 c.c. of concentrated sulphuric acid. Fill to the mark with chloroform. Shake up and allow to stand (away from light, since light gradually destroys the blue-green colour) for fifteen minutes.

Make up the standard for comparison with 5 c.c. of the weaker cholesterol standard, 2 c.c. of acetic anhydride and 0·1 c.c. of concentrated sulphuric acid, and then chloroform to the 10 c.c. mark, and also allow to stand for fifteen minutes away from light. Compare in the colorimeter with the standard set at 20 mm.

Calculation. The 5 c.c. of chloroform extract taken correspond to 0·3 c.c. of blood.

The 5 c.c. of standard taken contain 0·5 mg. of cholesterol.

Hence $0{\cdot}5 \times \frac{\text{Reading of standard}}{\text{Reading of unknown}}$ gives the number of milligrams of cholesterol in 0·3 c.c. of blood. Multiply by 100/0·3 to get the amount per 100 c.c. of blood.

Example. Reading of standard at 20 mm. ; reading of unknown (mean), 17·5 mm. Hence $20/17{\cdot}5 \times 0{\cdot}5 \times 100/0{\cdot}3$ gives the result—190 mg., or 0·19 gm., cholesterol per 100 c.c. blood.

EXERCISE XXIX

ESTIMATION OF CERTAIN BLOOD SERUM CONSTITUENTS

(Two Three-hour Periods)

Calcium [1]

THE principle of this method is one frequently employed in calcium determinations on a larger scale. The calcium is precipitated as oxalate, and the oxalate is then dissolved in acid and titrated against very dilute permanganate. Since normal blood serum only contains 10 to 11 mg. of calcium per 100 c.c. (while certain pathological bloods contain much less),[2] errors that can be disregarded in larger-scale operations become of importance. Two errors especially must be considered: the calcium oxalate must be freed from the soluble oxalate used as precipitant, or else too high values are obtained; the calcium oxalate itself, although usually regarded as very insoluble in water, is sufficiently soluble to cause appreciable loss and too low values when it is subjected to several washings. Exact conditions of analysis are so chosen that these positive and negative errors balance each other.

Experiment 1. Eight or ten cubic centimetres of blood are drawn from an arm vein **without the use of an anticoagulant** into a centrifuge tube and allowed to clot. After about an hour the clot is broken up with a glass rod and the tube centrifuged. Four or five cubic centimetres of clear yellow serum should be obtained.

[1] After Clark and Collip's modification (*J. Biol. Chem.*, 1925, LXIII., 461) of the Tisdall procedure (*J. Biol. Chem.*, 1923, LVI., 439).

[2] Values below normal are found in cases of tetany associated with diminished parathyroid function, and in certain types of rickets. High values are occasionally met with, as in the condition of generalised osteitis fibrosa, associated with increased parathyroid function.

Pipette 2 c.c. of this serum accurately into a centrifuge tube. (Preferably duplicate the whole procedure with a second 2 c.c. if there be sufficient serum.) Add 1 c.c. of a saturated solution of ammonium oxalate, stir up with a thin glass rod, wash off the rod with 1 c.c. of doubly distilled water (see below) and allow the tube to stand for fifteen minutes, during which time the precipitate of calcium oxalate will form completely and commence to settle. Then centrifuge fifteen minutes. Pour off the supernatant fluid, add 2 c.c. of 2 per cent. ammonia solution (made up with doubly distilled water; the calcium oxalate is a little less soluble in this ammonia solution than in distilled water), stir, wash off the rod again with 2 c.c. of the 2 per cent. ammonia solution, centrifuge fifteen minutes, pour off the supernatant liquid, again wash with the ammonia water and once more centrifuge. Pour off the supernatant liquid, allow to drain on to filter paper for fifteen minutes, and finally wipe off the mouth of the inverted tube with dry filter paper.

Add 4 c.c. of N/1 sulphuric acid, heat on an electric plate to 70° to 72° C. and titrate against N/200 potassium permanganate contained in a 5 c.c. burette graduated to hundredths. This burette should end in a fine point so that a small drop or part of a drop can be used at will. The titration is ended when the merest tinge of pink colour persists for one minute. After each addition of permanganate the tube must be stirred well with a thin glass rod.

THE PERMANGANATE SOLUTION. Dissolve 4 gm. of pure potassium permanganate in 1 litre of redistilled water. This is an approximately N/10 solution. Make up 50 c.c. of this to 1 litre with redistilled water. The resulting approximately N/200 solution deteriorates slowly and must always be standardised before use. It is conveniently titrated against N/100 oxalic acid (0·630 gm. of pure oxalic acid dissolved in 1 litre of redistilled water).

Pipette accurately 2 c.c. of this oxalic acid into a centrifuge tube, add 4 c.c. of N/1 sulphuric acid, heat to 70° to 72° C. and titrate against the permanganate as already described.

Calculation. One cubic centimetre of N/100 oxalic acid corresponds to 1 c.c. of N/100 solution of a calcium salt, and therefore to 1 c.c. of an N/100 solution of calcium ions [40/(2 × 100) gm. per litre] which contains 0·2 mg. of calcium per cubic centimetre.

Two cubic centimetres of an accurate solution of N/200 potassium permanganate correspond to 1 c.c. of N/100 oxalic acid, whence 1 c.c. should correspond to 0·1 mg. of calcium.

The factor for the permanganate is given by—

Number of cubic centimetres of permanganate used for 2 *c.c. of N/*100 *oxalic acid divided into* 4.

Hence, to determine the calcium present, multiply the number of cubic centimetres of permanganate used for the calcium oxalate by the factor and then by 0·1. The result is the number of milligrams of calcium in 2 c.c. of serum. Multiply by 100/2, *i.e.*, by 50, to get the value per 100 c.c. of serum.

Example. In the standardisation of the permanganate 4·483 c.c. were used for 2 c.c. of the N/100 oxalic acid solution.

In the titration of the calcium oxalate 1·614 c.c. were used.

The factor is 4/4·483, *i.e.*, 0·892.

$1{\cdot}614 \times 0{\cdot}892 \times 0{\cdot}1 = 0{\cdot}144$ mg. calcium per 2 c.c. serum. Multiplying by 50, the final result is—

7·2 mg. per 100 c.c. serum.

Note. Redistilled water must be used for accurate work, since ordinary distilled water frequently contains non-negligible traces of calcium salts. To somewhat more than a litre of distilled water in a 2 litre distillation flask, preferably of Pyrex or some similar hard glass, add a few crystals of potassium permanganate and a few drops of concentrated sulphuric acid. Distil, using a block tin condenser, and collect in a steamed-out large Erlenmeyer flask. Use the middle fraction of the distillate.

PHOSPHATASE

Blood contains one or more phosphatases, enzymes which hydrolyse organic to inorganic phosphate. Phosphatase can be conveniently estimated on either blood serum or plasma. Since the pure enzymes have not been prepared, the amounts present can only be expressed in terms of empirical units. King and Armstrong have recently published a convenient method suitable for clinical use.[1]

[1] *Can. Med. Assoc. J.*, 1934, XXXI., 376 ; 1935, XXXII., 379.

Reagents

1. *Buffered Substrate.* This consists of 0·005 *M* phenyl phosphate in 0·05 *M* veronal. Dissolve 10·3 gm. of sodium veronal (sodium salt of di-ethyl malonyl urea) and 1·09 gm. of disodium phenyl phosphate in water and dilute to 1 litre. Preserve in a well-stoppered bottle with a few drops of chloroform. Keep in an ice chest when not in use. (The solution will keep for at least one month.)

2. *Phenol Reagent of Folin and Ciocalteu.*[1] Introduce into a 1,500 c.c. flask 100 gm. of sodium tungstate, Na_2WO_4, $2H_2O$, 25 gm. of sodium molybdate, Na_2MoO_4, $2H_2O$, 700 c.c. of water, 50 c.c. of 85 per cent. phosphoric acid, and 100 c.c. of concentrated hydrochloric acid, and heat under a reflux condenser for ten hours. Remove the condenser and add 150 gm. of lithium sulphate, 50 c.c. of water, and a few drops of bromine. Boil (without condenser) for fifteen minutes to remove excess bromine. Cool, dilute to 1 litre, and filter. There should be no greenish tinge. Keep corked. *Dilute this reagent* 1 *in* 3 *for use.*

3. *Sodium carbonate* (Na_2CO_3), 20 per cent. solution.

4. *Stock Standard Phenol Solution.* Dissolve 1 gm. of crystalline phenol in 0·1 *N* hydrochloric acid, and make up to 1 litre with 0·1 *N* hydrochloric acid. Determine the exact strength of this solution.[2] It keeps indefinitely.

[1] *J. Biol. Chem.*, 1927, LXXIII., 627.

[2] To determine the strength of the *stock phenol solution*, transfer 25 c.c. to a 250 c.c. flask, add 50 c.c. of 0·1 *N* sodium hydroxide, heat to 65° C., add 25 c.c. of 0·1 *N* iodine solution, stopper the flask, and allow to stand at room temperature for thirty to forty minutes. Then add 5 c.c. of concentrated hydrochloric acid, and titrate the excess of iodine with 0·1 *N* thiosulphate solution. Each c.c. of 0·1 *N* iodine solution is equivalent to 1·567 mg. of phenol.

To make 0·1 *N iodine solution* place 1·27 gm. of pure resublimed iodine in a 100 c.c. graduated flask, dissolve 1·8 gm. of potassium iodide in 15 c.c. of water and add this to the flask (using it to wash in the last trace of iodine if necessary), and shake occasionally until the iodine is dissolved. This may require some time; if necessary, a trace of potassium iodide crystals can be added to facilitate complete solution. Then dilute to the mark with distilled water. Mix well. Keep stoppered in the dark.

To make a 0·1 *N sodium thiosulphate solution*, dissolve 25 gm. of pure sodium thiosulphate in water and dilute to 1 litre (using boiled distilled water throughout). Standardise against potassium hydrogen iodate. Dissolve 0·3249 gm. of the latter in 100 c.c. to give an exactly 0·1 *N* solution. Take 25 c.c. of this solution, add about 1 gm. of

5. *Diluted Stock Phenol Solution.* Dilute the stock solution No. 4 so that the diluted solution contains exactly 10 mg. per 100 c.c. (For example, if solution 4 contained exactly 100 mg. per litre, the dilution would be exactly 1 in 10. If the solution contained 94·5 mg. the dilution would be 1 in 9·45 or 10·58 in 100.) This solution keeps in the ice box at least three months.

6. *Standard Phenol Solution and Reagent.* To 5 c.c. of diluted stock standard (No. 5) add 15 c.c. of phenol reagent (No. 2 diluted), and then water to 50 c.c. The solution contains 1 mg. of phenol per 100 c.c. It must be made up daily as required.

Experiment 2. Draw 10 c.c. of blood from an arm vein and allow to clot. The clot is then loosened and centrifuged. The serum is poured off into a centrifuge tube and again centrifuged to get rid of any suspended cells. (If necessary, it can be kept overnight in an ice box.)

(A) *Enzymic Decomposition of Phenyl Phosphate.* Transfer to each of two test-tubes [1] 10 c.c. of the buffered substrate (No. 1). Place the tubes in a water-bath or oven at 37° C. for a few minutes to warm the solution to that temperature. Then as quickly as possible add exactly 0·5 c.c. of serum to each, stopper, mix, and allow to remain in the bath (or oven) exactly thirty minutes. Then add at once 4·5 c.c. of diluted phenol reagent (No. 2), mix, and filter.

Pipette 10 c.c. of the filtrate (in each case) into a clean test-tube, add 2·5 c.c. of 20 per cent. sodium carbonate solution, mix, and place these tubes in water-bath (or oven) for five minutes to develop the maximum blue colour.

(B) *Control.* Duplicate (A), except that the two tubes are not heated, but to each 4·5 c.c. of the diluted phenol reagent are added *immediately after* addition of the 0·5 c.c. of serum. The solutions are then filtered, 2·5 c.c. of sodium

potassium iodide crystals, and a few c.c. of dilute hydrochloric acid, and titrate the liberated iodine against the thiosulphate. When the solution is pale yellow add 1 c.c. of 1 per cent. (soluble) starch solution, and then titrate until the blue colour of the starch-iodide just vanishes. Calculate the factor for the thiosulphate solution. *E.g.*, if 25 c.c. of thiosulphate are required, the solution is exactly 0·1 *N*. If 24·70 c.c. were required, the factor would be 1·012, and the thiosulphate 0·1012 *N*.

[1] The determination is duplicated to ensure greater accuracy.

carbonate added to 10 c.c. of the filtrate, and the tubes warmed for five minutes as in (A).

(C) *Standard.* Place 10 c.c. of No. 6 reagent (standard phenol solution and reagent) in a test-tube, and add 2·5 c.c. of 20 per cent. sodium carbonate solution. Warm for five minutes as with (A) and (B).

(D) *Comparison.* The four solutions of (A) and (B) (unknowns) are compared with the standard by placing the *unknown* on the left side of the colorimeter, set at 30 mm., and the standard on the right side, and matching the colours.

Calculation. The unit adopted for phosphatase activity is the amount of phosphatase in 100 c.c. of serum which will liberate 1 mg. of phenol from the phenyl phosphate under the standard conditions.[1]

Since the tubes of (A) and (B) contain 15 c.c., while only 10 c.c. of the filtrates are taken for comparison with 10 c.c. of the standard, the value of phenol for each, calculated for 100 c.c. of serum, is obtained by

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \text{Strength of standard} \times \frac{15}{10} \times \frac{100}{0{\cdot}5}$$

and, since the strength of the standard is 0·1 mg., and the unknown is set at 30, this formula becomes

$$\frac{\text{Reading of standard}}{30} \times 0{\cdot}1 \times \frac{15}{10} \times \frac{100}{0{\cdot}5}$$

which is, numerically, the " Reading of standard."

The means of the two values of (A) and (B) are taken, and their difference gives the phosphatase activity of the serum.

Note. When the serum contains more than 30 units of phosphatase, but less than 60, the unknown should be set at 15 mm., and the final value multiplied by 2. With still higher enzyme content it is found that the products of the reaction, especially the inorganic phosphate, retard the reaction, leading to too low values. In such cases it is desirable to dilute the serum with normal saline to such an extent that the number of units in the diluted serum will not exceed 60, correcting for the dilution in the calculation.

[1] The unit employed by King and Armstrong is numerically almost exactly equal to that of Jenner and Kay (*Brit. J. Expt. Path.*, 1932, XIII., 22), although the latter is expressed in terms of phosphorus set free in inorganic phosphate.

Normal values lie between 4 and 13. High values (up to 100 or more) are found in generalised bone disease, such as generalised osteitis fibrosa and rickets, in cholecystitis and cholelithiasis without obstruction, and in infective and obstructive jaundice.

EXERCISE XXX

ESTIMATIONS OF CERTAIN BLOOD PLASMA CONSTITUENTS

(Two Three-hour Periods)

Chlorides

THE chloride figure for whole blood is of no special significance, since it is a variable depending upon the relative volume of cells and plasma. Normal plasma contains about 0·6 per cent. expressed as sodium chloride, while normal red cells contain only 0·3 per cent., similarly but incorrectly expressed. With the usual cell volume of about 40 per cent. of the whole blood, normal whole blood consequently contains about 0·48 per cent. (sodium) chloride. Any conditions leading to reduction of the red cells of the blood consequently give an increased value for whole blood, and such changes may mask the really significant changes in the plasma and cell chloride values.

The figure for plasma chloride can be easily determined and has some clinical significance. That for cell chloride also is of some clinical importance, but must be determined directly on the cells for accuracy; this determination is more difficult. Two methods are available for plasma chloride determinations: the Whitehorn, similar to that used for urine (*cf.* Exercise XXIII.), and the Van Slyke, somewhat more accurate, but requiring more time.

Preparation of Material. Blood is taken from an arm vein, oxalated and centrifuged. The clear plasma is then ready for analysis by either the Whitehorn or the Van Slyke method.

Experiment 1. The Whitehorn Procedure. Precipitate the plasma proteins by taking two volumes of plasma, sixteen volumes of distilled water, one volume of sodium tungstate and one volume of 2/3 N sulphuric acid. Filter.

Pipette accurately 10 c.c. of the filtrate into a porcelain basin. Add, accurately measured with a pipette, 10 c.c. of a standard silver nitrate solution (see below), stir thoroughly, add approximately 10 c.c. of concentrated nitric acid and stir again. Allow to stand five minutes. The silver chloride separates as a curdy precipitate. Add as much powdered ferric alum as will go on a silver threepenny or five-cent piece. Titrate against standardised thiocyanate solution (see below) until a salmon-pink colour just persists for at least fifteen seconds.

Standard Silver Nitrate Solution. Dissolve 2·905 gm. of pure silver nitrate in distilled water and make up exactly to 1 litre. One cubic centimetre of this solution is equivalent to 1 mg. of sodium chloride.

Thiocyanate Solution. Dissolve 1·4 gm. of ammonium thiocyanate in 1 litre of water. Standardise against the silver nitrate solution, taking 10 c.c. of this, 10 c.c. of concentrated nitric acid, the amount of iron alum indicated above, and titrating as directed above. The factor is based on the theoretical relationship of 1 c.c. of silver nitrate being equivalent to 1 c.c. of thiocyanate. Hence this factor is given by

$$\frac{10}{\text{Number of cubic centimetres of thiocyanate used}}.$$

Note. The nitric acid should not be pipetted.

Calculation. Multiply the number of cubic centimetres of thiocyanate by the factor. Subtract the result from 10 (the number of cubic centimetres of silver nitrate used). The difference gives the number of milligrams of chloride (expressed as sodium chloride) in 10 c.c. of plasma filtrate, *i.e.*, in 1 c.c. of plasma. Multiply the result by 100 to get the corresponding figure for 100 c.c. plasma.

Example. 9·68 c.c. of thiocyanate are required for 10 c.c. of silver nitrate. Hence 1 c.c. is equivalent to 10/9·68, *i.e.*, to 1·033 c.c. of silver nitrate; 1·033 is the factor.

4·56 c.c. of thiocyanate are required for the plasma

filtrate titration. This is equivalent to $4{\cdot}56 \times 1{\cdot}033 = 4{\cdot}71$ c.c. silver nitrate.

$10 - 4{\cdot}71 = 5{\cdot}29$, and since 1 c.c. silver nitrate is equivalent to 1 mg. NaCl,

1 c.c. plasma contains 5·29 mg. NaCl, or

100 c.c. plasma contain 529 mg. or 0·53 gm. NaCl.

Experiment 2. THE VAN SLYKE PROCEDURE.[1] Pipette 1 c.c. of plasma into a large Pyrex test-tube. Add 3 c.c. of the special silver nitrate-nitric acid reagent (see below). Insert a filter funnel in the mouth of the test-tube. Boil in a beaker two-thirds filled with water for two hours, adding water to the beaker as necessary. At the end of this time the tube contains a white silver chloride precipitate and a clear light yellow liquid. Cool. Add 6 c.c. of 5 per cent. ferric alum. Titrate with standardised thiocyanate until the salmon-pink colour just persists.

SPECIAL SILVER NITRATE-NITRIC ACID REAGENT. Dissolve 8·495 gm. of pure silver nitrate in the minimum amount of distilled water and make up exactly to 1 litre with pure concentrated nitric acid. This gives an N/20 solution of silver nitrate in nitric acid. From the equation

$$AgNO_3 + NaCl = AgCl + NaNO_3$$

it follows that 169·9 gm. of silver nitrate correspond to 58·5 gm. of sodium chloride, whence 1 litre of the silver nitrate solution corresponds to 58·5/20 or 2·925 gm. of sodium chloride and therefore

One cubic centimetre of the special reagent corresponds to 2·925 mg. of sodium chloride.

SPECIAL THIOCYANATE REAGENT. An approximately N/50 solution of ammonium thiocyanate is made up by dissolving 1·52 gm. of pure ammonium thiocyanate in 1 litre of water. This is standardised by titrating 3 c.c. of the special silver reagent plus 6 c.c. of ferric alum against it. These 3 c.c. of an N/20 reagent should obviously require $3 \times 50/20$ or 7·5 c.c. of an N/50 reagent.

Note. It is found by experience that in order to get a distinct end-point in the titration at least 1 drop of thiocyanate in excess has to be added. Hence 0·04 c.c. is subtracted from the burette reading in all titrations by this method.

[1] Van Slyke, *J. Biol. Chem.*, 1923, LVIII., 523.

Calculation. The factor for thiocyanate is given by

$$\frac{7{\cdot}5}{\text{Number of cubic centimetres of thiocyanate used} - 0{\cdot}04}.$$

To calculate the (sodium) chloride present in the plasma multiply the number of cubic centimetres of thiocyanate (— 0·04) by the factor. Subtract the result from 7·5. This gives the number of cubic centimetres of N/50 silver nitrate used (not N/20).

Since for N/20 silver nitrate 1 c.c. corresponds to 2·925 mg. NaCl, for N/50 silver nitrate 1 c.c. corresponds to two-fifths of this, 1·17 mg.

Hence multiply by 1·17 to get the number of milligrams of NaCl per 1 c.c. plasma, or by 117 to get the number of milligrams per 100 c.c. plasma.

Example. For the thiocyanate standardisation 7·61 c.c. were used. Hence the factor is 7·5/(7·61 — 0·04), or 7·5/7·57, *i.e.*, 0·991.

For the 1 c.c. of plasma 2·62 c.c. of thiocyanate were used.

$$\begin{aligned} 2{\cdot}62 - 0{\cdot}04 &= 2{\cdot}58, \\ 2{\cdot}58 \times 0{\cdot}991 &= 2{\cdot}56, \\ 7{\cdot}50 - 2{\cdot}56 &= 4{\cdot}94, \\ 4{\cdot}94 \times 117 &= 578 \text{ mg. or} \end{aligned}$$

0·58 gm. NaCl per 100 c.c. plasma.

Estimation of Inorganic Phosphate

A number of methods are available, amongst which may be mentioned those of Briggs,[1] Benedict and Theis,[2] and Fiske and Subarrow.[3] We have found the method of Benedict and Theis very satisfactory. The reaction develops a blue colour which is easily matched in the colorimeter.

Special Reagents

Molybdate Solution. Add to 20 gm. of pure molybdic acid (MoO_3, free from ammonia) 25 c.c. of 20 per cent. sodium hydroxide solution, and warm gently until solution is complete. Cool and dilute to 250 c.c. Filter if necessary.

[1] Briggs, *J. Biol. Chem.*, 1924, LIX., 255.
[2] Benedict and Theis, *J. Biol. Chem.*, 1924, LXI., 63.
[3] Fiske and Subarrow, *J. Biol. Chem.*, 1925, LXVI., 375.

Before use dilute 1 to 1 with concentrated sulphuric acid. (The diluted reagent is stable for a few days.)

Bisulphite-hydroquinone Reagent. Dissolve 15 gm. of sodium bisulphite and 0·5 gm. of hydroquinine in water and make up to 100 c.c.

Standard Solution. Make up a stock solution of 0·4394 gm. of pure potassium dihydrogen phosphate in 1 litre of water, and add a little chloroform as preservative. Make a standard solution from this as required by diluting 5 c.c. to 100 c.c. with water, adding chloroform to this solution also. 5 c.c. of the diluted solution contains 0·025 mg. of phosphorus.

Experiment 3. Dilute 2 c.c. of blood plasma or serum with a little water in a 10 c.c. volumetric flask. Add 4 c.c. of 20 per cent. trichloracetic acid and make up to the mark with water. Allow to stand ten minutes, and then filter through ashless filter paper.

Place 5 c.c. of the filtrate in a test-tube, add 3 c.c. of water, 1 c.c. of the molybdate reagent (diluted with sulphuric acid), and 1 c.c. of the bisulphite-hydroquinone reagent. Mix. Stopper loosely, and place for ten minutes in a boiling water-bath, with a simultaneously prepared standard containing 5 c.c. of diluted standard solution.

Compare in the colorimeter, setting the standard at a convenient depth.

Calculation. The 5 c.c. of plasma filtrate corresponds to 1 c.c. of plasma. Hence—

$$0{\cdot}025 \times \frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \frac{100}{1} = \text{mg. P per 100 c.c. plasma.}$$

Example. Standard set at 20 mm. Mean reading of unknown 15·7 mm. Hence 1 c.c. plasma contains 0·025 × 20/15·7, equalling 0·032 mg. P, and 100 c.c. contains 3·2 mg.

Note. The plasma or serum used must be fresh, and should not be allowed to remain long in contact with trichloracetic acid, otherwise lipoid phosphate may have decomposed.

Estimation of Plasma Proteins

The following method [1] has been tested for some time as a clinical procedure, with satisfactory results. It

[1] Cameron, Guthrie and White, *Can. Med. Assoc. J.*, 1936, XXXV., 32.

depends on a reaction between tyrosine radicals in the plasma proteins and a special phosphomolybdotungstic acid reagent, which develops a deep blue colour, very easily matched.

Special Reagents

Phenol Reagent.[1] Dissolve 100 gm. of sodium tungstate, Na_2WO_4, $2H_2O$, and 25 gm. sodium molybdate, Na_2MoO_4, $2H_2O$, in 700 c.c. water. Add 50 c.c. of 85 per cent. phosphoric acid, and 100 c.c. concentrated hydrochloric acid. Reflux eight hours. If the resulting solution is green, add a trace of bromide and boil off excess. Filter off any precipitate.

Tyrosine Standard. Dissolve 0·200 gm. of pure tyrosine in 1 litre of 0·1 *N* hydrochloric acid, to give a 0·02 per cent. solution.

In addition, the following are required: 1 per cent. sodium chloride, 10 per cent. sodium hydroxide, saturated solution of ammonium sulphate (56 gm. crystallised salt in 100 c.c. of solution), saturated solution of sodium carbonate, 20 per cent. trichloracetic acid, and a 2·5 per cent. solution of anhydrous calcium chloride.

Experiment 4. Blood is taken as usual from an arm vein, prevented from clotting by addition of oxalate (care being taken to avoid excess) and centrifuged. To 1 c.c. of the plasma is added 9 c.c. of 1 per cent. sodium chloride, giving 10 c.c. of *diluted plasma*, of which 1 c.c. equals 0·1 c.c. blood plasma.

(*A*) *Total Protein.* Transfer 1 c.c. of diluted plasma to a 15 c.c. centrifuge tube. Add 4 c.c. water and 1 c.c. 20 per cent. trichloracetic acid. Mix thoroughly (*cf.* Note 1, below). After fifteen minutes centrifuge, and then pour off the liquid, draining it off as much as possible. Add to the precipitated protein 1·0 c.c. of 10 per cent. sodium hydroxide, and heat on the boiling water-bath for thirty minutes, with a filter funnel in the mouth of the tube to lessen evaporation. Remove, and add in the order named 7·0 c.c. of water, 1 c.c. of phenol reagent, and 3 c.c. of saturated solution of sodium carbonate. Mix by inversion.

Standard (used for all comparisons). Add to 2 c.c. of 0·02 per cent. tyrosine 1 c.c. of 10 per cent. sodium hydroxide

[1] Wu, *J. Biol. Chem.*, 1920, XLIII., 208; 1922, LI., 33.

and 5 c.c. of water in a centrifuge tube, then 1 c.c. of phenol reagent and 3 c.c. of saturated solution of sodium carbonate. Mix by inversion.

After unknown and standard have stood at least thirty minutes to permit maximum development of colour, centrifuge five minutes (neglect any precipitate) and compare the solutions in a colorimeter with the *standard* set at 10 mm.

Albumin. To 4 c.c. of the *diluted plasma* add 4 c.c. of saturated solution of ammonium sulphate, giving half saturation. Mix thoroughly, and allow to stand (corked) in an oven at 37° C. for twenty minutes, to facilitate filtration. Filter. *The filtrate must be clear.* If necessary, filter through the same filter paper until clear. Take 2 c.c. of filtrate (equal to 0·1 c.c. of the original plasma), add 3 c.c. of water and 1 c.c. of trichloracetic acid, and proceed as for total protein.

Fibrinogen (estimated as fibrin). Take 1 c.c. of *undiluted* plasma in a centrifuge tube, add 10 c.c. of 1 per cent. sodium chloride, then 1 c.c. of 2·5 per cent. calcium chloride solution. Stir thoroughly, leave the stirring rod in the solution, and allow to stand twenty minutes. A fairly firm clot forms. Whirl the stirring rod. The clot clings to the rod, and by a little manipulation can be worked into a ball and compressed. Transfer to a clean centrifuge tube, add 5 c.c. or more of 1 per cent. sodium chloride, knead the fibrin thoroughly with a glass rod, centrifuge if necessary, and drain off the solution. Add 1 c.c. of 10 per cent. sodium hydroxide, and proceed as for total protein.

Procedure when serum is used. Take 1 c.c. of serum, add 9 c.c. of 1 per cent. sodium chloride, and mix. Estimate total protein (in this case only albumin plus globulin) and albumin as for plasma.

Note 1. After addition of trichloracetic acid the mixing must be thorough. A glass rod can be used, and then washed off with a drop or two of 3·3 per cent. trichloracetic acid. (The concentration of trichloracetic acid used is only just sufficient to ensure complete precipitation of the protein.)

Note 2. The necessary determinations can be carried out concurrently with a single standard, if timed to permit completion of all comparisons within a few minutes.

Note 3. In cases of second stage nephritis or nephrosis, when very low albumin values may be expected, double the quantity of filtrate can be taken for estimation, and a subsequent correction made in the calculation.

Calculation. The colour developed is not exactly proportional to the concentration of protein in the solution, but a simple correction establishes such proportionality. If the standard is set at 10 mm., and the reading of the unknown protein solution is x mm., then the value y mm., determined from the equation

$$y = 1{\cdot}1\,x - 1$$

is directly proportional to the concentration of protein in the unknown.

The (protein)/(tyrosine) factor varies for different proteins. Since certain other constituents of the protein molecule, besides the tyrosine radicals, react chromogenically with the phenol reagent, the factor has had to be determined by direct measurement. For human plasma albumin it is 11·6, globulin 10·2, and fibrinogen 10·7. Since the proportion of these constituents varies in different plasmas, there is no fixed value for total protein, but the factor approximates to 11·0.

The calculation is therefore made as follows :—

Albumin. The standard solution containing 0·0004 mg. of tyrosine, and set at 10 mm., is compared with the albumin in 0·1 c.c. of plasma, giving the reading x mm., corrected to y mm. by the above equation. Then the amount of albumin in 100 c.c. of plasma is given by—

$$0{\cdot}0004 \times \frac{10}{y} \times 11{\cdot}6 \times \frac{100}{0{\cdot}1} = \frac{46{\cdot}4}{y}.$$

Fibrinogen. The comparison is with the fibrin in 1 c.c. of plasma. Hence the amount in 100 c.c. is—

$$0{\cdot}0004 \times \frac{10}{y} \times 10{\cdot}7 \times \frac{100}{1} = \frac{4{\cdot}28}{y}.$$

Globulin. The calculation is indirect. The corrected readings for total protein, albumin, and fibrin are divided into 10, giving "inverted" readings, which are directly proportional to the degree of developed colour. Correct the fibrin value to that for 0·1 c.c. plasma, and subtract the sum of the albumin plus fibrin "inverted" values from the corresponding figure for total protein. The difference corresponds to the "inverted" figure for globulin ($10/y$).

The amount of globulin in 100 c.c. of plasma is therefore given by—

$$0{\cdot}0004 \times \frac{10}{y} \times 10{\cdot}2 \times \frac{100}{0{\cdot}1} = 4{\cdot}08 \times \frac{10}{y}.$$

Example. The respective x readings for a sample of blood plasma were found to be, for albumin 12·9, for total protein 7·5, and for fibrin 14·7. Hence :—

Albumin, corrected reading, 13·19 ; inverted 0·758
Fibrin, corrected reading, 15 2 ; inverted 0·066 (for 0·1 c.c.)

Total : 0·824

Total protein, corrected, 7 25 ; inverted 1·379

Difference : 0·555

Albumin :	46·4/13 19	equal to	3·52 gm.	per 100 c.c.
Globulin :	4·08 × 0·555	,, ,,	2·26	,, ,,
Fibrinogen :	4·28/15·2	,, ,,	0·28	,, ,,
Total protein :			6·06	,, ,,

Note. Estimations of albumin and globulin are of value in certain pathological conditions. In nephrosis, and in streptococcus and staphylococcus infections, the albumin content decreases markedly, and may fall below the value for globulin, which is much less affected.

EXERCISE XXXI

DETERMINATION OF BLOOD BASES (SO-CALLED ALKALI RESERVE)

(One or two Three-hour Periods)

KNOWLEDGE of the "alkali reserve" is often of importance clinically. It can be determined by several methods of varying value.

Indirect Methods. The Marriott method is a rough procedure for determining the carbon dioxide tension of alveolar air. It is useful clinically, since it can be employed with all patients, whether they are able to co-operate or not. The Fridericia method is a more accurate means of determining the percentage of carbon dioxide in alveolar air, but it requires the co-operation of the subject, and consequently its clinical use is somewhat limited.

Direct Methods. The Van Slyke procedure determines the carbon dioxide combining capacity of a sample of plasma by accurate analysis. The method is more difficult than the others, but co-operation of the patient is unnecessary, so that it can be used clinically without limitation, while results obtained with it are accurate.

The above three procedures should be demonstrated to groups of students, who should then carry out individually at least the first two.

The Marriott Method

Air is **rebreathed** so that its composition becomes virtually that of alveolar air. This "alveolar air" is then passed through a solution of sodium carbonate

coloured with a suitable indicator, such as phenolsulphonephthalein, which shows definite colour changes over a considerable range of *pH* values. The percentage of carbon dioxide present in the sample of alveolar air determines a specific mixture of carbonate and bicarbonate. Each such mixture has its own specific *pH* value, and therefore gives a definite colour tint with the indicator. This resulting colour is matched against a series of sealed tubes containing phosphate mixtures and the indicator, these standard tubes being already standardised to correspond with definite tensions of carbon dioxide. The result is therefore obtained directly in terms of carbon dioxide tension (pressure).

Normal (dry) alveolar air has a carbon dioxide content of from 5·5 to 6 per cent. The latter value, expressed in terms of tension, gives a CO_2 tension of

$$\frac{6}{100} \times \frac{760}{1} \text{ mm. mercury, } i.e., \text{ 45·6 mm.}$$

With this tension the indicator used shows a light or grey-yellow colour, with no trace of red. With lessening tensions of CO_2 a tinge of red appears and grows stronger the less the tension.

The alveolar carbon dioxide is determined by the CO_2 carried by the blood passing through the lung capillaries, and this in turn is mainly determined by the alkali present to hold it as (carbonate and) bicarbonate. Decreasing alveolar CO_2 values, shown by a reddish tinge in the test, indicate decreased alkali reserve in the blood, and since this mainly occurs in conditions of acidosis, such results generally indicate an acidosis. The *degree of acidosis* is roughly measured by the Marriott test.[1]

Experiment 1. A rubber bag of about 1,500 c.c. capacity is joined to a rubber tube, to which is attached a clip, and into which is inserted a sterilised glass mouthpiece. Through this, by means of an atomiser or similar rubber bulb, about

[1] W. M. Marriott, *J. Am. Med. Assoc.*, 1916, LXVI., 1594. The complete apparatus is supplied by Hynson, Westcott, and Dunning, Baltimore, Md., U.S.A.

600 c.c. of air are blown into the bag. The subject's nose is closed by compression of the observer's fingers or by a nose clip. Before and during collection of the alveolar air the subject, if capable of co-operation, must breathe normally ; *i.e.*, there must be no unusually deep inspiration or expiration. The mouthpiece is inserted at the end of expiration ; the subject inspires and completely deflates the bag, then expires into the bag, and repeats this four or five times, the whole rebreathing period occupying twenty seconds or more. Each respiratory movement must be ordered by the observer, provided co-operation is possible by the subject. Finally, at the end of **expiration** the bag is clamped off at the tube and the mouthpiece removed from the mouth.

(With patients unable to co-operate, and especially when the breathing is shallow, the time of rebreathing must be prolonged to thirty seconds or more.)

The mouthpiece is then replaced by a similar glass tube drawn off to a fine capillary point ; this is inserted in a test-tube containing the indicator and of the same bore as the standard sealed tubes, and air from the bag is forced through in a steady stream until there is no further colour change. The test-tube is then corked (to prevent loss of carbon dioxide) and matched with the standard tubes against a ground glass surface in the simple comparison apparatus provided. The matching gives the reading directly in millimetres of carbon dioxide tension.

Note. This method, with the indicator usually employed, does not measure CO_2 tension above 45 mm.

Note. The same sample of indicator solution can be used many times.

The Fridericia Method

Exactly 100 c.c. of alveolar air are measured off in a special glass apparatus at a definite temperature. The gas is then treated with 10 per cent. sodium hydroxide solution and the residual volume of gas measured at the same temperature. The difference is the volume of carbon dioxide present in 100 c.c. of air, so that the result is expressed directly, the number of cubic centimetres absorbed giving the corresponding volumes per cent.[1]

[1] Fridericia, *Hospitalstidende*, 1914, LVII., 585 ; Poulton, *Brit. Med. J.*, 1915, II., 392.

Experiment 2. Rinse out the special apparatus (Fig. 20) with dilute hydrochloric acid, and then with tap-water, and finally drain as free of water as possible. No water must collect above either tap.

Open tap A, and turn the three-way tap B so that the ends M and N are connected. The subject, after a **normal** inspiration, inserts the open end M into his mouth, compresses his nose and expires slowly through the apparatus for as long a time as possible.

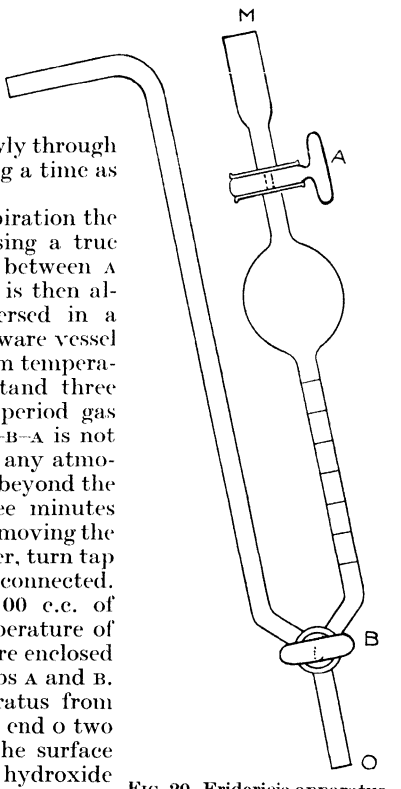


FIG. 20. Fridericia apparatus.

At the end of this expiration the tap A is turned, enclosing a true sample of alveolar air between A and N. The apparatus is then almost completely immersed in a large beaker or earthenware vessel containing water at room temperature, and allowed to stand three minutes. During this period gas diffusion in the tube N–B–A is not rapid enough to permit any atmospheric air to penetrate beyond the tap B. After the three minutes have elapsed, without removing the apparatus from the water, turn tap B so that N and O are connected. In this way exactly 100 c.c. of alveolar air at the temperature of the surrounding water are enclosed in the space between taps A and B. Now remove the apparatus from the water and insert the end O two or three inches below the surface of a 10 per cent. sodium hydroxide solution in a large beaker, the whole apparatus being sloped as in the figure (to lessen any possibility of gas escaping from the portion A–B when the tap is again turned).

Suck up sodium hydroxide solution into the tube N–B to

about six or seven inches above the tap. Then turn the tap B to connect A and N and, by blowing in gently at the end N, force 3 or 4 c.c. of solution into the space A–B. Again turn the tap B to its position connecting N and O. Remove the apparatus from the sodium hydroxide solution, dip in water to cleanse the open tube from sodium hydroxide and then invert several times. (Carbon dioxide absorption is very rapid.) Again place the apparatus in the large vessel containing water. Water rises towards N, filling the bore of the tap. Turn tap B under water, so as to connect A and O, and then nearly completely immerse the apparatus for three minutes. Then lift the apparatus vertically and as rapidly as possible take a reading when the liquid inside and outside the tube A–B is at the same level (*i.e.*, when the gas in A–B is at atmospheric pressure). The lower part of the tube is graduated in cubic centimetres, so that the actual reading gives the amount of liquid in the tube, and therefore the amount of carbon dioxide that has been absorbed. All measurements are made with gas saturated with water vapour, so that that can be neglected, while no change of barometric pressure occurs during the test.

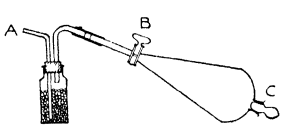
Note. If the Marriott and Fridericia tests have been carried out on the same subject the results should be comparable.

The Van Slyke Method

This is carried out in a special apparatus designed for gas analysis by Dr. D. D. Van Slyke.[1] Oxalated plasma is brought into equilibrium with normal alveolar air, and then a definite volume is decomposed with sulphuric acid, and the carbon dioxide liberated is measured. This volume, referred to standard tables, gives the amount of base present in the plasma.

[1] Van Slyke, *J. Biol. Chem.*, 1917, XXX., 289 ; 347. The apparatus has been employed for determination of oxygen of the blood and haemoglobin (*cf.*, *e.g.*, Van Slyke and Stadie, *ibid.*, 1921, XLIX., 1). Van Slyke and Neill (*ibid.*, 1924, LXI., 523) have devised a still more precise *manometric* procedure which has been utilised for the determination of carbon dioxide, oxygen, and other gases in blood, and of methaemoglobin, blood-urea, blood-glucose, etc. (*cf.* also Van Slyke, *ibid.*, 1925, LXVI., 409 ; 1927, LXXI., 235 ; LXXIII., 695 ; Harington and Van Slyke, *ibid.*, 1924, LXI., 575 ; Van Slyke and Hawkins, *ibid.*, 1928, LXXIX., 739).

Experiment 3. Five or six cubic centimetres of blood are drawn from an arm vein, oxalated, centrifuged and the plasma transferred to a clean, dry, pear-shaped separating funnel (Fig. 21). The narrow end of the funnel is then connected with a small bottle filled with glass beads. With the tap open the (normal) operator, after a normal inspiration, expires as long as possible into the tube A. (The beads prevent such a condensation of moisture from the breath of the operator as might materially dilute the plasma in the bulb.) At the end of the expiration stopcock C is inserted, and then tap B is turned. The funnel is then rotated on its side, so as to expose as large a surface of plasma as possible, for at least two minutes. It is then set down vertically, so that the plasma drains thoroughly towards the tap B. The plasma has now been brought into equilibrium with an atmosphere containing 5·5 to 6 per cent. of carbon dioxide, and the bases present in



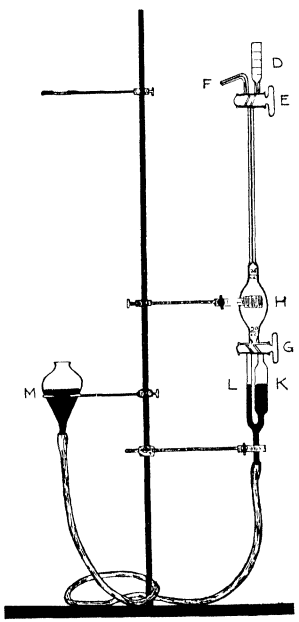


FIG. 21. Van Slyke blood gas apparatus. About one-ninth natural size.

the plasma have taken up the maximum amount of the gas possible under the conditions. The plasma is therefore ready for analysis.

The special gas apparatus (Fig. 21) is washed out twice with 2 per cent. ammonia solution, introduced through the cup D, pulled down into the apparatus by opening the tap E and lowering the mercury reservoir M, and finally expelled

through F by reversing the stopcock E and raising M. The apparatus is then completely filled with mercury just to the top of the capillary above the bore of stopcock E (leading to D). Close tap E. A trace of (ammonia) water will remain above the mercury.

Unless the apparatus has just previously been used, it

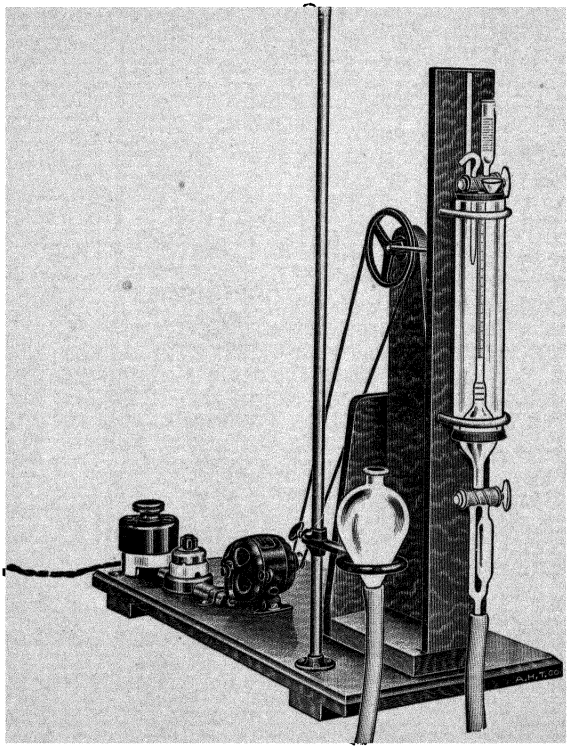


FIG. 22. Blood gas apparatus, Van Slyke precision model.

should be at this time tested for possible leakage by lowering the reservoir M to such a point that the mercury falls to the bottom of the bulb H, allowing it to remain in this position for two or three minutes, and then slowly raising M. If no leakage has occurred, the mercury should completely fill

the interior. If a gas bubble remains, regrease the taps and make certain that the rubber tubing is in good condition and properly wired. Then repeat until convinced that there is no leakage. Proceed as follows :—

Add 1 c.c. of distilled water (measured roughly) to the cup D. Pipette accurately 1 c.c. of plasma into D, keeping the tip of the pipette below the level of the water already there. (It is essential to keep the plasma from contact with atmospheric air, otherwise carbon dioxide is given up from the plasma.) Add a drop of caprylic alcohol. With the bulb lowered cautiously, turn the tap E so that the plasma, water and alcohol slowly run in. Turn the tap off while liquid still fills the capillary. Add 1 c.c. of 5 per cent. sulphuric acid to D. Carefully open the tap and run in acid until the mercury meniscus stands accurately at the mark 2·5 c.c. in H. Close E.

Lower M until the mercury in H stands at the level 50 c.c. (the mark just below the bulb). Carbon dioxide is evolved. Close tap G. Invert the apparatus carefully fifteen to twenty times.[1] Equilibrium is set up between the carbon dioxide in the 47·5 c.c. of gas space and that in the 2·5 c.c. of liquid phase.

Lower the reservoir M to about 30 in. below the tap G. The point can be judged as that at which the mercury below G has just commenced to fall. Open G so that H and K are connected. The liquid falls into K. While there are still a few drops of liquid above the tap reverse G so that L and H are connected and raise the reservoir M steadily until the mercury level in M coincides with that in H. Neglect the small amount of liquid and read the gas volume (to 0·001 c.c.). Note the room temperature and the barometric pressure. Correct the observed volume to normal barometric pressure as follows :—

$$\text{Gas volume} \times \frac{\text{actual barometric pressure}}{760} = \text{corrected volume.}$$

The number of cubic centimetres of carbon dioxide reduced to 0° and 760 mm. mercury pressure, held bound as bicarbonate by 100 c.c. of plasma, is determined by refer-

[1] It is preferable, especially for accurate routine work, to use an apparatus fixed in an electrically driven shaker, such as the precision model of the A. H. Thomas Company shown in Fig. 22. Five minutes' moderately rapid shaking with this apparatus is sufficient. A Shohl trap, just above the pressure tubing, prevents leakage from the tubing affecting the result. The trap is not shown in the figure.

ring this corrected volume to tables that have been determined by Van Slyke from actual measurements on the distribution of carbon dioxide under similar conditions to those in this experiment. An abbreviated table follows :—

Table for Calculation of Carbon Dioxide Combining Power of Plasma

Corrected gas vol.	C c. of CO_2 reduced to 0° and 760 mm. bound as bicarbonate by 100 c.c plasma.			Corrected gas vol.	C c of CO_2 reduced to 0° and 760 mm. bound as bicarbonate by 100 c c. plasma.		
	15°	20°	25°		15°	20°	25°
0·20	9·1	9 9	10·7	0·55	42 9	43 3	43·8
0·25	13·9	14 7	15 5	0 60	47 7	48·1	48·5
0·30	18 8	19 5	20 2	0 65	52·6	52·8	53·2
0·35	23 6	24 2	24 9	0 70	57 4	57 6	57 9
0 40	28·4	29 0	29 6	0 75	62·3	62 4	62·6
0 45	33·2	33 8	34 3	0·80	67·1	67 2	67·3
0·50	38·1	38 5	39 0	0 85	71 9	72·0	72 1

Note. Abnormal results obtained by any of these three methods can be interpreted by aid of the following table (after Hawk and Bergeim) :—

Interpretation of Results in Measurements of Blood Bases and Alveolar Carbon Dioxide

Method			Condition.
Marriott.	Fridericia	Van Slyke	
Alveolar CO_2 tension in mm. mercury.	Alveolar CO_2 in volume per cent	Bicarbonate CO_2 in vol. per cent.	
Over 45 to 35	6·8 to 5·0	80 to 53	Normal.
35 to 25 .	5·0 to 3·5	53 to 40	Mild acidosis without symptoms.
25 to 20 .	3·5 to 2·7	40 to 30	Moderate to severe acidosis (usually with clinical symptoms).
Below 20 .	Below 2·7	Below 30	Severe acidosis (usually with marked symptoms, even to air hunger).

EXERCISE XXXII

DETERMINATION OF THE BASAL METABOLIC RATE

(One Two-hour Period)

ACCURATE basal metabolic rate determinations must include measurement of the respiratory quotient, and involve at least collection of the expired air of the individual under examination during a measured time, accurate measurement of the total volume so collected, and accurate gas analysis of a sample of this air in a Haldane apparatus or some one of its modifications. Training in such procedures is unnecessary for the student of medicine who does not propose to proceed to post-graduate research work, although if time is available a demonstration of the methods is perhaps not undesirable.

A demonstration of the clinical procedure should be given, if any one of the various types of clinical apparatus is available. The details in the following experiment apply to types based on that of S. R. Benedict.[1] A useful home-made apparatus can be constructed on the lines laid down by Benedict and Benedict.[2]

Before such a demonstration the student should read the sections dealing with basal metabolism in a theoretical text-book.[3]

Basal metabolism is the heat production of the individual at rest mentally and physically, but not asleep. It must be stressed, therefore, that this demonstration

[1] S. R. Benedict, *Boston Med. and Surg. J.*, 1918, CLXXVIII., 667.

[2] Benedict and Benedict, *Boston Med. and Surg. J.*, 1923, CLXXXVIII., 567.

[3] *E.g.*, Cameron's "Biochemistry," 5th edit., Chapter XVI. For a complete account of the subject see Du Bois' "Basal Metabolism in Health and Disease," 3rd edit. (Lea and Febiger, Philadelphia, 1936).

cannot comply with at least one essential condition for an **accurate** measurement: the absence of all distracting factors, such as a class of observing students. Hence other necessary factors for accuracy, such as rest, post-absorptive conditions, etc., can also be neglected, and the demonstration will show only the method of using the apparatus, the making of the actual readings, and the calculation of the results from them.

DEMONSTRATION

Experiment 1. A student should volunteer as subject for the test.

The data required are sex, age, height, weight, and oxygen consumption per minute. Measure his height and weight and allow him to rest on a bed or couch in a recumbent position for a few minutes.

A diagrammatic sketch of the apparatus is shown in Fig. 23.

Fill the bell B of the apparatus with commercial oxygen (over 90 per cent. oxygen, the residue being nitrogen) from a tank through the pet-cock C. The bell contains a vessel nearly filled with medium-sized soda-lime granules, through which the circulating air must pass. Connect the subject to the apparatus by the mouthpiece M, and close his nose with a clip, thus closing the apparatus. Air circulation is directed by the flutter valves V_1 and V_2, and is indicated by the arrows. (In certain makes of apparatus air circulation is brought about by a motor-driven fan within the apparatus.)

The subject breathes normally, and the counterpoised bell **rises** and falls with each **expiration** and inspiration. A pointer, P, connected to the counterweight, indicates the volume in the bell on a scale S. Readings are taken at the end of **expiration,** these being more constant than those at the end of inspiration. At any given zero time (one-half to one minute after the subject is connected to the apparatus) take three consecutive readings. Consider their mean as the initial volume at this time. After exactly ten minutes from this zero again take three consecutive readings; their mean gives the volume at the end of the ten-minute interval, and the difference is the amount of oxygen used up in this period

(the nitrogen being unaffected and the carbon dioxide and water-vapour being practically all absorbed by the soda-lime).

Some of the more recent types of clinical apparatus have automatic recording attachments, which, if working correctly, give a more accurate story of the rate of

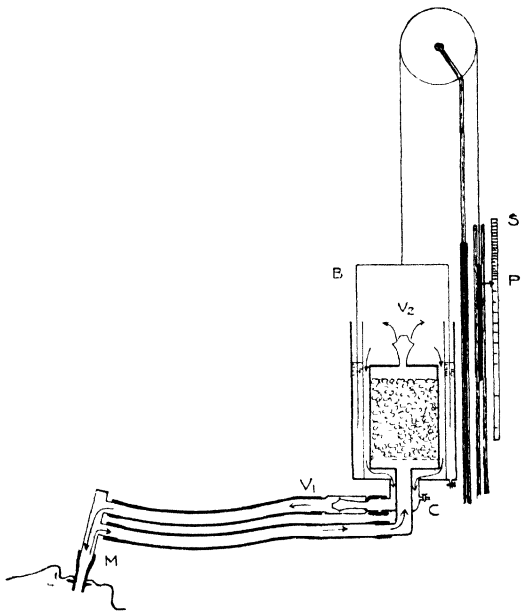


FIG. 23. Diagram of clinical apparatus for the determination of the basal metabolic rate (after Roth).

absorption. When these are used, periods during which the rate of absorption is fairly constant should be employed for the computation.

To obtain accurate results with all such clinical instruments, it is necessary not only that the correct preliminary treatment of the subject shall have been carried out, but also that the assumption that the

respiratory quotient during the actual test is 0·82 be a true one. (This is not always the case; in diabetes mellitus this quotient under basal conditions may be much lower than 0·82.)

The essential value to be determined is the percentage difference from normal. The normal value depends mainly upon the surface area, age and sex, as is shown in the following table (based on Boothby and Sandiford's modification of the Aub and Du Bois standards):—

Age.	Heat Production per Square Metre of Body Surface per Hour.		Oxygen Consumption per Square Metre of Body Surface per Minute.	
	Males.	Females.	Males.	Females.
years	cal.	cal.	c.c.	c.c.
6	52·7	50·7	182	175
8	51·2	48·1	177	166
10	49·5	45·8	171	158
12	47·8	43·4	165	150
14	46·2	41·0	160	142
16	44·7	38·5	154	133
18	42·9	37·3	149	129
20–24	41·0	36·9	142	128
25–29	40·3	36·6	139	126
30–34	39·8	36·2	137	125
35–39	39·2	35·8	135	124
40–49	38·0	35·2	131	122
50–59	36·9	34·3	128	118
60–69	35·7	33·6	123	116

In order to utilise such values clinically, an approximation to the body surface is derived from the height and weight, while oxygen consumption corresponding to the heat values (shown in the last two columns of the table) is determined from the relation—

One litre of oxygen is used up in the production of 4·83 *calories under basal conditions and with a respiratory quotient of* 0·82.

Based on numerous measurements of the actual body surface, height, and weight of many individuals, formulae have been devised to correlate the surface to the other measurements. None of these is perfect, since they

do not take into account sufficiently the widely differing bodily form of different individuals. The formula of D. and E. F. Du Bois is one of the most accurate, and can be taken for illustration :—

Surface area in square centimetres = (*weight* in kilograms) $^{0.425}$ × (*height* in centimetres) $^{0.725}$ × 71·84.

This is easily solvable by logarithms—

Log. A = 0·425 × log. W + 0·725 × log. H + 1·8564.

Instead of employing such formulae, shorthand procedures are usually adopted, utilising tables furnished with the clinical instruments, but these tables are calculated from the above or similar data and formulae.

Example (carried out under correct basal conditions).

Male, age twenty-six years, height 67 in., weight 159 lb.

Initial series of readings, 5,750, 5,730, 5,730, mean 5,740 c.c.

After ten minutes 2,900, 2,890, 2,840, mean 2,880 c.c.

This gives a difference of 2,860 c.c., and a measured consumption of 286 *c.c. per minute.*

(All the readings are assumed to have been corrected to 0° C. and 760 mm. mercury barometric pressure.)

Calculation of the normal Oxygen Consumption.

67 in. = 170·2 cm. ; 159 lb. = 72·2 kg.

Hence the log. of the surface area

= 0·425 × 1·8585 + 0·725 × 2·2310 + 1·8564

= 4·2638

= log. of 18,360,

and the surface area is 18,360 sq. cm. or 1·84 square metres.

The oxygen consumption for a male of twenty-six years is from the table 136 c.c. per minute per square metre.

Hence the normal oxygen consumption is

136 × 1·84 = 250 c.c. per minute *for this individual.*

Calculation of the Basal Metabolic Rate.

Normal oxygen consumption per minute = 250 c.c.

Actual oxygen consumption found = 286 c.c.

$$\text{B.M.R.} = \frac{100 \times (286 - 250)}{250} = +14 \text{ per cent.}$$

Note 1. Largely on account of the fact that calculation of surface area from height and weight is merely an approxima-

tion, a considerable margin of safety must be allowed for normality. Usually results within ± 15 per cent. are considered as within normal limits. Naturally increase in body temperature due to increased tissue consumption of oxygen (due to various causes) necessitates correction; there is an increase of 7·2 per cent. in the basal rate for each degree Fahrenheit above normal. Sub-normal temperatures do not require a corresponding correction.

Note 2. Under the conditions of a demonstration like that above, in which the subject has not been fasting for the necessary period of twelve to sixteen hours, has probably taken some degree of exercise until within a few minutes of the test, and is not resting mentally, results of + 30 per cent. and over will usually be obtained.

DEMONSTRATION

Experiment 2. By measuring the rate before and one hour after a meal, or before and immediately after strenuous exercise, an approximation to the effect of digestion and absorption, or of exercise, on oxygen consumption can be obtained.

EXERCISE XXXIII

CHEMISTRY OF VITAMINS AND DETERMINATION OF A GROWTH CURVE [1]

UNTIL further advances have been made in the isolation of pure vitamins, few chemical tests can be utilised to illustrate their properties and to differentiate between them. Biological tests are necessary, and these are time-consuming. This exercise, therefore, includes only a few notes on some chemical tests which appear to be of significance, and directions for the determination of the growth curve of a small mammal, a type of test frequently employed.

VITAMIN A. This is believed to be a derivative of carotene. Both carotene (and related pigments) and material rich in vitamin A give a striking reaction with antimony or arsenic chloride. In absence of carotinoid pigments the reaction can be adapted to the determination of relative richness of animal and plant material in A content.

Experiment 1. Add 1 c.c. of pure arsenic chloride to 1 drop of cod-liver oil in a test-tube and shake. The oil dissolves to a clear blue solution, which in a few seconds changes to purple, and then gradually fades (Rosenheim and Drummond's test). Repeat, using a benzine (petroleum ether) extract of carrots instead of the cod-liver oil (or a chloroform extract of carrots dried at 105° C. to constant weight). A slate-blue colour results.

Experiment 2. Dissolve 3 gm. of antimony trichloride in 10 c.c. of chloroform. Allow to stand and decant from any residue. Dissolve some cod-liver oil in chloroform to give a 20 per cent. solution. To a few drops of the cod-liver oil

[1] Read Cameron's "Biochemistry," 5th edit., sections on vitamins in Chapters III. and XVII.

solution add 2 or 3 c.c. of the antimony reagent. An intense blue colour is produced which is much more permanent than that given by arsenic trichloride (Carr and Price's modification [1]). Repeat with the chloroform extract of dried carrots.

Note. If even a small amount of water is present, no positive result is obtained.

Experiment 3.[2] Take some cod-liver oil (or other oil known to be rich in vitamin A) and dilute about 1 to 20 with chloroform. Add to 1 to 2 c.c. of this solution in a dry test-tube 1 c.c. of freshly prepared 0·5 per cent. solution of pyrocatechol in chloroform, and 2 to 3 c.c. of a cold saturated solution of antimony trichloride in chloroform. Immediately place the tube in a water-bath at 60° C., and keep there for two minutes. A red-violet colour develops, resembling dilute permanganate solution. Under similar conditions pure carotene gives a deep blue coloration.

Note. The chloroform used must be free from water and alcohol.

Measurement of a Growth Curve. When a diet deficient in some essential constituent is fed to an animal, one of the commonest effects is a slowing of the rate of growth. To demonstrate such an effect within reasonable time a small and rapidly growing animal is requisite. Young rats are pre-eminently suitable. Such growth responses follow various kinds of deficiencies—of calcium, of certain amino-acids, of certain vitamins. A type of experiment is outlined below; the principle holds for all such experiments. Since it requires several weeks, if time and other facilities do not permit that it be attempted by students individually, at the start of the experiment the procedure should be explained, and subsequently the animals and their growth charts exhibited from time to time.[3]

[1] For the literature and critical studies of the reaction, see Norris *et al.*, *J. Biol. Chem.*, 1929, LXXXIII., 469; 1929–30, LXXXV., 477; 1930, LXXXVII., 139; LXXXIX., 421, 589.

[2] Rosenthal and Erdélyi, *Biochem. Zeitschr.*, 1933, CCLXVII., 119.

[3] For a complete experimental study of vitamin deficiencies, and an account of the literature relating to them, see Hawk and Bergeim, "Practical Physiological Chemistry," 10th edit., Chapter XXIX. (J. and A. Churchill, London, and Blakiston, Philadelphia, 1931).

Experiment 4. Select two young rats about three weeks old, of the same sex and litter, and place them in separate cages. (Provided it can be easily kept clean, the nature of the cage is not very material, except in experiments in which it is desirable to collect urine and faeces. A wire false bottom, of large enough mesh to permit faeces to pass through, is necessary, and, if a wooden cage is used, the back and front should be of fine wire mesh, and the bottom should be cleaned out at frequent intervals, and sprinkled with fresh sawdust. Identical cages should be used for the two rats.) Administer unlimited supplies of water and of the following special food mixture :—

Casein (purified ; free from vitamins)	18 per cent.
McCollum-Davis salt mixture .	4 ,,
Irradiated dried brewer's yeast	10 ,,
Starch	68 ,,

The yeast can be irradiated from any convenient source of ultra-violet radiation, and supplies vitamins B, B_1 (or G), and D. The McCollum-Davis salt mixture is made up in the following proportions :—

Calcium lactate	35·15 gm.
Calcium hydrogen phosphate . . .	14·60 ,,
Dipotassium hydrogen phosphate . .	25·78 ,,
Sodium dihydrogen phosphate (monohydrate)	9·38 ,,
Sodium chloride	4·67 ,,
Anhydrous magnesium sulphate . .	7·19 ,,
Ferric citrate	3·19 ,,

The mixture is ground to a fine powder, thoroughly mixed, and stored in a dry container.

Administer daily to one rat, from a medicine-dropper or pipette, 1 drop of pure olive oil (free from vitamin A) and to the other rat, similarly, 1 drop of a good sample of cod-liver oil (containing vitamin A). The rats swallow the oil readily. Weigh the rats every third day (to the nearest 0·5 gm. ; they can be weighed in large beakers and the weight of the beaker subtracted).

Plot the weights on squared millimetre paper, against the time from the commencement of the experiment (ordinates, 1 mm. equal to 1 gm. body-weight ; abscissae, 1 mm. equal to one day). Note that after two or three weeks the rat on

the diet deficient in vitamin A is growing at a definitely slower rate (initially it contains some store of the vitamin), while, if the experiment is prolonged, ultimately the body-weight will become stationary and even decrease, while the rat will exhibit signs of xerophthalmia (at first a haemorrhagic area, freed from hairs, surrounding the eyes, and later keratinisation and infection of the eye itself, which becomes encrusted and closed). If time permits, when growth tends to become stationary, cod-liver oil can be substituted for the olive oil; the rate of growth immediately changes towards normal, and xerophthalmia, if it had developed, disappears.

VITAMIN C (ASCORBIC ACID). The following experiment [1] illustrates the marked reducing power of this vitamin.

Experiment 5. Immerse a slice of fresh ox or sheep adrenal in 0·5 per cent. silver nitrate solution for about fifteen minutes. Within a few minutes the surface of the cortex becomes blackened, due to deposition of metallic silver; the medulla may be but little affected.[2]

Estimation of Ascorbic Acid

The estimation of this vitamin in both urine and blood is relatively easy, and is now frequently performed for clinical purposes. It depends upon the rapid reduction of the dye 2.6.dichlorphenolindophenol by the vitamin in acid solution.[3]

Special Reagent

Crush, and dissolve in a little water in a 50 c.c. volumetric flask one tablet of the dye 2.6.dichlorphenolindophenol (Hoffmann-La Roche) which has been standardised to be equivalent to 1 mg. of ascorbic acid. Dilute with water to the 50 c.c. mark; 1 c.c. of this solution is equivalent to 0·02 mg. of ascorbic acid. This dilution is used for urine.

[1] Szent-Györgyi, *Biochem. J.*, 1928, XXII., 1393.

[2] Harris and Ray (*Biochem. J.*, 1933, XXVII., 2006) state that the ox adrenal medulla contains substances which inhibit the reaction, although ascorbic acid is present.

[3] Harris and Ray, *Lancet*, 1935, i., 71, 228; Farmer and Abt, *Proc. Soc. Exp. Biol. Med.*, 1935, XXXII., 1625; 1936, XXXIV., 146.

It deteriorates rather rapidly and must be made up freshly at least every second day.

For blood estimations a diluter solution is prepared by taking 10 c.c. of this reagent in a 100 c.c. volumetric flask, adding water to the mark, and mixing. 1 c.c. of the dilute solution is therefore equivalent to 0·002 mg. of ascorbic acid.

Experiment 6. *Ascorbic Acid in Urine.* A freshly-voided specimen of urine is measured, and to it is added 10 per cent. of its volume of glacial acetic acid. Estimation can then be delayed twelve hours if necessary, although it is better to carry it out as soon as possible for very accurate results.

Fill a 5 c.c. micro-burette with the acidified urine. Pipette 1 c.c. of the dye-solution into a centrifuge tube, add 1 drop of glacial acetic acid, and titrate immediately, running in urine until the pink colour of the dye is just discharged. The whole period of titration must not exceed two minutes.

Calculation of Result. Divide 0·02 by the number of cubic centimetres of urine used, and multiply the result by the total volume in cubic centimetres of the urine sample plus the added acetic acid. The result is the number of milligrammes of ascorbic acid in the total volume of urine (before acidification).

Example. The volume of a specimen of urine was 150 c.c. Hence 15 c.c. of glacial acetic acid were added, giving a total volume of 165 c.c.; 1 c.c. of dye-solution required 1·35 c.c. of the acidified urine, whence the ascorbic acid content of the sample was :—

$$\frac{0{\cdot}02}{1{\cdot}35} \times 165 = 2{\cdot}4 \text{ mg. in the 150 c.c.}$$

Note 1. With highly coloured urines it is usually advantageous to run a rough estimation first, and then to repeat the titration with a fresh amount of dye-solution, using as control another centrifuge tube containing 1 c.c. of water and the approximate volume of urine required in the rough estimation.

Note 2. If more than 4 c.c. of urine are required, the titration should be repeated, using 0·5 c.c. of dye-solution, equivalent to 0·01 mg. of ascorbic acid. On the other hand, if less than 0·5 c.c. of urine is required, the urine should be diluted, and the titration repeated.

Note 3. To determine the excretion of ascorbic acid over a twenty-four hour period, each sample of urine as voided

must have 10 per cent. of its volume of glacial acetic acid added to it, while at least two titrations of pooled samples will be necessary, since as has been pointed out samples cannot be correctly titrated when kept for more than twelve hours.

Experiment 7. *Ascorbic Acid in Blood Plasma.* Blood is taken from an arm vein, oxalated, and centrifuged. To minimise the oxidation of ascorbic acid, the plasma must be obtained as quickly as possible, and then its proteins must be immediately precipitated.

Transfer 2 c.c. of plasma to a centrifuge tube, add 4 c.c. of distilled water, and 4 c.c. of a 5 per cent. solution of glacial metaphosphoric acid. Mix well, allow to stand five minutes, and then centrifuge. Usually 5 or 6 c.c. of clear supernatant fluid are obtained. Pipette 2 c.c. of this clear fluid into each of two centrifuge tubes, and titrate directly with the diluted dye-solution, placed in a 5 c.c. micro-burette. The end point is the first faint pink colour.

Note. Since the end point is rather delicate, it is advantageous to use a blank solution as control, and to carry out the titration in duplicate.

Calculation. The number of cubic centimetres of dye-solution used, multiplied by 0·002, gives the amount of ascorbic acid present in 2 c.c. of diluted plasma (0·2 c.c. of plasma). Hence multiplying by 100/0·2 gives the number of milligrammes in 100 c.c. of plasma.

Example. The titration required 1·45 c.c. of dye-solution. Hence $1{\cdot}45 \times 0{\cdot}002 \times 100/0{\cdot}2$ equals 1·45 mg. ascorbic acid per 100 c.c. plasma (so that the actual titration figure expressed in milligrammes is the amount per 100 c.c.).

EXERCISE XXXIV

EXAMINATION OF A TWENTY-FOUR HOURS' SPECIMEN OF URINE

(*Two Three-hour Periods*)

THE student should carry out this Exercise on a twenty-four hours' specimen of his own urine for several reasons. If properly carried out, it illustrates the difficulties of complete collection, a point to be borne in mind when later, in practice, he gives instructions to have a twenty-four hours' specimen of a patient's urine collected, and suspects that the total quantity has not been obtained. He should appreciate better after this Exercise that many of the quantitative data of a urine are of no value unless applied to a twenty-four hours' specimen. Further, the Exercise affords a useful revision of a number of the previous determinations.

Collection. **All** the urine excreted in a twenty-four hours' period must be collected, no more and no less. Hence the bladder should be emptied at a certain time, say 8 a.m., and this sample rejected. All later samples until (in this case) 8 a.m. on the next day must be added together conveniently in a Winchester bottle.

Preservative. All urine samples that are not used for immediate test must always have a preservative added. Toluene is the best preservative. In this twenty-four hours' collection 25 c.c. of toluene should be poured into the **clean** Winchester bottle before collection starts.

Examination. Measure the volume accurately by means of a 1,000 c.c. graduated cylinder. Note the colour, the odour (which will, however, be largely masked by the toluene) and the reaction to litmus and congo-red paper.

Note if the urine be clear or cloudy. If cloudy, filter off a specimen and test the clear filtrate for specific gravity. Check the accuracy of the urinometer employed by measuring

distilled water with it. (The result should be 1·000.) Test also the clear filtrate for presence of reducing substances (if present, ascertain whether glucose or not), of albumin, of acetone and acetoacetic acid, and of indican. Examine the residue, if any, under the microscope. Remember that normal urines on standing frequently deposit amorphous precipitates of phosphates.

Carry out the following quantitative estimations : acidity by titration, acidity by *pH*, ammonia and amino-acids by Malfatti's method, urea by the urease method, ammonia by the aspiration method, total nitrogen by Kjeldahl's method, and creatine.

Express the results obtained in the following tabular form :—

Report on Twenty-four Hours' Sample of Urine

Sample of urine collected between.....a.m. of the.....day of.....and.....a.m. of the.....day of.....19...., and preserved in toluene.

Total volume.....c.c. Colour.....
Odour ..... Reaction to litmus.....
Transparency ..... Reaction to congo-red.....
Specific gravity .....
Reducing substances.....
Glucose .....
Acetone .....
Acetoacetic acid .....
Albumin .....
Indican.....
pH.....
Total acidity corresponds to.....c.c. of N/10 acid.
Urea-N.....gm. per 100 c.c., and therefore.....gm. per total volume.
NH_3-N.....gm. per 100 c.c., and therefore.....gm. per total volume.
Amino-acid-N.....gm. per 100 c.c., and therefore .....gm. per total volume.[2]

[1] Mention tests employed for glucose, if reducing substances are found present.

[2] The amino-acid nitrogen can be calculated from the differences between the results by Malfatti's procedure and the ammonia determination by aspiration.

Total N.....gm. per 100 c.c., and therefore.....gm. per total volume.

Creatinine.....gm. per 100 c.c., and therefore..... gm. per total volume.

Calculate from these results the *nitrogen partition* in the specimen of urine, taking the total N as 100 per cent. Tabulate thus :—

Urea-N	per cent.
Ammonia-N	,,
Amino-acid-N . . .	,,
Creatinine-N	,, [1]
Rest-N	,, [2]
Total . .	100 ,,

Calculate the creatinine coefficient, *i.e.*, the excretion of creatinine per kilogram body-weight per twenty-four hours, noting in the stated result whether this is a true value or not, *i.e.*, whether the diet during the twenty-four hours of collection was meat-free or not. Compare the result with normal values.

When considering the results obtained during this Exercise, the student should compare all values obtained with the limits of normal values. **Any abnormalities, such as positive tests for glucose, other reducing substances, or albumin, should at once be reported to the instructor,** who can decide if the tests are accurate, and whether further tests are desirable.

[1] To get the creatinine-N figure, multiply the result for creatinine by the weight ratio of its nitrogen content ($N_3 : C_4H_7N_3O$), *i.e.*, by 0·372.

[2] The rest-N includes the nitrogen present in uric acid, hippuric acid, and all other nitrogenous compounds not estimated in the above list.

EXERCISE XXXV

APPLICATIONS OF BIOCHEMICAL PROCEDURES TO MEASUREMENT OF CERTAIN TISSUE FUNCTIONS

MANY of the quantitative procedures that have been described are widely used in the diagnosis and control of pathological conditions, although training in the technique of these procedures can be more easily and as suitably given with normal material (urine and blood). Certain clinical applications of such procedures can, however, be most clearly illustrated when suitable abnormal (clinical) material is available. Some of these are described in this exercise, dealing with a few of the functional tests for the liver, the kidneys and the pancreas.[1]

TESTS OF LIVER FUNCTION

The multiplicity of functions carried on by liver tissues has led to many functional tests, each of which is only concerned with one specific function, so that results from any one such test may have little bearing on the function of the liver as a whole. Only a few of these tests will be mentioned.

Carbohydrate Tolerance Tests. The liver normally converts both fructose and galactose to glycogen. Fructose and galactose tolerance tests have been devised, similar to that described for glucose (Exercise XXVII.,

[1] For further details of this aspect of the subject the student is referred to such texts as Fowweather's "Clinical Chemical Pathology" (London, J. and A. Churchill, 1929), Hawk and Bergeim's "Practical Physiological Chemistry," 11th edit. (London, J. and A. Churchill, and Philadelphia, Blakiston, 1937), Nicholson's "Laboratory Medicine," 2nd edit. (Philadelphia, Lea and Febiger, 1934), Stewart and Dunlop's "Clinical Chemistry in Practical Medicine," 2nd edit. (Edinburgh, Livingstone, 1937), and Harrison's "Chemical Methods in Clinical Medicine," 2nd edit. (London, J. and A. Churchill, 1937), and also the papers in the literature cited in the text.

p. 164). While the dosage varies, 50 gm. of fructose and 40 gm. of galactose are usually given. The blood sugar values obtained during the test should not exceed the normal maximum values found after meals. The results of these tests are open to some criticism, and the tests cannot be applied to diabetics. Positive results may be of significance; negative results permit no definite conclusion as to the normal functioning of the liver.

Tests concerned with the Excretory Function of the Liver. The liver excretes the bile pigments; in conditions in which this function is impaired the excretion will be decreased. The bile pigments thereupon accumulate in the blood and the plasma takes on a deeper colour. The degree of bile pigmentation can be ascertained by determination of the icterus (bile) index or by the Van den Bergh test.

Determination of the Icterus Index [1]

A specimen of blood is matched against a standard solution of potassium bichromate.

Special Reagent. Dissolve exactly 0·1 gm. of potassium bichromate in water, transfer to a graduated litre flask, add 4 drops of concentrated sulphuric acid, and add water to the mark. Keep in a brown glass bottle to prevent the colour being affected by light.

Experiment 1. Collect in a centrifuge tube, without an anti-coagulant, 4 or 5 c.c. of blood from the arm vein of a patient with jaundice, and allow it to clot and the clot to stand until the serum begins to separate and then centrifuge (haemolysis must be avoided). Measure out 1 c.c. of the serum accurately into a test-tube and run in 0·9 per cent. sodium chloride solution from a burette until the colour of the diluted serum approximates to that of the standard in a similar test-tube. Compare in a colorimeter.

Calculation. The icterus index is given by the equation

$$\text{Icterus index} = \frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \frac{\text{Volume of diluted serum}}{1}.$$

[1] Meulengracht, *Deutsch. Arch. klin. Med.*, 1920, CXXXII., 285 Maue, *Surgery, Gynecol. Obstetrics*, 1922, XXXIV., 752.

Example. 1 c.c. of serum required 19 c.c. of the sodium chloride solution. The standard was set at 30 mm. and the diluted serum matched at 26·8 mm. Hence

Icterus index equals $30/26{\cdot}8 \times 20 = 22{\cdot}4$.

Note. The index is measured in arbitrary units (the reagent itself has an index of 1). The normal range is 4 to 6. Values above 15 are generally found in actual jaundice, while in the intermediate range they are usually to be attributed to latent jaundice (increase in blood bilirubin without definite clinical symptoms). Serum also contains the pigment carotene, and in certain conditions (diabetes, or, with children especially, a diet rich in vegetables, such as carrots or spinach, and therefore relatively rich in carotene) the blood carotene may increase to such an extent as to give an icterus index definitely above normal. The blood sample should be taken before breakfast, since alimentary lipaemia following a meal prevents a clear serum being obtained.

Determination of Blood Bilirubin by Van den Bergh's Method [1]

Bilirubin reacts with Ehrlich's diazo reagent to give a reddish-violet colour, proportional in intensity to the amount of bilirubin present. Bilirubin is too costly for the preparation of standard solutions, but the colour can be matched against a solution of cobalt sulphate (whose value in terms of pure bilirubin has been determined by Van den Bergh).[2]

Standard Solution.[3] Dissolve 1·3 gm. of anhydrous cobalt sulphate in 50 c.c. of distilled water. Add slowly, keeping the solution cool, 40 c.c. of concentrated hydrochloric acid, and then water to 100 c.c. Allow to stand twenty-four hours before use, and keep well stoppered and away from light. The colour of the solution is equivalent to that of 0·4 mg. of diazotised bilirubin per 100 c.c.

Special Diazo-reagent. Dissolve 1 gm. of sulphanilic acid in distilled water, add 15 c.c. of concentrated hydrochloric acid, and make up to 1 litre. Dissolve, separately, 3·75 gm.

[1] Thannhauser and Andersen, *Deutsch. Arch. klin. Med.*, 1921, CXXXVII., 179.

[2] Quoted by McNee and Keefer, *Brit. Med. J.*, 1925, II., 52.

[3] White, *Brit. J. Exp. Path.*, 1933, XIII., 76.

of sodium nitrite in water and make up to 1 litre. When required, mix 25 c.c. of the first solution with 1 c.c. of the second (the mixture does not keep).

Experiment 2. Add to 1 c.c. of blood serum, obtained as in Experiment 1 (or an accurately measured smaller volume if the bilirubin content is known to be large), in a graduated centrifuge tube 0·5 c.c. of the diazo-reagent, and, after fifteen minutes, 2·5 c.c. of 95 per cent. alcohol and 1 c.c. of saturated solution of ammonium sulphate, mixing well after each addition. The contents of the tube separate into three layers, a clear reddish-violet layer of azo-bilirubin on top, then a (coloured) layer of precipitated protein, and, below, a colourless layer of ammonium sulphate. Measure the volume of this colourless layer and subtract it from the total volume (the difference is usually about 4 c.c.). Transfer the clear-coloured supernatant liquid to a colorimeter and compare against the standard.

Calculation. Since the standard is equivalent to a solution of bilirubin containing 0·4 mg. per 100 c.c., the number of milligrammes per 100 c.c. serum is given by

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \frac{\text{(Total volume} - \text{volume of colourless layer)}}{\text{Original volume of serum}} \times 0{\cdot}4.$$

Example. Total volume after centrifuging was 4·8 c.c.
The volume of the colourless layer was 0·8 c.c.
Hence difference : 4·0 c.c.
With standard set at 20 mm. the unknown solution matched at 17·6 mm.
Hence $20/17{\cdot}6 \times 4{\cdot}0/1 \times 0{\cdot}4 = 1{\cdot}8$ mg. per 100 c.c. serum.

Note. The normal limits, according to Van den Bergh, are from 0·1 to 0·2 mg. per 100 c.c. The true normal maximum is probably somewhat higher. Modifications of the (qualitative) Van den Bergh test have been used to differentiate between different types of jaundice, with what justification is still uncertain.

TESTS FOR UROBILINOGEN IN STOOLS

Faeces are normally coloured by pigment derived from bilirubin. Absence of such pigment (as in so-

called " clay-coloured " stools) is of doubtful significance as far as liver function is concerned, but does indicate interference with the flow of bile from liver to intestine—obstruction of the bile ducts. The following delicate test, due to Watson,[1] is useful in determining whether or not urobilinogen (with other bile pigments associated with it) is completely absent from such stools.

Reagents

Ehrlich's reagent is prepared by the addition of 10 gm. of paradimethylaminobenzaldehyde, 75 c.c. of concentrated hydrochloric acid, and 75 c.c. of distilled water.

In addition crystallised ferrous sulphate and 10 per cent. sodium hydroxide are required.

Experiment 3. Transfer about 2 gm. of the mixed stool to a small mortar, add a small amount of water from a measured volume of 40 c.c., and grind thoroughly with a pestle. Add the remainder of the water, grind again, and decant the supernatant fluid into a conical flask. To the material left in the mortar add 20 c.c. of 10 per cent. sodium hydroxide, grind again, transfer the contents to the flask, and shake the latter vigorously for a few minutes.

Weigh out about 5 gm. of finely ground ferrous sulphate, and transfer to a suction flask equipped with a length of pressure tubing attached to the side tube. Dissolve the ferrous sulphate in 25 c.c. of water, and add to this, in small portions and with shaking, the contents of the conical flask. Cork tightly and evacuate with suction. When a fair vacuum has been produced, clamp the rubber tube, disconnect from the pump, and place the flask in the dark for an hour. Then filter about 5 c.c. into a test-tube and add the Ehrlich reagent drop by drop until cloudiness just disappears.

Normal stools give immediately a pink to red colour. A very deep red produced immediately probably indicates an abnormal amount of urobilinogen, whilst a faint pink may indicate a subnormal content. True clay-coloured stools give no coloration, indicating complete absence of urobilinogen.

[1] *Arch. Int. Med.*, 1931, XLVII., 698, and personal communication.

TESTS OF KIDNEY FUNCTION

Amongst the chief functions of the kidneys are the removal of certain waste products of metabolism, of which urea, creatinine and uric acid are of outstanding importance, and the maintenance of certain constituents of the organism in metabolic equilibrium and the whole organism in osmotic equilibrium; this is done by removal of water and inorganic salts. The normal kidney performs work in effecting a marked concentration. For example, a normal blood urea figure is 0·03 per cent., while the corresponding normal urine concentration is from 1 to 2 per cent. The damaged kidney loses to varying degree this power of concentration, while, in addition, it exhibits decreased ability to pass excretory substances through its tissues.

Numerous tests of kidney function have been devised. Increases in blood urea, in blood uric acid, and in blood creatinine, yield valuable information (although it must be remembered that circulatory disturbances also affect the concentrations of such constituents). Uric acid responds first to kidney dysfunction, urea follows, while creatinine, a compound which passes through animal membranes with ease, is one of the last to be dammed back by a markedly damaged kidney. Estimation of these in blood, especially of urea and creatinine, yields a critical insight into the extent of a definite nephritis.

It is more difficult to confirm a suspected mild nephritis, although obviously in this, as in other pathological conditions, early diagnosis is of greatest value. Experiments on animals have demonstrated that more than half the kidney tissue can be removed before an increase in blood urea occurs.

In Mosenthal's test [1] with a patient on a normal diet (three meals, with no solids nor liquids between meals) urine is collected from 8 a.m. to 8 p.m. at two-hour intervals

[1] Mosenthal, *Boston Med. Surg. J.*, 1914, CLXX., 245; *Ohio State Med. J.*, 1922, XVIII., 348.

and from 8 p.m. to 8 a.m. as one sample. On each specimen the volume, specific gravity, chlorides, and sometimes total nitrogen, are determined. Normally the extremes in specific gravity should show at least a difference of 0·009, and the night urine and at least one specimen of the day urine should show 1 per cent. of sodium chloride (and of total nitrogen). The night urine volume should not exceed 400 c.c. Diminished kidney function increases the volume of night urine, lessens the spread of specific gravity values (which tend to be low) and lessens the output of chloride and total nitrogen. The test is not too specific for kidney conditions and requires careful cognisance of the actual meals eaten.

In Maclean's urea concentration test, and modifications such as those of Calvert [1] and Fowweather,[2] a solution containing a definite quantity of urea is administered, and the rate of elimination of this added urea is determined with or without consideration of the blood urea value. Damaged kidney function lowers this rate. In De Wesselow's chloride concentration test the maximum chloride concentration in three one-hourly specimens of urine is determined following administration of 4 gm. of potassium chloride in 200 c.c. of water. In Rowntree and Geraghty's phenolsulphonphthalein (phenol red) test [3] 1 c.c. of a slightly alkaline solution containing 6 mg. of the non-irritant dye is injected intramuscularly or preferably intravenously, and the initial time of appearance in the urine is noted, and the rate of excretion determined colorimetrically. From 60 to 85 per cent. of the dose is normally eliminated in the first two hours. Lowered kidney function prolongs the excretion.

One of the most delicate tests for kidney function, with a sound scientific basis, is the urea clearance test of Van Slyke and his collaborators.[4]

The Urea Clearance Test. The principle of the test consists in determining the rate of removal of urea from blood to urine; the result is expressed as the volume of blood which, at this rate of excretion, would be completely denuded of urea in one minute (the *urea clearance*).

[1] Calvert, *Brit. Med. J.*, 1925, I., January 10th.
[2] Fowweather, *loc. cit.*, p. 58.
[3] Rowntree and Geraghty, *J. Pharmacol.*, 1910, I., 579; *Arch. Int. Med.*, 1912, March, p. 284.
[4] Möller, McIntosh and Van Slyke, *J. Clin. Invest.*, 1928–29, VI., 427.

Experiment 4. The subject, avoiding previous vigorous exercise and resting throughout the test, takes a moderate meal (preferably the breakfast meal) without coffee (a diuretic). The bladder is emptied at a definite time and the urine rejected. Shortly before the expiry of one hour from the commencement of the test an oxalated sample of blood is obtained. The bladder is emptied at the end of the first and second hours precisely, and urea is determined in the blood and the two urine samples (the amount of urinary ammonia is relatively negligible, and also, according to present theory, represents blood urea converted to ammonia by the kidneys, so that no correction need be applied for it).

Calculation. When, in an adult, the rate of urine excretion exceeds 2 c.c. per minute, there exists a direct proportionality between the blood urea content and the rate of urea excretion. If B is the number of milligrammes of urea per 100 c.c. of blood, U the corresponding number of milligrammes per 100 c.c. of urine, and V the volume of urine in cubic centimetres excreted per minute, then U/B is the degree of concentration effected by the kidneys, and hence represents the number of cubic centimetres of blood, which contain the same amount of urea as 1 c.c. of urine, whence UV/B represents the number of cubic centimetres of blood which would be cleared of urea in one minute (the urea clearance). This value is termed the *maximum clearance*, C_m, and, in normal adults, the average value found for it is 75 (c.c. per minute). Hence the relative percentage value found in any test in which more than 2 c.c. per minute is excreted is given by

$$\frac{UV}{B} \times \frac{100}{75}.$$

When the normal rate of urea excretion is less than 2 c.c. per minute (in an adult), the direct proportionality between blood urea content and rate of excretion no longer holds. The change takes place fairly sharply at about the limiting figure mentioned. With the lowered rate of excretion the blood urea clearance is found to vary on the average in proportion to the *square root* of the urine volume. For the purpose of comparison a standard urine volume has been taken as 1 c.c. per minute (in close approximation to the actual average excretion for a normal adult). If the

observed urea clearance is C and the *standard clearance* is C_s, then

$$C_s = C\sqrt{1/V}$$

where V is the observed volume per minute. Since the observed clearance is equal to UV/B, it follows that

$$C_s = \frac{UV}{B}\sqrt{\frac{1}{V}} = \frac{U}{B}\sqrt{V}.$$

The mean value for the *normal standard clearance* is 54 (c.c. per minute), whence the relative percentage value found in any test in which less than 2 c.c. of urine is excreted per minute is given by

$$\frac{U}{B}\sqrt{V} \times \frac{100}{54}.$$

The test can be applied to children by multiplying the urine volumes by the factor (normal surface area of adult, 1·73 sq. mm.)/ (actual surface area of child).

Van Slyke and his collaborators have shown that the test correctly indicates abnormal kidney function in cases which present normal blood urea values. Values below 70 per cent. of normal strongly suggest diminished function. Maximum clearance and standard clearance values are affected to approximately an equal extent.

The square roots of the values of V over the significant range are—

V	0·2	0·3	0·4	0·5	0·6	0·7	0·8	0·9	1·0	
$\sqrt{V}$	0·45	0·55	0·63	0·71	0·78	0·84	0·89	0·95	1·00	
V	1·1	1·2	1·3	1·4	1·5	1·6	1·7	1·8	1·9	2·0
$\sqrt{V}$	1·05	1·10	1·14	1·18	1·23	1·27	1·30	1·34	1·38	1·42

Examples. (Obviously urea-nitrogen figures can be substituted for those of urea.)

Case 1. Excretion below 2 c.c. per minute. Blood urea-N was 16 mg. per 100 c.c. (high normal).

1st hour :

urine volume, 30 c.c., equal to 0·50 c.c. per minute (V),
urine urea-N 1,257 mg. per 100 c.c. (U),

$$\text{hence } \frac{U}{B}\sqrt{V} \times \frac{100}{54} = \frac{1{,}257}{16} \times 0{\cdot}71 \times \frac{100}{54} = 103 \text{ per cent.}$$

2nd hour :
urine volume, 25 c.c., equal to 0·42 c.c. per minute (V),
urine urea-N 1,280 mg. per 100 c.c. (U),

$$\frac{U}{B}\sqrt{V} \times \frac{100}{54} = \frac{1{,}280}{16} \times 0{\cdot}65 \times \frac{100}{54} = 96 \text{ per cent.}$$

Mean *standard clearance* = 99 ,,

Case 2. Excretion above 2 c.c. per minute. Blood urea-N was 54 mg. per 100 c.c. (high).

1st hour :
urine volume, 140 c.c., equal to 2·33 c.c. per minute (V),
urine urea-N 291 mg. per 100 c.c. (U),

$$\frac{UV}{B} \times \frac{100}{75} = \frac{291 \times 2{\cdot}33 \times 100}{54 \times 75} = 17 \text{ per cent.}$$

2nd hour :
urine volume, 140 c.c., equal to 2·33 c.c. per minute (V),
urine urea-N 307 mg. per 100 c.c. (U),

$$\frac{UV}{B} \times \frac{100}{75} = \frac{307 \times 2{\cdot}33 \times 100}{54 \times 75} = 18 \text{ per cent.}$$

Mean *maximum clearance*, 17 per cent. (in agreement with the high blood urea).

Case 3. Blood urea-N was 15 mg. per 100 c.c. (normal).

1st hour :
urine volume, 25 c.c., equal to 0·42 c.c. per minute (V),
urine urea-N 781 mg. per 100 c.c. (U),

$$\frac{U}{B}\sqrt{V} \times \frac{100}{54} = \frac{781 \times 0{\cdot}65 \times 100}{15 \times 54} = 63 \text{ per cent.}$$

2nd hour :
urine volume, 17 c.c., equal to 0·28 c.c. per minute (V),
urine urea-N 735 mg. per 100 c.c. (U),

$$\frac{U}{B}\sqrt{V} \times \frac{100}{54} = \frac{735 \times 0{\cdot}53 \times 100}{15 \times 54} = 49 \text{ per cent.}$$

Mean *standard clearance*, 56 per cent. (suggesting a pathological condition not revealed by the blood urea figure).

TESTS OF PANCREATIC FUNCTION

Two tests of pancreatic function are interesting from the biochemical standpoint, the determination of the amylase content of urine and the determination of the fat of the faeces.

The Diastatic Index of the Urine.[1] Under normal conditions traces of pancreatic amylase are absorbed (with other enzymes) from the small intestine, and so reach the urine through the kidneys. In damaged kidney conditions the amount normally reaching the urine decreases. On the other hand, in acute inflammatory conditions of the pancreas (acute pancreatitis) the enzymes of the juice may pass directly from the secreting cells to the blood stream and a much-increased urine content result.

Experiment 5.[2] Dilute a sample of urine from a case of suspected acute pancreatitis with nine times its volume of 0·9 per cent. sodium chloride. Make up the following urine-saline mixtures in a series of test-tubes, using pipettes graduated to 0·1 c.c. Add to each mixture (when the series is complete) as rapidly as possible 2 c.c. of 0·1 per cent. starch solution and place in a water-bath at 37° C. for exactly thirty minutes.

Tube No.	Diluted urine.	NaCl 0·9 per cent.	Starch 0·1 per cent.	Equivalent vol. of undiluted urine.	Diastatic unit value.
	c.c.	c.c.	c.c.	c.c.	c.c.
1	1·0	0·0	2·0	0·10	20
2	0·8	0·2	2·0	0·08	25
3	0·4	0·6	2·0	0·04	50
4	0·2	0·8	2·0	0·02	100
5	0·1	0·9	2·0	0·01	200

Cool by almost filling the test-tube with cold water (the dilution tends to stop further amylase action) and add to

[1] " Diastase " is an old name for any starch-splitting enzyme.

[2] After Wohlegemuth, *Biochem. Zeitschr.*, 1908, IX., 1. Cohen and Dodds (*Brit. Med. J.*, 1924, I., 618) have suggested a somewhat more complex and more accurate procedure, and Willstätter, Waldschmidt-Leitz and Hesse (*Zeitschr. Physiol. Chem.*, 1923, CXXVI., 143) a titrimetric procedure, both of which could be adapted.

each tube 2 drops of iodine solution (in potassium iodide) and shake. Certain of the tubes will show the blue starch-iodide colour (some undigested starch remaining) others only a slight yellow colour from the iodine (all starch digested). Note where the dividing line occurs; the "diastatic activity" of the urine is between the limits indicated by the two tubes concerned.

Calculation. The diastatic units are arbitrary, being the number of cubic centimetres of 0·1 per cent. starch solution which are hydrolysed to sugar by 1 c.c. of an amylase solution in thirty minutes. Hence where x is the number of cubic centimetres of starch solution so changed and y is the volume of (undiluted) urine used, x/y gives the number of diastatic units (as shown in the last column on p. 224).

Example. Tube 4 gave a negative iodine reaction, tube 5 a positive blue colour. Hence the diastatic value of the undiluted urine lies between 100 and 200 units.

Note. In normal individuals the limits of value are 6 to 25 units, but with cases of acute pancreatitis values of 100 are frequently found. In kidney conditions values below 6 may be found, and if it be desired to apply the test in such cases undiluted urine should be used. The test is of no value in chronic pancreatic conditions.

The Fat Content of the Faeces. When, as a result of pancreatic lesions, there is a deficiency of the enzymic activity of the juice, such deficiency can frequently be detected by a change in the fat composition of the faeces. Faeces normally contain a small percentage of fat (both neutral fats and fatty acids and soaps). With diminished lipolytic activity in the intestine the percentage of total fat is increased, and the proportion of fat hydrolysed (fatty acids and soaps) to total fat is diminished. Determinations of total fat, hydrolysed fat and water are necessary, the last since the water content of faeces is so variable that it may easily mask other variations. All estimations should be made on a thoroughly mixed sample of faeces.

Experiment 6. (A) *Estimation of water content.* Weigh out accurately in a closed weighing bottle about 5 gm. of faeces. Evaporate to constant weight on a water-bath in a fume chamber. (This gives an approximate figure. For accuracy final heating should be carried out to constant weight in an oven at 105° C.)

(B) *Estimation of Total Fat.* Powder the dried faeces and transfer completely to a stoppered 100 c.c. graduated cylinder, washing in with water to the 30 c.c. mark. Add 2 c.c. of concentrated hydrochloric acid. Shake thoroughly (soaps are converted to fatty acids). Add 20 c.c. of ether and shake at frequent intervals for thirty minutes. Allow the two layers to separate completely, then pipette 10 c.c. of the ethereal solution (representing with sufficient accuracy one-half of the total ether extract) into a weighed evaporating basin, evaporate off the ether on a hot water-bath (in absence of a flame), and then dry the residue in an oven at 110° C. to drive off all trace of hydrochloric acid. Cool and weigh. The difference in weight gives the total fat.

(C) *Estimation of Hydrolysed Fat* (fatty acids and soaps). Dissolve the residue from (B) in hot benzene, add a few drops of phenolphthalein, and run in N/10 sodium ethoxide (prepared by dissolving freshly cut metallic sodium in absolute alcohol, and standardised as with ordinary sodium hydroxide solution) drop by drop until a red colour is just produced.

Calculation. The water content is given by difference in weights of the fresh and dried faeces and should be expressed in percentage. It is customary to represent the fatty acids in terms of stearic acid. One c.c. of N/10 sodium ethoxide corresponds to 28·4 mg. of stearic acid. An example will illustrate the general calculation most easily.

Example. 5·025 gm. of fresh faeces gave 1·310 gm. of dried residue. Hence the percentage of solid matter was 1·310/5·025 × 100, *i.e.*, 26·1 per cent.

The 1·31 gm. of dried faeces gave a residue from ether of 0·462 gm. (total fat), which, dissolved in ether, required 6·70 c.c. of sodium ethoxide to turn phenolphthalein red. Hence the total fat equalled 0·462/1·31 × 100, *i.e.*, 35·3 per cent. of the dry faeces, and therefore 35·3 × 26·1/100, or 9·2 per cent. of the fresh faeces. The hydrolysed fat, as stearic acid, amounted to 28·4 × 6·7 mg., or 0·190 gm. Hence the proportion of hydrolysed fat to total fat was 0·190/0·462 × 100, or 41 per cent., and the percentage of hydrolysed fat in the total solids of the faeces is 41 × 35·3/100, or 14·5 per cent.

Note. The method outlined tends to give somewhat too high results for hydrolysed fat, due to the initial drying. In normal faeces the hydrolysed fat usually exceeds the

neutral fat fraction, while the total fat seldom exceeds 25 per cent. of the total solids. In conditions involving deficiency of pancreatic lipase the neutral fat fraction is in excess of the amount hydrolysed, while the total fat may markedly exceed 25 per cent. of the total solids.

APPENDIX I

REAGENTS AND MATERIAL REQUIRED FOR THE COURSE

THE composition of many of the special reagents has been detailed in the text. Page references are given in the following lists in these cases.

Reagents on the students' benches should be as few in number as possible. Qualitative reagents only occasionally required should be provided on wall shelves conveniently arranged about the laboratory. A set of such reagents should be provided for each ten or twelve students. Special reagents for quantitative work and material subject to decomposition should only be issued as required. The same applies to material required for special demonstrations. The following system works satisfactorily :—

BENCH REAGENTS

Acids (on a glass shelf)

Acetic acid, $CH_3 . COOH$, *glacial*, pure, sp. gr. 1·06.

Acetic acid, dilute, 3 per cent. (27 c.c. of glacial acetic acid made up to 1 litre with water).

Hydrochloric acid, concentrated, sp. gr. 1·16 (366 gm. HCl per litre).

Hydrochloric acid, dilute, 10 per cent. (270 c.c. of concentrated acid per litre).

Nitric acid, concentrated, sp. gr. 1·42 (991 gm. HNO_3 per litre).

Nitric acid, dilute, about 2*N*, sp. gr. 1·07, 12 per cent. (133 c.c. of concentrated acid to 1 litre).

Sulphuric acid, concentrated, sp. gr. 1·84.

Sulphuric acid, dilute, about 2*N*, 10 per cent. (56 c.c. of concentrated acid to 1 litre).

Bases and Salt Solutions

Ammonia, 15 per cent. (one volume of water to one volume of concentrated ammonia, sp. gr. 0·88).

Ammonium molybdate. (Prepare by dissolving 100 gm. of molybdic acid in 144 c.c. of ammonia, sp. gr. 0·90—obtained

by diluting four parts of concentrated ammonia with one part of water—and 271 c.c. of water, and pouring the solution with constant stirring into 490 c.c. of concentrated nitric acid and 1,148 c.c. of water. Keep in a warm place until a portion heated at 40° C. deposits no yellow precipitate. Decant from any sediment into glass-stoppered bottles.)

Ammonium sulphate, saturated solution.

Barium chloride, 3 per cent.

Benedict's qualitative reagent for sugar. (Dissolve 173 gm. of sodium citrate and 100 gm. of anhydrous sodium carbonate in 800 c.c. of water. Transfer, filtering, if necessary, into a 1,000 c.c. graduated cylinder. Dissolve 17·3 gm. of crystallised copper sulphate in 100 c.c. of water, and pour this slowly with stirring into the cylinder. Make up to 1 litre.)

Copper sulphate, $CuSO_4$, 5 H_2O, 1 per cent.

Iodine solution (in potassium iodide), *N*/10. (Dissolve 30 gm. of potassium iodide in 250 c.c. of water, and in this dissolve 13 gm. of pure iodine. Dilute with water to 1 litre.)

Silver nitrate, 1 per cent.

Sodium carbonate, *N*/2, 2·6 per cent. (71·5 gm. of crystallised sodium carbonate, Na_2CO_3, 10 H_2O, or 26·5 gm. of anhydrous sodium carbonate, dissolved in water to 1 litre).

Sodium hydroxide, 2*N*, 8 per cent. (80 gm. of purified stick sodium hydroxide dissolved in water, and diluted to 1 litre).

ADDITIONAL REAGENTS

Solids

Ammonium chloride.
Ammonium oxalate.
Ammonium sulphate.
Bleaching powder (calcium hypochlorite).
Copper oxide (red, CuO).
Copper sulphate (cryst.).
Charcoal (animal).
Iodoform.
Magnesium (powder).
Magnesium sulphate (cryst.).
Metaphosphoric acid.
Oxalic acid (cryst.).
Potassium hydrogen sulphate.
Potassium hydroxide (stick).
Potassium nitrate (powdered).
Potassium oxalate (powdered).
Phenylhydrazine mixture (see p. 10).
Phloroglucinol.
Sand.
Soda lime.
Sodium carbonate (anhydrous).
Sodium chloride.
Sodium hydroxide (stick).
Sodium nitrite.
Sodium nitroprusside.
Sulphur (flowers).
Wax, paraffin.

Solutions

Acetic acid, *N*/10 (5·4 c.c. glacial acetic acid to 1 litre).

Boracic acid, 0·4 per cent.

Hydrochloric acid, 0·4 per cent. (11 c.c. of concentrated acid to 1 litre) (approximately *N*/10).

"*Combined hydrochloric acid*" (protein hydrochloride), 0·4 per cent. (Dissolve 5 gm. of Witte's peptone in 100 c.c. of 0·4 per cent. acid and allow to stand for twenty-four hours.)

Hydrobromic acid, *N*/10.

Lactic acid, *N*/10, 0·9 per cent. (10·5 c.c. of syrupy 85 per cent. lactic acid to 1 litre).

Lactic acid, 0·5 per cent. (6 c.c. of 85 per cent. acid to 1 litre).

Lactic acid, 0·1 per cent.

Nitric acid, yellow. (Nitric acid that has stood for some time, or to which have been added a few crystals of sodium nitrite.)

Nitric acid, *N*/10 (6 c.c. of concentrated acid to 1 litre).

Oxalic acid, *N*/10 (6·3 gm. of crystallised oxalic acid $(COOH)_2$, 2 H_2O, dissolved to 1 litre in water).

Sulphuric acid, *N*/10 (2·8 c.c. of concentrated acid to 1 litre).

Ammonium carbonate, saturated solution.

Ammonium chloride, saturated solution.

Ammonium hydroxide, concentrated, sp. gr. 0·88 (31 gm. NH_3 per litre).

Ammonium oxalate, saturated solution.

Ammonium sulphide, saturated solution. (Saturate concentrated ammonia with hydrogen sulphide.)

Ammonium thiocyanate (13 gm. of ammonium thiocyanate to 1 litre).

Bleach (calcium hypochlorite), saturated solution.

Calcium chloride, saturated solution.

Calcium chloride, 2 per cent.

Ferric alum, saturated solution.

Ferric chloride, 10 per cent.

Ferric chloride, 2 per cent.

Ferrous sulphate, 10 per cent. (freshly made up as required).

Hydrogen peroxide, 3 per cent.

Lead acetate, *neutral*, 10 per cent.

Lime water, saturated solution.

Magnesium sulphate, saturated solution.
Mercuric chloride, about N/2 (7 gm. to 100 c.c.).
Potassium bichromate, 2·5 per cent.
Potassium carbonate, saturated solution.
Potassium ferricyanide, 10 per cent.
Potassium ferrocyanide, 10 per cent.
Potassium oxalate, 20 per cent.
Potassium permanganate, 8 per cent.
Sodium carbonate, saturated solution.
Sodium carbonate, 5 per cent.
Sodium carbonate, 2 per cent.
Sodium carbonate, 1 per cent.
Sodium carbonate, 0·5 per cent.
Sodium chloride, 10 per cent.
Sodium chloride, 1 per cent.
Sodium fluoride, 2 per cent.
Sodium hydroxide, 40 per cent.
Sodium hydroxide, 1 per cent.
Sodium hypobromide (25 c.c. of bromine added to 250 c.c. of 40 per cent. sodium hydroxide; this solution should be fairly fresh).
Sodium nitrite, 1 per cent.
Sodium phosphate, Na_2HPO_4, 0·1 per cent.

N.B. Where more than one concentration of a solution is required, only the strongest need be placed on the shelves; it is convenient, however, to issue all such solutions.

Organic Liquids, etc.

Acetic anhydride.
Acetone.
Alcohol, ethyl, 95 per cent.
Alcohol, ethyl, 70 per cent.
Amyl alcohol.
Chloroform.
Diacetyl.
Ether.
Formaldehyde (40 per cent.).
Glycerol.
Oleic acid.
Olive oil.
Pepsin solution (commercial, 1 per cent.).
Peptone, 1 per cent.

Rennet (commercial).
Sodium citrate, saturated solution.
Starch, 1 per cent.
Sucrose, 20 per cent.
Toluene.

Special Reagents

Alcoholic sodium hydroxide (prepared by dissolving about 2 gm. of cleaned metallic sodium in 1 litre of absolute or 95 per cent. ethyl alcohol, or else by dissolving 20 gm. of stick sodium hydroxide in 1 litre of 95 per cent. ethyl alcohol).
Barfoed's reagent (see p. 10).
Benzidene, saturated solution in glacial acetic acid.
Bial's reagent (see p. 15).
Bromine water, saturated solution.
Chlorine water, saturated solution.
Cross and Bevan's reagent (see p. 22).
Denigès' reagent (see p. 67).
Diphenylamine, 20 per cent. solution in 95 per cent. alcohol.
Esbach's reagent. (Dissolve 10 gm. of picric acid and 20 gm. of citric acid in 1 litre of water.)
Fehling's solution A and B (see p. 8).
Glyoxylic acid (see p. 39).
Guaiac solution (0·5 gm. of guaiac resin dissolved in 30 c.c. of 95 per cent. ethyl alcohol).
Günzberg's reagent (see p. 155).
Hanke and Koessler's reagent. (Make up two stock solutions, the first containing 9 gm. sulphanilic acid and 90 c.c. of concentrated hydrochloric acid to the litre and the second being a 5 per cent. sodium nitrite solution. When the reagent is required, transfer 3 c.c. of each solution to a 100 c.c. flask, cool in ice-water for five minutes, add 12 c.c. of the nitrite solution, again cool for five minutes, and add water to the 100 c.c. mark. The solution when ready for use must be kept on ice, and will not keep more than twenty-four hours.)
Hubl's iodine reagent (see p. 35).
Mercuric sulphate (10 gm. $HgSO_4$ dissolved in 100 c.c. of 5 per cent. sulphuric acid).
Millon's reagent (see p. 38).
Molisch's reagent (see p. 8).
Nessler's reagent. (To 100 gm. of mercuric iodide and 70 gm. of potassium iodide in a 1 litre flask add 400 c.c. of

water, and rotate until the iodides have dissolved. Dissolve 100 gm. of stick sodium hydroxide in 500 c.c. of water, cool to room temperature or below, and add this solution to the flask with constant shaking. Add water to the mark and mix. On standing a brownish-red precipitate usually forms. Decant from this into a stoppered bottle.)

Ninhydrin solution (0·1 per cent. solution of triketohydrindene hydrate).

Nylander's solution (see p. 9).

Obermayer's reagent (4 gm. ferric chloride dissolved in 1 litre of concentrated hydrochloric acid).

Osmic acid (1 per cent.).

Phosphomolybdic acid. saturated solution.

Phosphotungstic acid. Dissolve 50 gm. of phosphotungstic acid and 30 c.c. of concentrated sulphuric acid in water and make up to 1 litre.)

Picric acid, saturated solution. (For purification see p. 123.)

Potassium mercuric iodide (2 per cent.). (Or saturate with 120 gm. of mercuric iodide a solution of 50 gm. of potassium iodide in 500 c.c. of water, and make up to 1 litre.)

Seliwanoff's reagent (see p. 13).

Sodium nitroprusside solution (5 per cent., freshly made up when required).

Sodium picrate (see p. 167).

Takayama's solution (see p. 78).

Tannic acid solution. (Dissolve 100 gm. tannic acid, 25 gm. sodium acetate, 25 gm. sodium chloride, and 50 gm. glacial acetic acid in water and make up to 1 litre.)

Thymol solution in alcohol, 5 per cent.

o-Tolidin, 4 per cent.

Trichloracetic acid, 10 per cent.

Uffelmann's reagent (see p. 88).

Uric acid reagent (*Folin's*). (Transfer 100 gm. of sodium tungstate to a 2 litre flask, add 750 c.c. of distilled water, and shake until dissolved (neglecting any trace of calcium salts remaining). Add 80 c.c. of syrupy phosphoric acid (85 per cent.). Insert a funnel into the mouth of the flask, and boil gently for two hours. If the resulting fluid is very dark in colour add a few drops of bromine, and boil until bromine is no longer given off. Cool, transfer to a 1 litre volumetric flask, and add water to the mark. Mix.)

Indicators

Clark and Lub series (*brom cresol green, chlorphenol red, brom phenol red, brom cresol purple, brom thymol blue, phenol red, cresol red*) (see p. 131).

Congo-red solution, 0·1 per cent.

Congo-red paper.

Litmus powder.

Litmus paper, red and blue.

Litmus solution. (Extract 10 gm. of finely powdered litmus with about 50 c.c. of boiling water, decant and make the solution up to 1 litre.)

Methyl red, 0·2 per cent. in 95 per cent. ethyl alcohol.

Phenolphthalein paper.

Phenolphthalein solution, 1 per cent. in 95 per cent. ethyl alcohol.

Töpfer's reagent (see p. 49).

Vanillin, 0·6 per cent. aqueous solution.

N.B. Congo-red paper can be made by saturating good filter paper with 0·1 per cent. congo-red solution, drying it in air, and cutting it into strips. Phenolphthalein paper can be similarly prepared.

Material for Testing, etc.

Agar-agar.
Albumin, blood, commercial.
Albumin, egg, commercial.
Bayberry tallow.
Beeswax.
Bone ash.
Butter.
Casein, commercial.
Cholesterol.
Citric acid.
Cotton-wool.
Dextrin.
Fibrin.
Gelatin.
Glucose (powdered).
Guano, pigeon's.
Gum arabic.
Haemoglobin, commercial.
Hemp seed.
Hippuric acid.
Inulin.
Keratin (ox-horn shavings).
Lactose (powdered).
Lard.
Palmitic acid.
Peptone, commercial.
Peptone, Witte's.
Sodium citrate.
Starch.
Stearic acid.
Sucrose.
Thyroid, desiccated.
Urea.
Urease tablets (or Soy-bean powder).
Uric acid.
Wheat flour.
Yeast, dried.

APPENDIX II

SOME USEFUL DATA

Atomic Weights of Elements of Biochemical Importance
(As revised to 1935)

Boron	B	10·82
Bromine	Br	79·92
Calcium	Ca	40·08
Carbon	C	12·00
Chlorine	Cl	35·46
Copper	Cu	63·57
Fluorine	F	19·00
Hydrogen	H	1·0078
Iodine	I	126·92
Iron	Fe	55·84
Magnesium	Mg	24·32
Manganese	Mn	54·93
Molybdenum	Mo	96·0
Nitrogen	N	14·008
Oxygen	O	16·0000
Phosphorus	P	31·02
Potassium	K	39·10
Silver	Ag	107·88
Sodium	Na	23·00
Sulphur	S	32·06
Tungsten	W	184·0
Uranium	U	238·14

Comparison of Metric and English Weights and Measures

1 metre	= 1·0936 yards	= 39·37 in.	1 yard	= 3 ft. = 0·9144 metre
1 cm.	= 0·394 in.		1 in.	= 2·54 cm.
1 kg.	= 1,000 gm.	= 2·2046 lb.	1 lb.	= 453·59 gm.
1 gm.	= 15·432 gr.	= 0·03527 oz.	1 oz.	= 437·5 gr. = 28·35 gm.
			1 gr.	= 0·0648 gm.
1 litre	= 1,000 c.c.	= 0·22 gallon (Brit.)	1 gallon (Brit.)	= 8 pints = 4·546 litres
		= 0·264 gallon (U.S.)	1 gallon (U.S.)	= 3·785 litres

1 c.c. = 0·0352 fluid oz. (Brit.) 1 fluid oz. (Brit.) = 8 fluid drachms
= 0·282 fluid drachm (Brit.) = 28·40 c.c.
= 16·9 minims 1 minim (Brit.) = 0·0592 c.c.
1 gallon (Brit.) of distilled water = 10 lb. = 70,000 gr.
1 gallon (U.S.) of distilled water = 8·33 lb.
1 litre of hydrogen gas at 0° C. and 760 mm. mercury pressure weighs 0·0899 gm.[1]

Essential Data for Normal Urine (usual limits) (Twenty-four Hours' Specimen, Adult Man)

Volume	1,200—1,800 c.c.
Specific gravity	1·015—1·025
pH	5·5 —8·0
Acidity corresponding to	200—600 c.c. N/10 acid
Total solids	45 —90 gm. (av. 70 gm.)
Total nitrogen	12 —18 gm.
Chloride	10 —15 gm. (as NaCl)
Phosphate	av. 2·5 gm. (as P_2O_5)
Sulphate	1·5 — 3·0 gm. (as SO_3)
Calcium	0·07— 0·3 gm.
Magnesium	0·06— 0·2 gm.
Potassium	1·5 — 2·5 gm.
Sodium	3·0 — 4·5 gm.
Ammonia	av. 0·7 gm.
Amino-acid-N	0·1 — 0·2 gm.
Urea	15 —30 gm.
Uric acid	0·5 — 1·0 gm. (av. 0·7 gm.)
Hippuric acid	av. 0·7 gm.
Creatinine	1·0 — 1·5 gm.
Creatine	0·0 —trace
Glucose	0·1 gm.
Protein	0·0 gm.

Essential data for Normal Human Blood

Whole Blood

Glucose[2]	0·08— 0·10 gm.	per 100 c.c. (fasting)
Non-protein-N	25 —35 mg.	,, ,, ,,
Urea-N	12 —15 mg.	,, ,, ,,
Uric acid	2 — 3·5 mg.	,, ,, ,,
Creatinine	1 — 2 mg.	,, ,, ,,
Cholesterol	0·14— 0·18 gm.	,, ,, ,,

[1] Different authorities still give different figures for the final integer.
[2] As determined by the original Folin-Wu procedure.

Plasma

Chloride (asNaCl)	0·58—0·63 gm. per 100 c.c. (fasting)	
Inorganic P	2·4 (adults)—6 (children) mg. per 100 c.c.	
Fibrinogen .	0·2 — 0·6 gm. per 100 c.c.	
Albumin . .	4·6 — 6 gm.	,, ,,
Globulin . .	1·8 — 2·7 gm.	,, ,,
Alkaline reserve.	50 —80 volumes per cent. CO_2	

Serum

Calcium . .	10 —11 mg. per 100 c.c.	

APPENDIX III

FIRST AID PROCEDURES [1]

THESE notes only apply to the **immediate** treatment of such injuries as may happen to a student in a biochemical laboratory through insufficient care by himself or other students.

In cases of severe injury, poisoning, etc., **call a physician at once.**

Burns from Flames. Sponge with 5 per cent. tannic acid solution. If severe enough to require a dressing apply a pad of plain gauze moistened with 2 per cent. picric acid solution.

Burns from Hot Objects, Scalds, etc. Apply sterile vaseline. Dress, if necessary, as above, using 0·5 per cent. picric acid solution.

Burns from Strong Acids, Bromine, Phosphorus, etc. Wash with much water, then with 5 per cent. ammonia solution. Dress, if necessary, as above.

Burns from Strong Alkalies, etc. Wash as for acid burns, but use 4 per cent. boric or acetic acid instead of ammonia. Dress and bandage as necessary.

Eye Burns. If due to acid or formaldehyde flush with large quantities of water, then with 5 per cent. sodium bicarbonate solution. If due to alkaline material use 4 per cent. boric acid solution instead of the bicarbonate. Finally, allow a

[1] The Fisher Scientific Co., Ltd., of Montreal, have issued a very useful "Laboratory Emergency Chart," which was prepared under the supervision of Dr. H. R. Moody, of the College of the City of New York, and Dr. A. O. Gettler, Toxicologist of the Medical Examiner's Office, New York City. A copy of this chart could usefully be placed in a conspicuous position in any biochemical laboratory, next to an emergency cupboard containing the materials required for treatment.

The brief notes in this Appendix are largely based upon this chart. Dr. B. J. Brandson, Emeritus Professor of Surgery in the University of Manitoba, kindly revised the first half of the appendix for us.

drop or two of castor oil or olive oil to remain in the eye as soothing agent.

Blisters from Burns. Puncture in two places near the edge with a needle *sterilised in the flame of a Bunsen Burner.* Press out the liquid. Bandage if necessary.

Cuts. Remove dirt and other foreign material. Wash thoroughly with 95 per cent. ethyl alcohol, or 2·5 per cent. tincture of iodine. If there is no severe bleeding, place a small piece of gauze directly over the wound and bandage tightly enough to stop bleeding. With copious bleeding apply a tourniquet on the side nearer the heart if the blood is arterial (scarlet, intermittent spurts) or on the distal side if the blood is venous (purplish, steady flow), and *call a physician.*

Fainting from Shock from Burns, Cuts, etc. Remove to fresh air, and place flat on back. Brisk rubbing of chest and extremities may be sufficient. *If conscious,* to prevent **collapse** administer 10 to 15 c.c. of brandy or whisky, or hot strong coffee, or cause inhalation of dilute ammonia, or apply cold packs to the head and warmth to the rest of the body.

Sterilisation of Cuts. When open cuts are accidentally brought into contact with pathological or other septic material, sterilise **immediately** with 95 per cent. ethyl alcohol, or 2·5 per cent. tincture of iodine.

Electric Shock producing unconsciousness. Artificial respiration as for drowning; administration of stimulants as for collapse.

Headaches due to Inhalation of Toxic Chemical Vapours. Fresh air, 5 to 10 grains of aspirin, and rest.

Careless Pipetting. This not infrequently introduces strong Acid or Alkali or other dangerous liquid into the mouth. If acid is so introduced, spit it out and wash out the mouth immediately with 5 per cent. sodium carbonate (or, if this is not *immediately* available, with tap water, and then with

the carbonate solution). If alkali is introduced, spit it out and wash out with 4 per cent. boric acid solution (or tap water first, if the solution is not *immediately* available). Finally wash copiously with water. If metallic solutions such as Benedict's solution be introduced into the mouth, spit out, and wash out copiously with tap water.

During many years' experience we have never seen a case in which such a liquid, accidentally introduced into the mouth, has been swallowed. To spit it out is almost a reflex action. The following notes should cover such a case, should it ever occur in a biochemical laboratory.

Mineral acids. Do not use stomach tube or emetic. Give calcined magnesia, milk of magnesia, or lime water, **immediately,** mixed in milk or any oleaginous or mucilaginous fluid that will act as a demulcent. Repeat the dose at short intervals and continue until neutralisation can be inferred. **Do not give carbonates.** If the acid be strong sulphuric acid, either give much water or none (on account of the heat developed). Ice may be given to relieve thirst or pain. Administer stimulants to combat collapse. *Call a physician.*

Strong alkalies. Do not use stomach tube or a powerful emetic. Give 5 per cent. acetic acid, or vinegar, or lemon juice, until it may be inferred that neutralisation is complete. Give butter or olive oil (to form soaps) and assist vomiting by draughts of slightly warm water. Stimulate, and combat collapse. Administer a demulcent. *Call a physician.*

Copper solutions (including Benedict's solution). Give white of egg in water, milk, much water, then 10 per cent. sodium hydrogen phosphate, then an emetic, and *call a physician.*

Cyanides : Speed is the essential. Administer 3 per cent. hydrogen peroxide in large amounts. Immediately evacuate the stomach. Stimulate and combat collapse. Apply artificial respiration. Whatever the condition, **call a physician at once.**

Mercury compounds. Administer white of egg in water, then give an emetic or empty the stomach with a stomach tube, then give 10 per cent. sodium hydrogen phosphate, milk, or a thin starch paste, and stimula ts to combat collapse. *Call a physician.*

Oxalic acid or oxalates. Administer milk of magnesia (or better still precipitated chalk suspended in water), and then

an emetic. Stimulate and combat collapse. Administer demulcents, raw egg, milk, etc. *Call a physician.*

Silver compounds. Administer 25 per cent. sodium chloride solution, then an emetic, or use the stomach tube, then give white of egg in water, or egg and milk. *Call a physician.*

Emetic. Where called for, administer one tablespoonful of powdered mustard previously mixed in enough warm water to form a thick cream.

Universal Antidote. When the poison is unknown, administer a mixture consisting of two parts of powdered charcoal, one of magnesium oxide, and one of tannic acid, giving one heaped teaspoonful in a small glass of warm water. *Call a physician.*

Inhalation of Toxic Vapours

Carbon monoxide, carbon dioxide, coal gas (illuminating gas), etc. Remove to fresh air. If unconscious, practise artificial respiration and *call a physician immediately.* If still conscious stimulate with brandy or whisky or ammonia.

Chlorine, bromine, nitrous fumes, etc. Remove to fresh air, place prone, face down, with head slightly lower than chest. Cause inhalation of dilute ammonia. Inhalation of alcohol or ether soothes the respiratory tract. *Call a physician* if the toxic effect is severe.

Ammonia. As for bromine, but cause inhalation of acetic acid (from moderately strong acetic acid solution).

Hydrogen sulphide. Cause inhalation of ammonia from 5 per cent. ammonium hydroxide. Administer milk, white of egg in water, or olive oil. Stimulate and combat collapse. Use artificial respiration if necessary. *Call a physician.*

Hydrogen Cyanide. Inhale chlorine from a weak chlorine solution. **Call a physician immediately.** Apply artificial respiration.

IN ANY CASE IN WHICH THE EFFECTS SEEM AT ALL SEVERE CALL A PHYSICIAN AT ONCE, PROCEEDING IN THE INTERVAL AS DIRECTED ABOVE.

INDEX

PRINTED IN GREAT BRITAIN BY THE WHITEFRIARS PRESS LTD.
LONDON AND TONBRIDGE