The Madras Veterinary College

A LABORATORY MANUAL

OF

PHYSIOLOGICAL CHEMISTRY

(Exclusively for the use of the members of the Madras Veterinary College Students' Co-operative Stores Ltd.)

PREFACE

This laboratory manual is intended only as a practical guide in Physiological Chemistry, to be followed by students in their practical work. The manual is divided into 2 parts, Part I containing mostly, qualitative analyses and Part II quantitative analyses.

Some amount of theoretical information is given in this Manual but no attempt is made to make it complete, even from the students' point of view. Standard text books should be consulted for this purpose and to help the student in this, a list of reference is given at the beginning of each lesson. The student is advised to go through these references and make complete notes of his own therefrom before hand, in order that he may have a good theoretical background which is necessary to appreciate the significance of the tests and analyses he makes.

This manual may be used as an observation note book. Any additional information and explanations given during demonstrations may also be taken down against the appropriate place. In addition, each student should provide himself with a standard Record book and complete details of each experiment should be written down in this with the help of this Manual and the references given. The Record books should be submitted for scrutiny and signature the next day.

The excercises given are adopted mostly from " Practical Physiological Chemistry " by S. W. Cole and " Practical Physiological Chemistry " by P. B. Hawk et al.

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PART I

LESSON I.

Technique of the determination of H-ion concentration of Fluids.

Theory:

Ref:

(1) Dukes's Physiology Chapter I-Reaction of a Solution

(2) Cole: Chapter I Sections A & B
„ Section L
„ Section O (general discussion)

The reaction of the medium is a factor of such importance in the determination of a large number of Bio-chemical Phenomena that it is essential for the student to be familiar with the conditions that affect it and with the methods for its estimation.

We possess two methods of measuring the pH:

(1) The electrical method–this is accurate but requires much special and costly apparatus.

(2) The colorimetric method–not so accurate but in most cases sufficient and more easily applied.

The electrical method of measuring the pH and the theory on which it is based.

When a rod of a metal is immersed in an aqueous solution of one of its salts, an electrical potential difference is built up between the metal and the solution, the P. D. depending upon (1) the particular metal, (2) the concentration of the ions of the metal in solution and (3) the temperature.

So, if it were possible to introduce a rod of H_2 into a solution and measure the potential differance between the rod and the solution, the P. D. can be used to calculate the concentration of Hydrogen in the solution. But Hydrogen is a gas and the P. D. developed under the above conditions cannot be measured with any degree of accuracy. The usual method is to introduce two electrodes of known potential into a solution and calculate the pH from the P. D. between the two electrodes. It is not necessary to enter into the calculations as the instrument shown to you is calibrated to indicate the pH directly.

The Colorimetric method.—Indicators are used in this method. These are substances that change in color with alterations in pH of the solution to which they are added. The range of an indicator is that part of the pH scale over which the eye can appreciate the change in color of the indicator. The range of some common indicators are given below.

ACID RANGE.

Thymol Blue	RED	YELLOW	1.2— 2.8
Methyl Orange	RED	YELLOW	3.1— 4.4
Methyl Red	RED	YELLOW	4.3— 6.1
Brom Thymol Blue	YELLOW	BLUE	6.0— 7.6
Phenol Red	YELLOW	RED	6.8— 8.4

ALKALINE RANGE.

Thymol Blue	YELLOW	BLUE	8.0— 9.6
Phenolphthalein	COLOUR LESS	RED	8.2—10.0

By using the above seven indicators, a range of pH 1·2 to 10 can be covered.

Technique.—A given volume of fluid is treated with a certain amount of a suitable indicator. The colour developed is compared with the color of the indicator added to standard buffers or with permanant coloured glass standard discs in suitable instruments. The result indicates the pH of the fluid.

Experiment 1.

1. (Demonstration) Determine the pH of the given solution using Beckman Model M. pH meter.

2. Fluids of pH 2 to 10 (approximate) are provided. Check the useful ranges of the following indicators; (*a*) Methyl Orange (*b*) Methyl Red, (*c*) Phenol Red, (*d*) Phenolphthalein. (Take one c.c. of different fluids in different test tubes, and to each add one drop of the indicator. Note the range of the pH through which the eye can appreciate a change in color. This is the useful range of the indicator.)

3. Three fluids A to C are provided. Making use of the above indicators determine the pH of each within 0.5 of the correct pH.

4. Determine the pH of the fluids D, E, F within O.1 of the correct pH by using a Comparator and buffer solutions. Check your result by using Hellige Comparator.

LESSON 2.

General Reactions of Carbohydrates.

Ref : Cole—CHAPTER VI.

The important groups of carbohydrates that will be studied are Simple sugars (Mono saccharides) typified by Glucose, Disaccharides such as Sucrose, Lactose and Maltose and Polysacharides such as Starch and Cellulose.

The various tests employed depend on both Chemical and Physical properties. On account of easy oxidisability, some bring about certain reductions and hence are called reducing sugars (Glucose, Lactose, Maltose) Some react with phenylhydrazine to form characteristic yellow crystalline Osazone which can be identified under the microscope. Some are fermented by yeast with the production of Ethylalcohol and Carbon-dioxide, so that evolution of gas is used as a test. All sugars are optically active. The simpler members of the group (Mono and Disacharides) are soluble in water. Starch forms a colloidal solution on boiling. Cellulose is characterised by extreme insolubility.

Experiment 2.

A. Tests on glucose.

(1) **Molisch's test** (*general test for carbohydrates*).

Add to 5 c.c. of glucose soln., in a t.t. 2 drops of Molisch's reagent (5% soln., of α Naphthol in alcohol) and shake. Pour carefully down the side of the inclined t.t., 5 c.c. of Conc. H_2SO_4. A reddish violet color is produced at the interface between the liquids. (a greenish color is to be neglected).

(2) **Reduction tests.**

(*a*) **Trommer's test.**—To 3 c.c. of glucose soln., in a t.t., add an equal volume of 40% NaOH. Add a drop of dil. soln. of $CuSO_4$. A deep blue solution is produced. Continue to add $CuSO_4$. until there is faintest turbidity. Boil. A yellowish-red precipitate of cuprous oxide seperates.

(*b*) **Fehling's test.** Mix 1 c.c. each of Fehling's Solns., Nos. 1 and 2. Note the appearance of a deep blue color due to the formation of a complex copper tartarate. Boil. No change of colour and no reduction should take place. If any does occur, the Fehling's soln. should be changed for a fresh one. If satisfactory, add 2 c.c. of glucose soln. and boil. A yellow or brownish red precipitate of Cuprous oxide is produced.

Fehling's soln. No. 1 :—34.65 gms. of crystalised $CuSO_4$ ($CuSO_4$ $5H_2O$) Dissolve in distilled water to make 500 c.c.

Fehling's soln. No. 2 :—125 gms. of KOH and 173 gms. of Sod. Pot. Tartarate dissolved in water and soln. made upto 500 c.c.

(*c*) **Benedict's test.**—Transfer 5 c.c. of Benedict's soln. to a t.t. and add exactly 8 drops of glucose soln. Boil for 2 minutes. Allow to cool slowly. A ppt. gradually forms throughout the solution red, yellow or green depending upon the concentration of glucose soln. used.

Benedict's soln.—17·3 gms. of $CuSO_4$ crystals, 173 gms. of Sod citrate, 100 gms. of anhydrous Na_2CO_3 dissolved in water and water to make 1 litre.

(*d*) **Nylander's test.**—Add exactly 10 drops of Nylander's soln. to 5 c.c. of glucose soln. in a t.t. and heat for 5 minutes. The soln. gradually darkens and turns black due to the precipitation of metallic bismuth.

(3) **Formation of Osazone.**—To 5 c.c. of glucose soln. add 10 drops of strong acetic acid. Add about 0·5 gms. of Phenylhydrazine hydrochloride and 1 gm. of Sod. acetate. Dissolved by warming and mix well. Keep in a boiling water bath for 1/2 hour. Remove the flame and allow to cool slowly in the water bath. A yellow crystalline precipitate of Phenyl glucosazone appears even before the soln. has cooled. Transfer some to a slide and examine under a microscope. Note the characteristic arrangement of the fine yellow needles in fan shaped aggregation, sheaves or crosses.

(4) **Fermentation of glucose** (*demonstration*).—Mix 1 to 2 gms. of dried yeast preparation with 20 to 25 c.c. of glucose by rubbing in a morter. Transfer to a saccharimeter. Place this in an incubator at 37°C. and allow to remain overnight. CO_2 collects in the apparatus. Fill the open end of the apparatus with dilute Sod. hydroxide. Close with thumb and mix well. Restore to the normal position and remove the thumb. The fluid almost completely fills the closed limb thereby showing the gas is CO_2. The fluid contains C_2H_5OH.

(5) **Optical activity** (*demonstration*).—Study the working of the Polarimeter. Note that with water in the tube the " equal shadow " position corresponds to the zero on the scale. Introduce 1% glucose soln. in the tube and note the rotation of the polarised beam to the right side. Make a rough calculation of the specific rotation of glucose.

Specific rotation is that rotation expressed in degrees produced by a solution containing 1 gm. of substance per c.c. of solution and 1 decimeter in thickness.

$[\alpha]_D = \alpha / pl.$

$[\alpha]_D$—is the specific rotation.

α—is observed rotation.

p—is gms. of substance per c.c. of of soln.

l—is the length of the tube in decimeters.

Experiment 3.

B. Disacharides.

(6) **Sucrose or cane sugar.**— Repeat the experiments 1 to 5 with 1% cane sugar. Note that Molisch's and fermentation tests are positive while the reduction and osazone tests are negative. However, prolonged boiling may give some reduction.

(7) **Specific color test for sucrose.**—Add 1 c.c. of 1% Sod. nitrite soln. to 5 c.c. of amino uracil soln. (0·2%—5 amino uracil in N/20 HCl) Mix. To 2 c.c. of the diazo uracil soln so formed, add 5 c.c. of 1% sucrose. Allow to stand. In about 2 minutes a clear blue color developes. Glucose, Maltose and Lactose give only a yellow brown color.

(8) **Inversion of cane sugar.** (demonstration)—Measure the rotation of a 5% solution of sucrose. Take 50 c.c. of this soln. in a porcelain dish add 2 c.c. of conc. HCl and heat on the water bath for 45 min. Cool. neutralise with 10% NaOH and make up to volume to 50 c.c. Again measure the rotation.

With the hydrolysed soln. carry out the Fehling's and Benedict's tests. They are positive.

(9) **Lactose.**—Repeat the experiments 1 to 5 with 1% soln. of lactose. Note all tests are positive except fermentation test. Compare the microscopic appearance of Lactosazone with that of Glucosazone.

(10) **Maltose.**—Repeat experiments 1 to 5 with 1% soln. of maltose Note that all the tests are positive. Compare maltosazone with glucosazone and lactosazone.

N.B.—Glucosazone crystals commence to seperate from the hot soln. but lactosazone and maltosazone crystals only commence to seperate when the soln. is allowed to cool. This fact helps to differentiate them.

Experiment 4.

C. Polysacharides.

(11) **Starch.**—Examine under the microscope starch granules from potato. Make a sketch of the granules. Compare with starch granules of rice and wheat. Run a drop of Iodine under the cover slip. Sketch

the characteristic shape of each. Note that the granules take on a blue colour on the addition of I_2.

(12) Filter a suspension of potato starch in cold water. Add a drop of Iodine to the filtrate. The blue reaction is not obtained, showing thus that starch is insoluble in cold water.

(13) Mix about 1 gram of starch with 10 c.c. of cold water in a t.t. Shake well to obtain a uniform suspension. Pour this into about 90 c.c. of boiling water. Keep boiling for 1 min. Cool. An opalacent colloidal soln. is obtained. (starch forms a colloidal soln. with boiling water).

(14) To 5 c.c. of starch soln. add a drop or two of dilute Iodine. A deep blue color is obtained. Warm the soln. (but do not boil). The blue color disappears. Now cool the soln. and the blue color reappears. (Note: the color is produced by the Iodine being adsorbed on the colloidal molecules of starch. Warming liberates the adsorbed Iodine and thus the color disappears. On cooling the Iodine is readsorbed and the color reappears).

(15) To 5 c.c. of starch soln. add five drops of 10% NaOH and a drop or two of dilute. Iodine soln. The blue color is not obtained. Treat with 1 c.c. of strong acetic acid. The blue color reappears. (Note: Free Iodine is necessary to give the blue adsorption compound with starch. NaOH removes free Iodine converting it into iodide and iodate. The acid liberates free Iodine from these and the color is obtained. *N.B.* Always neutralise an alkaline soln. before testing for polysacharides.)

(16) Treat 5 c.c. of cold starch soln. with an equal bulk of saturated ammonium sulphate soln. Shake the tube and allow it to stand for 5 min. The starch is precipitated. Filter through a dry paper. Test the filtrate with a drop of Iodine. Practically no blue color is obtained, showing that starch is precipitated by 1/2 saturation with ammonium sulphate.

(17) **Hydrolysis of starch by acids** —Measure 20 c.c. of the 1% starch soln. you have prepared into a small beaker. Add 5 c.c. of a 1 in 5 dilution of strong HCl into this and mix well. Divide into 5 equal portions and place them into test tubes. Immerse the tubes into boiling water bath and note the time. Keep the water boiling. Remove one of the tubes 1, 5, 8, 12 and 20 min. from the time they were placed in the water bath. In each case pour one half of the fluid into a t.t. and add about 5 c.c. of distilled water and then Iodine drop by drop noting the appearance after each addition. To the remaining soln. add an equal volume of a mixture of

Fehling's solns, Nos. 1 and 2, and boil. Tabulate your results in the following form.

Minutes of	Iodine reaction	Reducing reaction.
1		
5		
8		
12		
20		

Note:—The experiment indicates that the starch is hydrolysed by the boiling acid to a reducing substance (glucose) and that there are intermediate substances (erythro-dextrins, achro-dextrins) which give brown color with Iodine.

Cellulose.

(18) **Solubility.**—Test the solubility of cellulose in water, dilute and concentrated acids and alkalies.

(19) **Iodine test.**—Add a drop of Iodine soln. to a few shreds of cotton. Cellulose differs from starch and dextrin in giving no color with Iodine.

(20) **Formation of amyloid.**—To a few shreds of cotton in a t.t. add 10 c.c. of 50% H_2SO_4. Note that the cotton dissolves slowly.

Cool half of this soln. and add an equal quantity of water. Amyloid seperates out as a gummy ppt. Add Iodine soln. A brown or blue color is produced.

Allow the other half to stand for 10 min. Dilute with equal quantity of water and keep in boiling water-bath for 15 min. Cool and neutralise with strong NaOH. Test the reducing action with Benedict's soln. Reducing sugar is formed. Glucose is formed from the cellulose by the action of the acid.

REVIEW.

Carbohydrate	Solubility	Molisch's test	Fehling's test	Benedict's test	Nylander's test	Iodine test	Osazone test	Rotation	Fermentation
Glucose	+	+	+	+	+	—	+	+D	+
Maltose	+	+	+	+	+	—	+	+D	+
Lactose	+	+	+	+	+	—	+	+D	—
Sucrose	+	+	—	—	—	—	—	+D	+
Starch	—	+	—	—	—	+	—	+D	—
Cellulose	—	—	—	—	—	—	—	—	—

LESSON 3.

General reactions of Proteins.

Ref: Cole—CHAPTER III.

Starling—CHAPTER IV.

The proteins form the most important constituent of living protoplasm and must always be present in the food. In structure they are very complex.

Composition.—All proteins contain Oxygen, Hydrogen, Nitrogen, Carbon and usually Sulphur and in some cases Phosphorus. A rough average is given below.

Carbon	about	53%
Oxygen	,,	23%
Nitrogen	,,	16%
Hydrogen	,,	7%
Sulphur	,,	1%

Certain other elements are contained in special proteins such as P in casein, Fe in Haemoglobin, I_2 in Iodothyroglobin. They also contain inorganic ions such as Na, K, Ca.

Proteins are compounds of certain amino acids mostly condensed together by peptide linkage. Their molecular weight is high and they characteristically exhibit colloidal properties. They do not diffuse through intact animal membranes and exert a small but constant osmotic pressure, which is of importance in the passage of fluids between blood and tissues. Free amino and carboxylic groups are present and so they can combine with acids and bases depending upon the pH of the medium. In this way they act as buffers. On hydrolysis, they break up into their constituent amino acids.

Classification.—Is based chiefly on the physical and partly on the chemical properties of the proteins. The three main divisions are: i simple, ii. conjugated, iii. Derived proteins or products of protein hydrolysis.

I. Simple proteins.

1. **Albumins.**—These are soluble in water and the soln. is neutral in reaction. They are coagulated by heat and precipitated from solutions by full saturation with ammonium sulphate. Eg: Egg albumin, serum albumin etc.

2. **Globulins.**—These are insoluble in pure water but soluble in dilute salt solutions. They are coagulated by heat and precipitated from solutions by half saturation with ammonium sulphate. Eg: Ovoglobulin of egg yolk, serum globulin.

3. **Sclero-proteins.**—These are characterised by marked insolubility. Eg: Keratin of horn and hoof.

4. **Histones.**—These are soluble in water and the solution is alkaline in reaction. Structure simpler than the above and contain only a fewer number of amino acids. Eg: Globin from Haemoglobin.

II. Conjugated proteins.

Proteins belonging to this class contain a prosthetic group in the molecule in some radical which is not amino acid in character.

5. **Phospho-proteins.**—Contain phosphate radicals but not as phospholipoids or nucleic acid. They are soluble in dilute alkalies. Eg: Casein of milk.

6. **Nucleo-proteins.**—Are present in all cell nucleii and are compounds of one or more molecules of protein with a nucleic acid. They are acid in character and soluble in dilute alkalies and dilute mineral acids.

7. **Gluco-proteins.**—have as prosthetic group carbohydrate radicals. Eg: Mucin.

8. **Chromoproteins.**—contain a coloured prosthetic group Eg: Haemoglobin.

III. Derived proteins and products of protein hydrolysis.

This class includes Metaproteins-proteoses, peptones, polypeptides, dipeptides and amino acids.

Amino acids.—Discussed in the lecture classes.

Proteins may be precipitated by:—

(i) Salts of heavy metals such as lead acetate, mercuric chloride and nitrate, ferric chloride, copper sulphate and zinc sulphate.

(ii) Alkaloidal reagents such as phosphotungstic, phosphomolybdic, ferrocyanide, tannic, picric, metaphosphoric and sulphosalycylic acids.

(iii) Alcohol.

Experiment 5.

General reactions of Proteins.

A. Precipitation tests.

(1) **Heat coagulation test.**—Add one drop of acetic acid (dilute) to 5 c.c. of egg white soln. Boil the upper layer of the soln. A cloudiness or precipitate indicates the presence of protein.

(2) **Heller's test.**—Take 2 c.c. of Conc. HNO_3 in a t.t. and layer on top an equal volume of egg white soln. taking care to avoid mixing of the two solns. A white ring appears at or immediately above the junction of the two fluids.

(The ring is due to the precipitation of metaprotein of the albumin and globulin which are insoluble in strong mineral acids).

Mix the two fluids. The ppt. dissolves in the dilute acid.

(3) **Precipitation by heavy metals.**—To 3 c.c. of the soln. in a t.t. add soln. of lead acetate, one drop at a time, until the ppt. is obtained.

(In many cases the precipitation is due to the adsorption of metallic kation by the negatively charged colloidal proteins).

(4) **Precipitation by alkaloidal reagents.**—To 5 c.c. of soln. add 2 drops of soln. of sulpho-salicylic acid. A white ppt. is obtained.

(Precipitation is due to the adsorption of the complex anion by the colloidal protein).

(5) **Precipitation by alcohol.**—Allow a few drops of soln. to fall into about 3 c.c. of strong alcohol. A ppt is formed due to the coagulation of albumin and globulin.

B. Color reactions of Proteins.

Most of the color reactions are due to the presence of certain amino acids in the protein molecule. The biuret test is due to the peptide linkage. The tests can be summarised as below.

Test.	Reagent	Color	Reacting Group	Amino acids giving the test.
1. Xanthoproteic	Strong HNO_3 followed by NH_3	Yellow Orange	Benzene Ring	Tyrosine, Phenylalanine, Tryptophane
2. Mercuric Nitrite test (Modified Million's)	Mercuric salt followed by nitrite.	Red	Phenol	Tyrosine
3. Aldehyde	Formoldehyde & $HgSO_4$ and strong H_2SO_4	Purple	Tryptophane	Tryptophane
4. Sulphur	Hot NaOH followed by lead acetate.	Black	Sulphide group	Cystine and Cysteine
5. Arginine	∝ Naphthol, NaOH and Sod. Hypochlorite	Red	NH_2 H. N: C NH (F.A.)	Arginine
6. Molisch's	∝ Naphthol followed by strong H_2SO_4	Purple	Carbohydrate	Carbohydrate group in protein molecule
7. Biuret	NaOH and trace of $CuSO_4$	Violet or Pink	Two peptide linkages	

Experiment 6.

Color reactions of Proteins.

(6) **Xanthoproteic reaction.**—To 3 c.c. of the protein soln. add about 1 c.c. of strong HNO_3. A white ppt. is formed. Boil for half a minute.

The ppt. turns yellow and partly dissolves to give a yellow soln. Cool under the tap and add strong NaOH soln. until the reaction is alkaline. The yellow color changes to orange.

The yellow color is due to the formation of a nitrosocompound of some aromatic compound containing the Benzene ring. This nitro compound is freely ionized in alkaline solutions. So the color is intensified on the addition of NaOH.

(7) **Mercuric Nitrite Test.**—To about 1 c.c. of the soln. add about an equal volume of 10% mercuric sulphate in 10% H_2SO_4. Boil gently for half a minute. A yellow ppt. is formed usually. Cool under the tap. Add one drop of a 1% soln. of Sod. nitrite. Warm gently. The ppt. or the soln. turns red.

The protein is ppted. by the mercury which then combines with the tyrosine. This compound becomes red on treatment with nitrous acid.

(8) **The aldehyde reaction for Tryptophane.**—Treat about 1 c.c. of the fluid with one drop of 1 in 500 formalin. Add one drop of 10% $HgSO_4$. Mix. Add about 1 c.c. of pure conc. H_2SO_4. Mix gently. A deep violet or purple soln. is obtained.

Tryptophane condenses with a large No. of aldehydes in the presence of strong Sulphuric acid and a trace of some oxidising substance (mercuric sulphate) to yield purple products.

(9) **The Biuret reaction.**—Treat about 3 c.c. of the soln. with an equal volume of 5% NaOH. Add a *single drop* of a 1% soln. of copper sulphate. A violet or pink color is produced.

Usually proteoses and peptones give a rose colour and other proteins a violet colour. If the soln. contains much ammonium sulphate it must be treated with excess of NaOH. The reaction is given by all substances containing two -CO-NH- groups attached to one another to the same N.atom or to the same corbonatom. So in addition to proteins the reaction is given by oxamide $CO{-}NH_2$ | $CO{-}NH_2$ and

Biuret $CO{-}NH_2$ | NH | $CO{-}NH_2$

and other compounds.

(10) **The arginine reaction.**—To about 3 c.c. of the soln. add about 1 c.c. of 5% NaOH and then 2 drops of 1% α – Naphthol. Add a single drop of 1% NaOCl. A bright red color soon developes.

This is specific for arginine, a basic amino acid found in all proteins The reacting group is HN-C$\begin{matrix}\diagup NH_2 \\ \diagdown NH\text{-fatty acid}\end{matrix}$. The reaction is very sensitive.

(11) **The sulphur reaction for cystine.**—Boil 2 c.c. of the soln. with 2 c.c. of strong NaOH for over a minute. Add a drop of lead acetate. The soln. turns brown or black due to the presence of lead sulphide.

The reaction is specific for cystine or cysteine. Sulphur from these is split off as sulphide on boiling and on oxidation of lead acetate, lead sulphide is produced and gives the colour.

(12) **Molisch's reaction for carbohydrates.**—Treat 3 c.c. of the soln. with 3 drops of 1% α - Naphthol in alcohol. Run about 3 c.c. of Conc. H_2SO_4 under the liquid. Very gently agitate if necessary, just to mix the liquids at the junction.

The reaction is due to the presence of a carbohydrate in the protein molecule. H_2SO_4 converts carbohydrate to furfural which condenses with α Naphthol to give the purple color.

LESSON 4.

General reactions of fats.

Ref :—Cole—CHAPTER VII

Starling—CHAPTER IV

The lipids consist of a number of classes of chemical compounds many of which are but slightly related or quite unrelated chemically, but all of which are allied by the physical properties of insolubility in water and fairly marked solubility in fats and fat solvents such as alcohol, acetone and ether.

Lipids may be classified as follows :—

1. Fats and fatty acids obtained from them
2. Waxes and fatty acids and higher alcohols obtained from them
3. Phospholipids (lipids containing a phosphate radical)
4. Glycolipids (those containing a carbohydrate radical)
5. Sterolipids (those with a sterol ring structure)
6. Chromolipids (lipids which are coloured)
7. Others still unclassified into any of the above

Our practical study is restricted to the first of the above classes only. i.e., *Fats and their derivatives.*

Fats are widely distributed in nature-in plants and animals. They are the most compact form of stored energy and at the same time, since they conduct heat very poorly, they act as insulators to the organism preventing undue loss of heat.

When fats are hydrolysed, they are broken down to trihydric alcohol-glycerol and a mixture of fatty acids. Of these, the most common are the saturated Stearic acid-$C_{17}H_{35}COOH$, and Palmitic acid $C_{15}H_{31}COOH$ and the unsaturated Oleic acid $C_{17}H_{33}COOH$. The three fatty acid radicals in a neutral fat are seldom the same. Different fats show great variations in the proportions of the various fatty acids obtained from them. Generally speaking, the physical properties of a neutral fat depend on the relative proportion of the fatty acids contained in it. Unsaturated acids make the fat soft or even liquid as in the case of oil. Saturated fats tend to produce hard fat.

When heated with alkalies or acids, fats are hydrolysed. This is termed saponification because, when alkalies are used soaps are formed. Soap forms a colloidal soln. in water but may be salted out on saturating the soln. with NaCl.

Although fats are insoluble in water, in the presence of emulsifying agents which reduce surface tension such as soaps, bile salts etc., they form stable emulsions. This fact is of considerable importance in digestion of fat.

Glycerol is not a lipid but a trihydric alcohol with a sweet taste.

Various methods have been described for the identification of fats and oils, the important amongst them :—

(1) Melting point or solidifying point.

(2) Saponification value–being the no., of mgms. of KOH that will be neutralised by the free or combined fatty acids of 1 gm of fat. This is a measure of the mean molecular weight of the fatty acids contained in the fat. The value decreases with increasing molecular weight.

(3) Iodine value–is the no. of gms of iodine absorbed by 100 gms of fatty material. Iodine is absorbed only at points of unsaturation and so, greater the iodine value the more unsaturated the fatty material.

Experiment 7.

Fats.

(1) Take a small portion of lard and test its solubility in (*a*) water, (*b*) ether, & (*c*) alcohol.

In each case, remove one drop of the soln. and put it on a paper. Allow it to evaporate and look for grease spot.

(2) Put in a t.t. a small piece of lard and about 15 c.c. of alcoholic potash and place in a boiling water bath. Continue heating until all fat disappears. The fat is now saponified.

(3) Put the contents in 10 c.c. of cold water in a beaker and add 5 gms of NaCl and mix. Soap floats on top.

(4) Remove the soap, dissolve in 15 c.c. of hot water and add about 3 c.c. of conc. HCl. Fatty acids will float on top.

(5) Remove some of these to a t.t. and add a soln. of Caustic potash and observe the fatty acids dissolving to form soap.

LESSON 5.

The quantitative estimation of sugars.

Estimation of reducing sugars by Benedict's method.

Principle.—Benedict's reagent for the reducing sugars contains Pot. thiocyanate as well as copper sulphate. And in the presence of the former a white ppt. of cuprous thiocyanate is formed in reduction instead of the usual red ppt. of cuprous oxide. The small amount of Pot. ferrocyanide also aids in keeping the cuprous oxide in solution. As the ppt. is white the loss of all blue tint in the soln. indicating complete reduction of copper is readily observed. The reagent is fairly stable and the method simple and accurate.

It has to be remembered, that in methods, which utilise a reduction procedure for estimating reducing sugars quantitatively, we cannot deduce the quantitative relationship from any equation but can only ascertain them emperically. Consequently, directions should be closely followed to avoid inaccuracy.

Benedict's reagent.

Copper sulphate (crystalline)	18.0 gms.
Sodium carbonate (anhydrous)	100.0 gms.
Sod. or Pot. citrate	200.0 gms.
Pot. thiocyanate	125.0 gms.
Pot. ferrocyanide 5% soln.	5.0 c.c.
Distilled water to make a total volume of	1000 c.c.

Dissolve the carbonate, citrate and the thiocyanate in enough water to make about 800 cc. with heat and filtering if necessary. Dissolve the copper sulphate seperately in about 100 c.c. of water and pour slowly into the other liquid with constant stirring.

Add the ferrocyanide soln., cool and dilute to 1000 c.c.

25 c.c. of the reagent are reduced by 50 mgms of glucose
67 „ of lactose
76 „ of maltose

Estimation of Sucrose.—Sucrose is not a reducing sugar and hence the technique requires some modification. Sucrose may be hydrolysed to yield a mixture of glucose and fructose, the latter being reducing sugars. The hydrolysis may be effected by boiling with HCl for 10 minutes.

25 c.c. of Benedict's reagent are reduced by the mixture of sugars obtained from 47.5 mgms. of Sucrose.

Experiment 8.

(1) *Estimation of glucose.*

Method.—Rinse out the burette with the glucose soln. whose sugar content is to be estimated, drain it carefully and then fill the burette with the same soln. Take an accurate reading.

Pippette out accurately 25 c.c. of Benedict's reagent into a 250 c.c. conical flask. Add about 5 gms of anhydrous Sod. carbonate and a few bits of porcelain and heat the mixture to boiling until the carbonate has entirely dissolved. Run in the glucose soln from the burette rather rapidly, half a c.c. at a time at half minute intervals until a chalk white ppt. forms and the blue color of the mixture begins to lessen perceptibly, after which, run in a few drops at a time, until the last trace of blue color disappears, which marks the end point. Take the final reading. The soln. must be kept vigourously boiling during the titration. If the mixture becomes too concentrated during the process add water from time to time to replace the volume lost by evaporation. Repeat to get nearly concurrent readings.

Calculation.—If V c.c. of the soln. are added to discharge the colour of 25 c.c. of Benedict's reagent then the glucose content of the given soln will be : $\frac{50 \times 100}{V \times 1000}$ gms per 100 c.c.

Precautions (*a*).—The mixture should be kept well boiling during the titration and the glucose soln. added at half minute intervals so that the reaction may be as complete as possible. However, undue concentration should be avoided as it may lead to spurting.

(*b*) The reaction should be completed within 6 minutes.

(*c*) The color may reappear on cooling and shaking with air. So the soln. should not be allowed to cool once the titration begins.

(*d*) The volume of the soln. added should not be less than 5 c.c. If the given soln. is so strong that the color is completely discharged before 5 c.c. have been added, suitably dilute the soln.

(2) **Estimation of Lactose and Maltose.**—Proceed as above but substitute the suitable factor for 50 in the above calculation.

(3) **Estimation of Sucrose.**—To 25 c.c. of the soln. add 10 c.c. of O. 1 N. HCl. Boil gently for 10 minutes. Cool thoroughly under the tap. Add 10 c.c. of O. 1 N. NaOH N. NaOH. Transfer quantitatively to a 100 c.c. graduated flask and make the volume upto the mark with water. Use the solution of invert sugar so obtained for titrations and calculate the amount of sucrose in the original soln.

LESSON 6.

The digestive juices and the action of certain enzymes.

Ref :—Cole—CHAPTER IX

Cameron—CHAPTER I (digestion)

—CHAPTER II (Catalysis)

Enzymes are colloidal organic catalysts produced by living organisms. They accelerate the velocity of certain reactions.

Properties of Enzymes (*a*).—Each enzyme acts on a particular substance (substrate) or a group of substances having some similarity in chemical composition or configuration. In many cases this similarity must be extremely close. This specificity in their action differentiates enzymes from inorganic catalysts such as acids which may act on substances as dissimilar as disacharides and proteins.

(*b*) The main chemical constituent of the purest enzymes that have been isolated seems to be similar to protein.

(*c*) The first stage in enzyme action appears to be adsorption of the substance by the enzyme. This is followed by activation of some or all of the adsorbed substrate.

(*d*) Like other catalysts, enzymes do not affect the final state of equilibrium. Accumulation of the products of reaction induce reverse reaction.

(*e*) For each enzyme, there is a definite temperature at which it acts rapidly. This is called the optimum temperature.

(*f*) There is an optimum pH for each enzyme.

(*g*) In some cases some substances markedly accelerate the action of the enzyme. They are called co-enzymes.

Action of Ptyalin.—Ptyalin is the salivary amylase present in man and pig. In other animals the saliva is of value as a lubricant in the act of deglutition. Ptyalin acts on starch and certain other polysaccharides, the chief end product being Maltose. The optimum pH for this enzyme is 6.7. Chloride ions are essential for the action of Ptyalin.

Experiment 9.

(1) Obtain diluted saliva as follows: warm some distilled water in a beaker to about 40°C (body heat). With a portion of this thoroughly rinse out the month. Now take about 20 c.c. of warm water in the mouth and move it about by the tongue for at least a minute. Collect the liquid thus obtained in a clean beaker. Mix well and filter.

(2) In a clean t.t. place 5 c.c. of freshly prepared 1% starch soln. and add 5 c.c. of dilute saliva. Mix well. Place a glass tube in the test tube and place in water bath at body temp. At intervals of half a minute remove a drop of the mixture and mix with a drop of iodine solution in a china dish. A blue color is produced at first but with drops added later the color becomes blue-violet, red-violet, red-brown and finally no increase of color is obtained.

When a drop of the mixture fails to give a color with iodine, boil a few c.c. with a little Fehling's soln. A marked reduction is obtained showing that the enzyme Ptyalin has converted starch into the reducing sugar, Maltose.

(3) **Determination of the chromic period.**—Measure 5 c.c. of the soluble starch into a t.t. Add 2 c.c. of a buffer soln. of pH 6·7 and 2 c.c. of 0·5% NaCl. Place the tube in a water bath maintained at 38°–40°C, for a few minutes. Have ready a series of test tubes each containing 3 c.c. of distilled water. To each tube add a couple of drops of iodine soln.

To the warmed starch add 2 c.c. of diluted saliva. Mix well and note the time. At intervals of 15 seconds transfer a drop or two of the digesting mixture to a tube containing dilute iodine and shake. Determine the time when additions cease to produce any color. This point which is the moment when the last trace of erythrodextrin is converted into achroodextrin and maltose is known as the achromic point. The time taken to reach this point (the chromic period) varies inversely as the activity of the enzyme and is a measure of the activity.

B. Pepsin and Renin.

Pepsin.—Is the proteolytic enzyme secreted by the chief or peptic cells of the gastric glands. These cells elaborate the zymogen-pepsinogen, which is converted to pepsin by HCl.

Pepsin acts only in a decidedly acid medium, the optimum pH being about 1.4. Alkaline solns. rapidly destroy it. The end products of peptic digestion are peptones and polypeptides.

Renin.—Is a specific enzyme of the gastric juice of the young animals. It has the specific property of clotting milk, due to the hydrolysis of casein to paracasein. In the presence of Ca ions, paracasein is precipitated as the Ca salt and holds within its meshes the other constituents of milk as a clot.

Experiment 10.

Place equal, small quantities of freshly washed fibrin in four t.t.s labelled A.B.C.D.

To A add 3 c.c. of Pepsin and 3 c.c. of 0.4% HCl.

To B add 3 c.c. of Pepsin and 3 c.c. of water.

To C add 3 c.c. of water and 3 c.c. of 0.4% HCl.

To D add 3 c.c. of Pepsin that has been boiled and cooled and 3 c.c. of 0.4% HCl.

Place the tubes in water bath at 40°C (body temp.) for 30 minutes. Note that in

A the fibrin swells up, then becomes transparent and finally dissolves.

B the fibrin is not altered.

C the fibrin becomes transparent but does not dissolve.

D the fibrin is like that in C.

(2) **Detection of pepsin by carmine fibrin method.**—To 5 c.c. of the ferment soln. add 5 c.c. of 0.4% HCl. Divide into two portions A & B. Boil B for a few minutes and cool. To each tube add a few flakes of carmine fibrin. Place the tubes in a water bath at a body temp. for 10 min. Shake and observe the color of fluid. In A it will be red while in B it will be almost or quite colorless.

(3) Take about 5 c.c. of milk in a t.t. and place it in a waterbath at 40°C for a few minutes. Add 1 c.c. of renin soln. Mix and replace in the water bath. Observe from time to time. Note that the milk soon sets to a solid mass.

(4) To 10 c.c. of milk add 3 c.c. of ammonium oxalate soln. (O.2N) to remove the Ca ions. Mix, divide into 3 equal parts, A, B. & C.

To A add 1 c.c. of N. $CaCl_2$ Soln. and 1 c.c. of renin.

To B add 1 c.c. of renin.

To C add 1 c.c. of boiled renin.

Place the tubes in water bath for 10 min. Note that A clots and that B & C do not.

Boil B to destroy the renin and cool under the tap. To B & C add 1 c.c. of normal $CaCl_2$. A flocculent ppt. of calcium paracaseinate is produced in B indicating that the enzyme has acted on the casein but Ca is necessary for the formation of the ppt.

C. Trypsin, Steapsin and Amylopsin.

Pancreatic juice shows the presence of at least three enzymes, Trypsin, Steapsin and Amylopsin.

Trypsin.—The pancreatic juice contains several proteolytic enzymes-protease, polypeptidase, a protaminase etc. One may disignate them collectively as trypsin.

Trypsin acts best in an alkaline medium, about pH 8 O. It can act on native protein or products of peptic digestion. The end products are amino acids.

Steapsin.—Hydrolyses fat into fatty acids and glycerol. Fat digestion is facilitated by emulsification of fat by soaps formed from the liberated fatty acids and by the bile salts.

Amylopsin.—Resembles ptyalin in many respects and hydrolyses starch and various dextrins to maltose.

Experiment 11.

1. Most favourable reaction for tryptic digestion.

Prepare 3 tubes as follows:—

(*a*) 3 c.c. of neutral pancreatic extract + 3 c.c. of water (ph 7.0).

(*b*) 3 c.c. of neutral pancreatic extract+3 c.c. of water+1 drop of phenolphthalein soln+ 0.5% Na_2CO_3 enough just to produce a faint pink color (pH 8·3).

(*c*) 3 c.c. of neutral extract of pancreas + 3 c.c. of 0.6% HCl (pH·3·0).

Add a small piece of fibrin to the contents of each tube and keep them at 40°C noting the progress of hydrolysis. At what reaction does trypsin act?

2. Determination of Amylase in the pancreatic extract.

Take in a t.t. 5 c.c. 1% soln. of strach. Add 2 c.c. of neutral pancreatic extract. Shake and place in water bath at 40°C for 30 min. Test the soln. for starch (iodine) and reducing sugars (Benedict's).

3. **Litmus milk test for steapsin.**—In each of two test tubes, introduce 10 c.c. of milk and a small amount of litmus soln. To one tube add 3 c.c. of neutral pancreatic extract and to the other 3 c.c. of the extract, boiled and cooled. Keep the tubes in a water bath at 40°C. and note any change in color that may occur. What is the result and how do you explain it?

LESSON 7.

Milk.

Ref—Cole—CHAPTER X

Cameron—CHAPTER VIII

Milk consists essentially of an emulsion of fine particles of fat in a watery liquid, whose chief solutes are proteins, lactose and inorganic salts. Since it contains most, if not all, of the vitamins, it contains all the essential food factors for the young animal. It is amphoteric in reaction. Its pH value is about 6.6. The average composition is:

	Human	Cow	Goat.
Water	87·5%	87%	87%
Protein	1·5 to 0·7%	4 to 2·5%	3·7%
Lipids	2 to 4%	2 to 4%	4·1%
Lactose	6 to 7·5%	3·5 to 5%	4·1%
Salts	0·2 to 0·3%	0·6 to 0·7%	0·9%

Milk proteins consist chiefly of Casein, lactalbumin and lactoglobulin. Casein amounts to about 80% of the total proteins.

Milk fat consists largely of oleates and palmitates with smaller amounts of glycerides of lower fatty acids. Small amounts of lectithin, cephalin and cholesterol are also present. Milk fat is formed from the neutral fat and cholesterol esters of the blood.

Lactose is formed in the mammary gland from glucose and galactose, the galactose being formed from glucose.

The inorganic salts of milk include K, Na, Ca, mostly in the form of phophates and also as chlorides. Mg and traces of iron are also present.

Many other constituents diffuse into the milk from blood. They include the vitamins, traces of urea and a number of amino acids.

***Experiment* 12.**

(1) Examine a drop of fresh cow's milk under the high power of a microscope. Notice the highly refractive fat globules of varying sizes, the smallest exhibiting brownian movement.

(2) Take the Sp. gr. of the milk with a lactometer. It varies between 1028 and 1034. The S. gr. is always expressed as the S. G. at 60°F. or 15°C. So the lactometer reading at any other temp. should be corrected by adding or subtracting O.1 for every degree F. or O·2 degree for every degree of C. above or below 60°F or 15°C.

(3) Estimate the reaction of the milk by using the following indicators.

(*a*) Methyl red. (range 4.3 red 6.1 yellow).

(*b*) Chlorphenol red (Range 4·8 yellow6·4 red).

(*c*) Phenol red (range 6·7 yellow-8·3 red).

The color will be yellow with (*a*), red with (*b*) and yellow with (*c*), showing that the reaction is between pH 6 and 7.

(*d*) Litmus paper-Red and Blue—Blue litmus paper is usually turned red and red litmus paper is turned blue-Milk is amphoteric in reaction.

(4) Pour a small t.t. full of milk into a small conical flask, add equal quantity of water and dil. acetic acid (1%) Mix well by swirling the contents. Filter. The filtrate should be clear. The ppt. consists of casein and fat. Filtrate contains the other constituents. Reserve both for subsequent exercises.

(5) Demonstrate the presence of protein in the ppt. Shake very small amounts with about 1 c.c. of water and apply the mercuric nitrite test and the aldehyde reaction.

(6) Demonstrate the presence of organic phosphorous in the ppt. (*demonstration*) Digest a small amount of the ppt. with about 12 drops of H_2SO_4. Cool. Add about 5 c.c. of distilled water. Add about 2 c.c. of ammonium molybdate soln. and boil. A yellow ppt. of ammonium phosphomolybdate indicates the presence of phosphorous in casein.

(7) Shake up a portion of the ppt. with ether. Apply grease spot test to the ethereal soln. Shows fat.

(8) To the filtrate from 4 add NaOH soln. till neutral to litmus and then 6 drops of dil. acetic acid. Boil. The fluid becomes turbid due to the coagulation of Lactalbumin and lactoglobulin. Filter.

(9) Test a portion of the above filtrate for lactose by the reducing test.

(10) To another portion of the filtrate from 8 add a few drops of Pot. oxalate soln. A ppt. or turbidity indicates the presence of Calcium.

Quantitative Analysis of Milk.

Lactose is estimated by using Benedict's quantitative reagent. To prevent the proteins of the milk interfering with the reaction, they are precipitated and the protein free filtrate used for titration.

Fat is estimated by the destruction of organic matter, other than fat, by H_2SO_4, and centrifuging of the acid soln. in a special tube, the % of fat being read off by means of a graduated neck of the tube.

Total inorganic salts are estimated by the usual method of incineration to constant weight.

Experiment 13.

(1) **Lactose.**—Mix 10 c.c. of milk with 10 c.c. of tungstic acid soln. Dilute with 30 c.c. of water. Mix well. Filter until clear. Titrate the clear filtrate against Benedict's quantitative reagent.

(2) **Fat.**—*Gerber's method*—Take 10 c.c. of 90% H_2SO_4 in the Gerber's tube. Add 11 c.c. of milk and mix very well carefully. Add 1 c.c. of amyl alcohol and if necessary, 1 in 3 H_2SO_4 to fill the tube up to the neck. Centrifuge for 5 minutes. Place the tube in water bath at 60 C. The gm % of fat in milk may be read off from the graduation.

Total Inorganic Salts. (*demonstration*).—Introduce about 5 c.c. of milk into a weighed silica crucible and quickly weigh to mgms. Expel major portion of the water by heating the open dish on a water bath and continue heating in the hot air oven till all the moisture is removed. Heat the dry solids over a very low flame (the dish should not be heated very strongly as the chlorides may volatalise) until a white or grey ash is obtained. Cool the crucible in a dessicator and weigh. Calculate the Total inorganic salts per 100 gms. of milk.

LESSON 8.

Blood.

Ref :—Cole—CHAPTER X

Cameron—CHAPTER IX

Quantitative data on blood composition (average)

Amount of Blood.—8–9·7% body weight.

Cellular elements.—30–40 Vol. % of blood.

S. Gr.—1040–1060.

Reaction.—pH 7·4.

Plasma composition.—Water about 91%

gases O_2, CO_2, N_2

Proteins.—6.7 gms. per 100 c.c. serum albumin and serum globulin form the bulk, the rest figrinogen.

Glucose

Lipids-Neutral fats, lecithin, cholesterol, Nonprotein nitrogenous substances-aminoacids, urea, uric acid, creatinine, creatin, ammonium salts etc.

Inorganic salts.-Chlorides, bicarbonates, sulphates and phosphates of Na, K, Ca, Mg, Fe.

Trace elements-Mn, Co, Cu, Zn, etc.

Enzymes, Harmones, Vitamins, Immune substances etc.

	Size		*Number*
Erythrocytes.—Elephant	9.28	Microns	———
Man	7.8	,,	5,400,000
Dog	7.1	,,	———
Ox	6.0	,,	6,400,000
Horse	6.0	,,	6,940,000
Cat	5.8	,,	———
Sheep	5.2	,,	8,120,000
Goat	4.1	,,	13,940,000
Muskdeer	2.1	,,	———

Composition.—Water 59–64%
Total solids 36–41%
Haemoglobin 30–33%
Stroma (lipids & proteins) .... 6—8%

Haemoglobin.—12–16 gms per 100 c.c. of blood in different animals.

The amount of iron in Hb———0·33%.

Molecular weight————68000 (which corresponds to 4 atoms of iron in the molecule).

1 gm. of Hb combines with 1·34 c.c. of CO_2.

This is in the ratio of one atom of iron to one molecule of CO_2 Therefore Hb may be represented by $HbFe_4$

Oxyhaemoglobin .... Hb $(FeO_2)_4$
Methaoemoglobin .... Hb $(FeOH)_4$
Carbonmonoxide Hb (carboxy Hb) .... Hb $(FeCO)_4$

On hydrolysis, Hb yields about 94% globin and 6% Haem. Addition of dil. alkali to haemoglobin leads to the production of haemochromogen which consists of one molecule of Haem united to one molecule of haemochromogen. On adjustment to neutrality these polymerise to Hb.

When Hb is heated with sodium chloride, and glacial acetic acid characteristic, minute, dark brown, rhomboid crystals of haemin are produced. (if haem is represented by X FeOH, haemin is X FeCl).

Haem is built-up from 4 pyrole nucleii. Each molecule of haem contains one atom of iron. It can combine with many nitrogenous substances. With native globin it forms haemoglobin. With others it forms haemochromogens.

When haem or any of its derivatives is dissolved in Conc. H_2SO_4 the atom of iron is split off and haematoporphyrin is formed. Haematoporphyrin is allied structurally to Bilirubin and chlorophyll—the respiratory pigment of the plants.

The absorption spectra of Hb and its derivatives.

When white light is passed through colored solns. some of the light is absorbed. In some cases, the light is absorbed in relatively narrow areas of the spectrum and absorption bands can be seen. The position of the bands are constant for a given pigment and their recognition is a valuable means of identification.

In dilute solutions, Oxyhaemoglobin shows two bands in the green (between D & E Fraunhofer's lines). As the concentration increases, the two bands get deeper and broader.

Carbonmonoxyhaemoglobin gives a similar spectrum but both bands are slightly shifted to the blue.

Reduced haemoglobin shows a single absorption band between D & E lines.

HbCO is not converted to Hb by weak reducing agents, such as ammonium sulphide, so that if a soln. shows two bands between D & E. lines it will continue to show the two bands after the action of ammonium sulphide if carboxy Hb is present. But only one band if oxy Hb is alone present.

***Experiment* 14.**

(1) Take 5 c.c. of blood in 3 tts. and add to the respective tubes

(*a*) 5 c.c of water

(*b*) 0·9% NaCl 5 c.c.

(*c*) half c.c. of ether and 5 c.c. of 0·9% NaCl

Note the change in each case.

(2) **Acid haematin.**—to 5 c.c. of dilute blood add 5 drops of strong acetic acid and heat. The color changes to brown due to the formation of acid haematin.

(3) **Alkaline haematin.**—To 5 c.c. of dilute blood add 5 drops of strong ammonia and heat. The color changes to brown due to the formation of alkaline haematin.

(4) Examine the spectra of Oxy Hb, Hb and Co Hb. How will you differentiate between a soln. of Oxy Hb and Co Hb.

(5) **Pseudoperoxidase reaction.**—(*a*) *Benzidine reaction.* To 3 c.c. of a saturated soln of benzidine in alcohol, acidified with acetic acid, add 2 c.c. of dilute soln. of blood and 1 c.c. of 3% H_2O_2. A blue color is produced.

(*b*) *Tr. Guaicum test.*—To 5 c.c. of a dilute soln of blood add one drop of Tr. Guiacum and 5 c.c. of 3% H_2O_2. A blue color is produced.

(In both the above tests Haem and its derivatives act as pseudoperoxydases and catalyse the oxidation of Benzidine or guiac resin by H_2O_2 to blue compounds. The peroxidases, like other enzymes, are destroyed by boiling. Haem compounds, however, continue to give the reaction even

after boiling. Because peroxidases are present in many body fluids, to confirm the pretence of blood, any sample giving a positive reaction should be boiled and cooled and tested again.)

(6) **Haemin crystals.**—Spread a small drop of blood on an area of about 1 sq. cm. on a slide and slowly evaporate till it is quite dry. To the film add a drop of O.1% soln. of KCI in glacial acetic acid. Cover with a slip and gently heat, cautiously just to boil. Immediately run another drop of reagent under the slip, allow to cool and examine under the microscope for haemin crystals. In testing suspected blood—stains on cloth etc, extract the material with a little dilute alkali, neutralise the soln. with dil acetic acid and concentrate it to dryness on the water bath. Treat a small quantity of the residue on a slide as described above, look for haemin crystals.

LESSON 9.

Bile

Ref:—Cole—CHAPTER XII

In most animals and man bile is secreted continuously by the liver cells. It passes along the bile capillaries, hepatic and cystic ducts to be stored in the gall bladder. Bile passes into the intestines from the gall-bladder when food arrives in the intestines. Gall bladder absorbs water from the bile and adds to it mucin secreted by the m.m.

Composition.—The bile is a highly complex fluid. The physiological significance of many of its constituents is unknown. Some are of use in digestion while others, for the most part, are probably merely waste products undergoing elimination. So bile is a secretion as well as an excretion.

Bile as secreted by the liver is distinctly alkaline (PH 8-9) but the bladder bile is less alkaline or may even be slightly acid in some animals, such as dog & cat. It is a ropy viscid substance with a bitter taste. It varies in color in different animals and may be yellow, brown or green or a mixture of these shades. The chief billiary components are: the bile salts; bile pigments; cholesterol; lecithin and mucin.

(*a*) **The bile salls.**—are the sodium salts of glycocholic ($C_{26}H_{43}NO_6$) and taurocholic ($C_{26}H_{45}NSO_7$) acids. The acids are the compounds formed by glycine and taurine respectively with cholic acid. Taurine is a sulphur containing compound related in structure to cysteine. Cholic acid belongs to the sterol group of compounds.

(*b*) **The bile pigments.**—are Bilirubin and Biliverdin. Bilirubin is the most important bile pigment and is derived from Haem of Haemoglobin. During the disintegration of senile R. B. Cs, the iron is split off and conserved in the liver for use in fresh R. B. C. formation. The iron—free pigmentary part is excreted in bile as bilirubin. Liver is not the only site of bilirubin formation—it is formed where ever there are cells of reticulo endothelial system. Bilirubin is reddish in color.

Biliverdin is the oxidation product of bilirubin and is greenish in color.

Bilirubin is the chief pigment of the bile in carnivora. Biliverdin is particularly abundant in the bile of herbivora and is the chief pigment of bile of birds.

Bile pigments passing into the intestines are converted there by bacterial reduction into stercobilin, which is excreted for the most part with the faeces, a small part being absorbed into the blood and excreted along with bile or as urobilin in urine.

(*c*) **Cholesterol and Lecithin.**—Cholesterol is a stero-lipid and lecithin is a phospholipid. These lipids form the "element constant" of the tissues. The ability of bile to hold cholesterol in solution is in a large measure dependant upon the bile salts. The origin and significance of these are obscure.

(*d*) **Mucin.**—Mucin is added to the bile in the gall bladder and is secreted by the m. m. of the gall-bladder.

***Experiment* 15.**

1. Bile salts.

(1) Half fill 2 test tubes with H_2O, and to each add respectively
(*a*) 2 c.c. of bile and 1 c.c. of oil.

(*b*) 2 c.c. of water and 1 c.c. of oil.

Shake vigourously to emulsify the oil. Set aside. Explain any difference you may notice.

(2) **Hay's test for bile salts.**—Take 5 c.c. of bile solution in a t.t. Sprinkle the surface with a few grains of dry flowers of sulphur and note that they fall through the liquid to the bottom of the tube. Repeat the test with water. Nothing will happen, the sulphur remains at the top of the surface.

(3) **Pettenkofer's test for bile salts**—To 5 c.c. of bile solution add one drop of 10% sucrose solution and mix. Layer under this 2 c.c. of Conc. sulphuric acid. Gently shake the tube just to mix the two fluids at the interface between them. A deep purple color develops at the interface.

II. Bile pigments.

(4) Over 2 c.c. of Conc. nitric acid in a t.t. layer 2 c.c. of bile soln. gently shake the tube as before. Note the play of colors (green is predominant) as the pigments are oxidised, by the acid, (from acid to bile—the colors are : yellow, red, violet, blue and green).

(5) **Huppert's test (Cole's modification of)**—Boil about 15 c.c. of bile soln. in a large t.t. Add 2 drops of a saturated soln. of Mag. Sulph. Add 10% soln. of $BaCl_2$ until no further ppt. is obtained. Boil again. Allow to stand for a short time. Pour off the greater part of supernatant fluid. Filter the reminder through a small filter paper. Remove the ppt to a dry t.t. Add about 4 c.c. of strong alcohol and shake vigorously. Add 3 drops of strong H_2SO_4 and 1 drop of 2% soln. of $KClO_3$, boil for half a minute and allow the $BaSO_4$ to settle. The presence of bile pigments is indicated by the alcoholic solution being colored greenish blue.

(Ex. 3 above: H_2SO_4 produces furfural from sucrose and cholic acid combines with this to produce the color).

(Ex. 5 above: Pigments are adsorbed by $BaSO_4$ but eluded by acid-alcohol and then oxidised by $KClO_3$).

LESSON 10

Urine.

Ref :—Cole—CHAPTER XIII

Cameron—CHAPTER XIV

Color and consistancy.—In most animals consistancy is watery but in the horse it is syrupy due to the presence of mucin. The color is usually yellowish. The pigments of urine are Urochrome and Urobilin. Urochrome is the chief pigment.

The average Sp. Gr. of urine of various animals.

Horse	1040
Ox	1032
Sheep and Goat	1030
Pig	1012
Dog	1025
Cat	1030
Man	1020

The determination of the Sp. Gr. for clinical purposes is done by Urinometer.

The Sp. Gr. depends upon the total amount of solids in the urine. This can be calculated roughly from the Sp. Gr. by Long's coefficient. The last 2 figures of the Sp. Gr. (expressed correct to 3 decimal places) multiplied by 2·6 gives the total solids in gms. per 1000 c.c. of urine.

Eg. If Sp. Gr. is 1017,

Total solids = 17X2·6 = 44·6 gms per 1000 c.c. or urine.

Reaction.—Reaction varies in the different species and even in the same animal from time to time depending upon food and metabolism. Carnivorous animals generally have an acid urine, herbivorous animals an alkaline urine and omnivores either an acid or alkaline urine depending upon the diet. This is so because, during metabolism, the sulphur and P abundant in the food rich in protein are oxidised to sulphuric and phosphoric acids, thus rendering urine acid, and the organic salts abundant in most of the food stuffs of plant origin are oxidised to alkaline carbonates, thus rendering the urine alkaline. During starvation, the urine is acid in every animal, because, then it lives upon its own tissues.

Amount.—Is variable depending upon many factors. Average values are :

Horse	5 litres
Cattle (cow)	14 litres
Sheep & goat, large dog and man	1 litre

Chemical composition of urine.—The principal constituents of urine are: water, urea, creatinine, purines, and allantoin, hippuricacid, ammonia, amino acids, sulphur compounds, inorganic salts and the pigments, urochrome and urobilin. Other abnormal constituents may be present in pathological conditions.

Urea.—Derived mainly from the amino radicals of the protein molecules. The urea content of the urine of animals is between 1·5 to 3 gms%. Urea nitrogen forms about 80% of the total nitrogen of the urine.

Ammonia.—Is produced in the kidney and is used in eliminating the acid products of metabolism. The source of NH_3 is the NH_2 of the protein. Usually the amount of ammonia in urine is only about a twentieth of the amount of urea but in conditions causing acidosis increased quantities of NH_3 are formed from proteins to eliminate the increased quantities of acid products of metabolism and to this extent the formation of urea from the proteins is decreased. Hence in such conditions the ratio of NH_3: Urea becomes narrow.

Creatine and Creatinine.—Creatine is formed in the muscles. During metabolism, a part is converted into is anhydride, creatinine, and is excreted in urine. In all mammals creatine is usually not found in the

urine of adult animals but is found in the urine of young growing animals, of female animals during pregnancy and a short while after parturition. Creatine is an important constituent of the urine of birds.

Purines and allantion.—The chief purine in the urine of man and anthrapoid apes is uric acid. In the urine of other mammals uric acid is largely replaced by its oxidation product, allantoin. The Dalmation dog is exceptional in that only about 1/3 of the uric acid of this animal is converted to allantion, the rest being excreted as uric acid. Uric acid or allantion is an end product of nucleo-protein metabolism of mammals, but it is an end product of the protein metabolism in birds, which cannot make use of the arginine cycle of urea formation.

Hippuric acid.—Is Benzoyl glycine. The food of herbivores contains comparatively much benzoic acid. This is conjugated with glycine to detoxicate it and excreted in urine. It is formed in the kidney.

Amino acids.—Traces of amino acids that have diffused from the blood may be detected in the urine.

Sulphur compounds.—Exist in 3 forms.

(1) Neutral sulphur compounds such as traces of amino acids—cystine, cysteine, methionine.

(2) Inorganic sulphates.

(3) Ethereal sulphates such as phenol—sulphuric acid, p—cresol sulphuric acid, indoxyl sulphuric acid (Indican) and Skatoxyl sulphuric acid. Phenol, p—cresol, indole & skatole are derived from proteins in the intestines, where they are decomposed by bacterial decomposition. These compounds are rendered harmless to the organism by conjugation with sulphuric acid and are excreted in urine in the form of their sulphates, The amount of ethereal sulphates in urine serves as an index of bacterial putrefaction in the body.

Inorganic salts.—The chief inorganic salts are the chlorides sulphates, phosphates and bicarbonates of Na, K. Ca & Mg. Traces of other salts may be present. Inorganic salts of urine are mostly derived from food and the bulk consists of NaCl.

Urinary pigments.—The chief pigment is urochrome. Traces of other pigments such as urobilin may also be present.

Experiment 6.

(1) Note. (*a*) color, (*b*) deposits, (*c*) reaction and (*d*) Sp. Gr. From the Sp. Gr. calculate the amount of solids in gms. per 1000 c.c. of urine.

(2) Demonstrate the presence of (*a*) *Chlorides*-$HNO_3 + AgNO_3$ + urine gives a white curdy ppt. of AgCl.

(*b*) *Sulphates*: $HCl, + BaCl_2 +$ urine gives a ppt. of $BaSO_4$.

(*c*) *Phosphates*: $HNO_3 +$ Amm. Molybdate + urine—on heating gives a yellow color or a yellow ppt.

(3) **Ammonia.**—Take 10 c.c. of urine in a t.t. and a few drops of phenolphthalein. Cautiously add dil Na OH till the color is faintly pink. Dip a glass rod in an aq. soln. of phenolphthalein-Boil the mixture in the t.t. and hold the glass rod in the issuing steam. The drop of phenlophthalein becomes pink owing to the evolution of NH_3.

(4) **Urea**—*Urease test*—Take about 10 c.c. of urine, add about 5 or 6 drops of phenol red and render the fluid slightly acid with weak HCl. The color is yellow. Divide into 2 portions. To one add very dilute NaOH till the color is pink. To this add just enough of the yellow solution to discharge the red color thus making the mixture neutral.

Prepare urease soln. by putting 1/2 gram of soya bean meal in about 5 c.c. of water and shaking very well. Neutralise if necessary with dil. HCl.

Mix the Neutral urine with neutral urease soln. and allow to stand at room temp. for 5 to 10 min.

The mixture turns red owing to the liberation of NH_3 from Urea.

(5) **Uric acid and urates.**—To 3 c.c. of urine add 2 drops of Folin's uric acid reagent, about 1 gm. of $Na_2 CO_3$ and mix. A deep blue color is produced.

(6) **Hippuric acid.**—Render about 5 c.c. of urine neutral and add 3 drops of neutral ferric chloride solution. The ferric salt of Hippuric acid is precipitated as a cream colored precipitate.

(7) **Creatinine.**—To 5 c.c. of urine add 10 drops of 5% NaOH and 4 drops of picric acid solution. A red colored tautomer of creatinine picrate is produced.

(8) **Indican.**—Mix equal quantities (5 c.c.) of urine and Obermeyers reagent. Add 1 c.c. of chloroform. Gently pour the contents from one t.t. into another 10 times. The chloroform is coloured blue.

LESSON 11.

Detection of Pathological Constituents of Urine.

Ref—Cole-CHAPTER XIII

Hawk—CHAPTER XXIX

Examination of urine is a useful diagnostic aid. Many of the substances considered below as pathological constituents of urine are present in small amounts in normal urine. Hence, their pathological

singnificance may be more a question of the amount present than of their actual presence in or absence from the urine. It is generally true, however, that the usual qualitative tests for the substances are of such sensitivity as to yield an essentially negative result when applied to normal urine, but to respond readily when unusual amounts are present. It is recommended that the tests be carried out on a portion of well mixed and properly preserved 24 hrs. urine and if this is not possible, on the urine passed for the first time in the morning.

(1) **Albumin.**—Albuminuria is a condition in which serum albumin or serum globulin appears in the urine. It may be *renal*-the albumin being excreted by the kidneys or *accidental*-the albumin being not excreted by the kidneys but arising from blood, lymph or some albumin-containing exudate coming into contact with urine at some point below the kidneys.

Albumin is present in the urine in such conditions as Nephritis, Cystitis etc.

(2) **Glucose.**—Traces may be present in the normal urine. Pathological glycosuria may be renal glycosuria or due to diabetes mellitus. Renal glycosuria is due to reduced renal threshold not accompained by hyperglycaemia. In diabetis mellitus there is hyperglycaemia, polyuria and glycosuria.

(3) **Acetone.**—Present in urine in the later stages of diabetes mellitus or other conditions associated with deficient or defective carbohydrate metabolism. In such cases, the terminal stages of fat metabolism are altered to the extent that ketosis develops.

(4) **Blood.**—Blood is found in the urine in haematuria and haemoglobinuria. Haematuria is caused by blood passing into urine because of some lesion of the kidneys or of the urinary tract below the kidneys. Haemoglobinuria is caused by conditions associated with extensive haemolysis such as piroplasmosis and also extensive burns. Biochemical tests described herein do not differentiate between haematuria and haemoglobinuria.

(5) **Bile.**—The constituents of bile are found in urine when bile duct is obstructed. The bile is absorbed into the lymphatics and passes into circulation, and reaches all parts of the body, the pigments causing a staining of the various tissues. The condition is known as Jaundice.

(6) **Sediments.**—Are either organised or unorganised. Organised sediments consist of casts of renal tubules, epithelial cells from different parts of the renal tract, pus, blood cells, spermatozoa, parasites etc.

Unorganised sediments vary with the reaction of the urine. In acid urine Calcium oxalate, Ca Hydrogen phophate etc. and in alkaline urine NH_4, $MgPO_4$, $CaCO_3$ etc are some of the unorganised sediments found.

***Experiment* 17.**

(1) Note (a) color, (b) deposits, (c) reaction, (d) Sp. Gr. of the urine. If urine is turbid, filter it before doing the following tests.

(2) **Albumin.**—(*a*) *Heller's test.* Take 2 c.c. of Conc. HNO_3 in a t.t. and layer on top an equal volume of urine taking care to avoid any mixing of the 2 fluids. A white ring at the interface between the fluids indicates presence of albumin. If positive confirm by the following.

(*b*) *Heat coagulation test.* Adjust the reaction about 5 c.c. of the urine to about PH 5·4 (the isoelectric point of albumin) by using chlorophenol as indicator. Boil the upper layer. A turbidity here indicates albumin or earthy phosphates. Add a drop or two of strong acetic acid to the hot soln. If the turbidity persists, albumin is present.

If albumin is present remove it by heat—coagulation and filteration before testing for sugar.

(3) **Sugar (glucose).**—(*a*) *Fehling's test.* Mix 1 c.c. each of Fehling's solns. 1 and 2, boil to test the reagent. Add 2 c.c. of the urine and boil again. A yellowish or brownish red ppt. of Cuprous oxide indicates the presence of glucose.

Fehling's test is not very reliable when used to detect sugar in urine. Some compounds such as glycuronates, uric acid etc. may produce a result similar to that produced by glucose. Hence if a positive reaction is obtained conduct the following tests.

(*b*) *Benedict's test* To 5 c.c. of Benedict's soln. in a t.t. add exactly 8 drops of urine and boil for 1 to 2 min. Allow the fluid to cool slowly. If glucose is present a ppt. gradually forms throughout the fluid red, yellow or green depending upon the quantity of glucose present.

(*c*) *Nylander's test.* Add exactly 10 drops of Nylander's soln. to 5 c.c. of urine in a t.t. and keep in a boiling water bath for 5 min. The soln. gradually darkens and turns black if glucose is present.

(4) **Acetone**—*Rothera's test.*—Saturate 5 c.c. of urine with $(NH_4)_2SO_4$. Add 1 drop of freshly prepared 5% soln. of sod. Nitroprusside and 2 c.c. of Con. NH_4OH. Mix and allow to stand undisturbed. A characteristic permanganate coloration indicates presence of acetone and or acetoacetic acid.

(5) **Bile salts.**—*Hay's test.*—Over 5 c.c. of urine sprinkle a few grains of flowers of sulphur. If bile salts are present the sulphur falls through the liquid to the bottom of the tube.

(6) **Bile pigments.**—*Huppert's test (Cole's modification of)*—Boil about 15 c.c. of urine. Aud 2 drops of saturated soln. of Mag. Sulph. Add 10% soln. of $BaCl_2$ until no further ppt. forms. Boil again. Allow to stand for a short time. Pour off the greater part of the supernatant

fluid. Filter the reminder through a small filter paper. Remove the ppt. to a dry t.t. Add about 4 c.c. of strong alcohol and shake vigorously Add 3 drops of strong H_2SO_4 and one drop of 2% $KClO_3$. Boil for half a minute and allow the $BaSO_4$ to settle. The presence of the pigments of bile is indicated by the alcoholic soln. being coloured blue or greenish blue.

(7) **Blood.**—*Tincture Guiacum test (Pseudoperoxidase reaction)*—To 5 c.c. of urine add 1 drop of Tr. guiacum and 2 c.c. of 3% H_2O_2. A blue color develops if blood is present. A positive reaction should be confirmed by repeating the test on 5 c.c. of urine, boiled and cooled. Why ?

(8) **Sediments.**—Centrifuge about 15 c.c. of the original urine (unfiltered) Pour off the supernatant fluid. Shake up the sediment with the few drops of the fluid remaining in the tube. Transfer a drop of this to a slide and cover with a slip. Examine under the low and high powers of a microscope for the presence of organised and unorganised sediment.

LESSON 12.

Analysis of Gastric contents.

Ref. Cole—CHAPTER IX—Section C.

An analysis of the material withdrawn from the stomach after a standard test breakfast is an usual diagnostic procedure used to study gastric function in human medicine. The method is applicable in comparative medicine as well, esp. in the dog and cat, though seldom used.

A few items of the information usually required are.

(i) free acidity, (ii) total acidity, (iii) total chlorides, (iv) mineral chlorides, (v) the active HCl=3–4 and (vi) Combined HCl = 5–1.

The HCl secreted by the parietal Oxyntic cells of the fundus, neutralises the alkaline salt of the saliva to form NaCl. It also combines with the proteins of the saliva and of the food to form acid-metaprotein compounds. These function as week acids. In addition, lactic acid is produced from carbohydrates by bacterial action. The amount of lactic acid produced increases when the secretion of HCl which inhibits the action of the bacteria decreases.

Experiment 18.

I. Estimation of free acidity (free HCl). *Topfer's method.*

Measure 5 c c. of the clear gastric filtrate into a clean dry t.t. Add 2 drops of Topfer's reagent. Titrate with 0·1 N soda until the bright pink color disappears. The correct end point is an orange color rather than

yellow. Read the burette after each drop when near the end point. From the results calculate the free acidity which is expressed in c.c. of 0·1 N percent.

II. Estimation of Total Acidity

To the fluid remaining in the tube after the above estimation, add 2 drops of phenolphthalein. Continue the titration until a faint color is produced. From the result calculate the total acidity in c.c. of 0·1 N percent.

III. Estimation of Total Chlorides. (*Whitehorn-Volhard method.*)

Principle.—A measured volume of the fluid is treated with a given volume (excess of standard silver nitrate in the presence of strong HNO_3. After the silver chloride has aggregated, the excess of silver nitrate is titrated with standard thiocyanate in the presence of iron alum, which gives a red color of ferric thiocyanate when the last traces of silver has been precipitated by the thiocyanate.

Method.—Measure 5 c.c. of the clear filtrate of gastric digest into a 100 c.c. conical flask. Add 10 c.c. of standard silver nitrate soln containing HNO_3. Stir well by rotating the flask and allow to stand for 5 min. for the silver chloride to aggregate. Add 1 c.c. of iron alum soln. and titrate with standard thiocyanate soln—until a salmon red color is obtained, which persists for 15 seconds. During titration gently rotate the flask just to mix the thiocyanate added, as too vigourous a shaking accelerates the disappearance of the pink color.

Standard $AgNO_3$ soln ≡ Standard thiocyanate ≡ 10 mg NaCl

Calculation.—If x c.c. of thiocyanate are used for the back titration, the amount of standard silver nitrate used up in precipitating the chlorides = 10x

Every c.c. of $AgNO_3$ soln ≡ 10 mg NaCl

The amount of chlorides in the gastric digest expressed as NaCl = (10–x)10 per 5 c.c. of the digest.

Total chloride content of the digest

$$= \frac{(10-x)10}{5} \times 100 = (10-x)\ 200 \text{ mg\%}$$

The estimation of mineral chlorides.

Principle.—A measured volume of the gastric digest is evaporated to dryness on a boiling water bath and then heated until the organic matter has been just carbonised. This will drive off the free and combined HCl, leaving behind only mineral chlorides. The residue is dissolved in water and the chloride content is estimated by the above method.

Method.—Measure 5 c.c. of the clear filtrate of the gastric digest into a porcelain crucible. Evaporate to dryness on a boiling water bath. Cautiously heat the crucible placed on a clay pipe triangle with the bunsen burner just to completely carbonise the organic matter. Do not attempt to burn away the carbon as it might result in loss of some of the chlorides.

Allow the crucible to cool thoroughly. Add 5 c.c. of distilled water and 10 c.c. of standard $AgNO_3$. Stirwell, allow to stand for 5 min. and then proceed as in the previous exercise.

N.B.—Total acidity includes HCl and other organic acids such as lactic acid.

Free acidity is practically the same as free HCl.

Physiologically active HCl = Total chlorides—mineral chlorides.

Combined HCl = Physiologically active HCl—free HCl.

LESSON 13.

***Experiment* 19.**

Detection of Substances of Physiological Interest.

1. Note the color, smell, consistency and reaction.

2. **Bile salts.**—Test by *Hay's test.* If positive, confirm by *Pettenkofer's test.*

3. **Bile pigments.**—Test by *Gmelin's test.* If positive, confirm by *Huppert's test.*

4. **Blood.**—Subject the solution to the *Tr. Guiacum test.* If positive, test by the spectroscope looking for the characteristic absorption bands.

5. **Urea.**—Test by *Urease test* as in the case of urine.

6. **Uric acid and urates.**—Detect the presence of these by *Folin's uric acid reagent.*

7. **Ammonia.**—Test for ammonia as in the case of urine.

1. **Proteins.**—Detect the presence of proteins in the given soln. by the mercuric nitrite test and the Biuret test. If protein is present, determine whether it is (*a*) Albumin, (*b*) proteoses, or (*c*) gelatin or (*d*) peptones, by the following tests.

(*a*) Make the soln. slighty acid by adding 2 drops of acetic acid to 10 c. c. of the soln. Boil the upper layer. A cloudiness is obtained if Albumins and globulins are present. If a positive reaction is obtained, boil the soln. in the t.t. and filter.

(*b*) To 6 c.c. of the filtrate from (*a*) or the original soln. if albumin and globulin are not present, add ammonium sulphate to saturation. Filter. To 3 c.c. of the filtrate add 5 c.c. of 40% Sod. hydroxide and one drop of 1% copper sulphate soln. If a postive Biuret reaction is obtained peptones are present. (peptones are the only proteins that are not precipitated by full saturation with ammon. sulphate) *Note* that the Biuret reaction has been modified above as is necessary when the soln. to be tested contains a large quantity of ammon. sulphate.

(*c*) Subject the filtrate from (*a*) or the original soln. if no albumins and globulins are present, to the aldehyde reaction for Tryptophane and Mercuric nitrite test for Tyrosine. Proteoses contain both these aminoacids and so both the reactions are very positive. Gelatin contains little tryptophane and so the aldehyde reaction is negative and the Mercuric nitrite test is only faintly positive.

N.B.—If albumins and globulins are present, remove them by heat coagulation and filter before proceeding to test for Carbohydrates.

(9) **Carbohydrates.**—Test by *Molich's test.* If a positive reaction is obtained, determine the nature of the carbohydrate, (starch, reducing or a nonreducing sugar, by the following).

(i) Take 5 c.c. of the soln. in a tt. If its reaction is alkaline, slightly acidify it with weak HCl. Add a drop or two of I_2 soln. A blue color indicates starch.

(ii) If (i) is negative, do Fehling's test for, reducing sugars. If positive confirm by Benedict's test.

(iii) If i and ii above are negative, to 5 c.c. of the soln. add 6 drops of Conc. HCl. Boil for 2 min. and cool. Now do the reduction tests. A positive reaction denotes the presence of a nonreducing sugar in the original soln.

(10) **Acetone.**—Detect the presence of this by *Rothera's test.*

N.B.—The various tests have been chosen and arranged in order to make the examination easy and avoid interferences from substances other than the one being examined for. This scheme of examination should be strictly followed.

PART II.

LESSON 14.

Urine—Quantitative Analysis

Ref: Cole—CHAPTER—XIV

In analysing a normal or a pathological sample of urine quantitatively for any of its constituents, it is particularly necessary that complete and exact 24 hours sample be obtained. Before any urine is taken for analysis its total volume should be measured and from this the daily output of the individual constituents determined. Quantitative analysis should be preceded by qualitative tests for pathological contituents.

Most of the selected methods described below are volumetric and in order to obtain reliable results, the student must study the correct method of using volumetric glass ware such as pipettes etc. The following are the references suggested:

(1) Cole—Appendix.
(2) Cameron and White—Practical Biochemistry Chapter XV
(3) Fowls (volumetric analysis) Chapter II

Colorimetry.—Many methods for quantitative analysis of blood, urine and other biological materials are based upon the production of colored solns. in such a way that the intensity or depth of color so obtained may be used as a measure of concentration of substances being determined. Such use of color as an index of concentration in analysis is colorimetry and the instrument used in color evaluation is called a colorimeter. In this connection, the following references are suggested:

(1) Hawk——Chapter XXIII.
(2) Cole——Appendix.

1. *Chlorides.*

Whitehorn—Volhard method.

Principle.—See estimation of total chlorides of Gastric digest.

Method.—Measure 5 c.c. of clear urine (filtered if necessary) into a 100 c.c. flask. Add 10 c.c. of standard Silver nitrate soln. containing HNO_3. Stir well by rotating the flask and allow to stand for 5 minutes for the silver chloride to aggregate. Add 1 c.c. of iron alum soln. and titrate with standard thiocyanate soln. until a salmon red color is obtained which persists for 15 seconds. During titration gently rotate the flask just to mix the thiocyanate added, as too vigourous a shaking accelerates the disappearance of the pink color.

Calculation.—See estimation of total chlorides in gastric digest.

2. *Sulphates.*

A—Inorganic sulphates.—by the Benzidine method of *Rosenheim and Drummond.*

Principle.—The urine is acidified with HCl and treated with excess of Benzidine hydrochloride. The sulphates are precipitated quantitatively. The precipitate is filtered off, washed free from acid with saturated soln. of Benzidine sulphate and suspended in hot water. Phenolphthalein is added and the mixture is titrated with standard soda. Benzidine sulphate being the salt of a strong acid and weak base, freely dissociates in soln. and the liberated strong acid can be titrated against soda. From the result, the sulphates can be calculated.

Reagents.

(1) *Benzidine Hydrochloride.*—2 gms. of Benzidinewith 2·5 c.c. of Conc. HCl to one litre of water.

(2) *Hydrochloric acid.*—Dilute 1 Vol. of Conc. HCl with 3 vols. of distilled water.

(3) *Saturated soln. of Benzidine sulphate.*

(4) O·I N NaOH.

Method.—Measure 25 c.c. of urine, filtered if necessary, into a 250 c.c. conical flask. Add 2 c.c. of HCl and 100 c.c. of Benzidine Hydrochloride. Mix and allow to stand for 10 minutes. Filter through a filter paper, repeatedly if necessary, until the filtrate is clear. Washout the flask with 10 c.c. of saturated Benzidine sulphate and wash the precipitate in the paper with this. Repeat the washing once more. Transfer the filter paper and the precipitate carefully to the same conical flask. Wash the funnel into the flask with about 50 c.c. of boiling water (distilled) Suspend the precipitate in the water by careful shaking. Add a few drops of saturated soln. of phenolphthalein and titrate the hot soln. with standard soda. From the result express the inorganic sulphates in mgms. of Sulphates per 100 c.c. of urine.

Calculation.—1 c.c. of O·1 N soda ≡ 4 mgms. of Sulphates.

If X c.c. of soda are used for titration, the inorganic sulphates in the sample of urine expressed as SO_3 = 4X × 4 mgms. per 100 c.c.

B. Total sulphates.—Measure 25 c.c. of urine into a conical flask, add 2 to 2·5 c.c. of HCl (1 in 4) and 20 c.c. of distilled water. Place the funnel in the neck of the flask and boil gently for 20 minutes. Cool well under the tap. Add 100 c.c. of Benzidine hydrochloride and proceed as directed above.

3. *Phosphates.*—(*inorganic*).

Colorimetric method.—(*Fiske and Subba Row*).—Phosphate reacts with molybdic acid to form phosphomolybdic acid. On treatment with 1-2-4-amino-naphthol-sulphonic acid phosphomolybdic acid is selectively reduced to produce a deep blue color. This color is then compared in a colorimeter with that of a suitable standard phosphate solution treated in the same way.

Reagents.

(1) *Molybdate I Reagent.*—Dissolve 25 gms. of ammonium molybdate in about 200 c.c. of water. In a 1000 c.c. volumetric flask place 500 c.c. of 10 N H_2SO_4 (450 c.c. of Conc. pure H_2SO_4 in 1300 c.c. of water) Add the molybdate soln. and dilute with washings to 1000 c.c. with water.

Amino naphthol sulphonic acid reagent.—To 195 c.c. of 15% soln. of sod. bisulphite add 0·5 gms. of 1-2-4-aminonaphthol sulphonic acid. Add 5-c.c. of 20% sod. sulphite soln. Mix well until dissolved. If necessary add more sod. sulphite soln. 1 c.c. at a time, but avoid excess. Keep cool in an amber coloured bottle. Keeps for 4 weeks.

(3) *Standard phosphate soln.*—5 c.c. contains 0·4 mgms. of phosphorus. Prepared by dissolving 0·351 gms. of pure monopotassium-phosphate in about 200 c.c. of water and 10 c.c. of 10 N. sulphuric acid in a 1000 c.c. volumetric flask and diluting to mark with water.

Method.—Measure into a 100 c.c. volumetric flask 1 to 2 c.c. of urine. Add water to bring the volume to nearly 70 c.c. and then 10 c.c. of Molybdate I reagent. Mix by gentle shaking. Add 4 c.c. of the amino naphthol sulphonic acid reagent. Mix—dilute to mark—stopper and invert several times.

Measure into another 100 c.c. vol. flask 5 c.c. of the standard phosphate soln. containing 0·4 mgms of P instead of urine. Proceed just as in the case of urine.

Allow both flasks to stand for 5 minutes.

Compare in a colorimeter with the standard at 20 mm.

Calculation.—Inorganic phosphates expressed as mgms.

$$\text{of phosphorus per 100 c.c. of urine} = \frac{800}{\text{Reading of unknown X vol. of urine.}}$$

N.B.—With the standard at 20, unknown should have a reading of 5 to 40. If the reading is outside this range, repeat the analysis using larger or smaller volume of urine.

4. *Urea.*

Hhpobromite method.

Principle.—Urine is treated with an alkaline solution of hypobromite and the amount of urea calculated from the volume of N_2 evolved. The reaction that takes place is as follows :

$$CO(NH_2)_2 + 3\,NaBrO + 2\,NaOH = N_2 + 3\,NaBr + Na_2CO_3 + 3H_2O$$

Therefore one gram of urea evolves 373 c.c. of nitrogen measured at N.T.P. In practice only 357 c.c. are evolved, the remainder being converted into some other nitrogenous compounds.

Apparatus.—*Doremus'* ureometer and *Dupre's* ureometer.

Reagents.—To 25 c.c. of 40% NaOH in a beaker add gradually 2.5 c.c. of bromine. mixing during addition and preventing much rise of temperature by keeping the beaker in cold water.

Method of analysis.

(1) **Using Doremus ureometer.**—Fill the apparatus with freshly prepared hypobromite soln. Take urine in the small burette if it forms a part of the apparatus or upto the mark in the special 1 c.c. pipette. Grudually introduce 1 c.c. of urine into the vertical limb of the apparatus-taking about 5 minutes to introduce this one c.c. If the quantity of nitrogen evolved is very small, another one or more c.cs. of urine may be similarly introduced. The instument is graduated in gms. of urea in the quantity of urine introduced. At the end of 10 minutes take the reading and calculate the quantity of urea in the sample of urine (gms. per 100 c.c.)

(2) **Dupre's ureometer.**—Place 25 c.c. of freshly prepared hypobromite soln. in the bottle and 5 c.c. of urine accurately measured in the small tube. Place the tube inside the bottle by means of a pair of forceps, taking care not to upset any urine into the hypobromite. Fit the rubber cork into the bottle and place this in the jar of water to cool. See that the gas burette is as low as possible, that the cylinder has sufficient water in it to reach the zero graduation of the burette and that the screw clamp is open. Leave the apparatus to cool to the temp. of water. Clamp the burette in such a position that the water in the burette is at zero mark. Screw tight the clamp on the rubber tubing.

Take the bottle out of the jar and gently tilt it so that the urine flows into the hypobromite. Gently shake the bottle from side to side, but keeping it vertical to prevent the froth from being carried over. Replace the bottle in the water for about 5 minutes to cool. Raise the burette until the level of the water inside is the same as outside. Prevent warming of the tube by the hand by holding it only at the top. Clamp

the burutte in this position and take the reading. The apparatus is graduated to give the amount of urea in gms. per 100 c.c. of urine.

5. Sugar.—(*glucose*).—Traces may be present in normal urine. In diseased conditions larger amount are present.

Benedict's method.

Principle.—Refer to the quantitative estimation of sugars.

Method.—Dilute 10 c.c. of urine to 100 c.c. (unless the sugar content is believed to be low, when it may be diluted 1 in 5 or even used undiluted) Place this in a 50 c.c. burette.

Pipette out, accurately, 25 c.c. of Benedict's reagent into a 250 c.c. flask, add about 5 gms. of anhydrous sod. carbonate, a few bits of broken porcelain and heat the mixture to boiling, until the carbonate has entirely dissolved. Run in the glucose soln. from the burette, rather rapidly 1/2 c.c. at a time at 1/2 minute intervals until a chalk white ppt. forms and the blue color of the mixture begins to lessen perceptibly, after which, run in a few drops at a time, until the last traces of blue color disappears, which marks the end point. Take the reading of the burette. The solution must be kept vigorously boiling during the titration. If the mixture becomes too concentrated, during the process, add distilled water from time to time to replace that lost by evaporation. Repeat to get nearly concurrent readings.

Calculation.—Calculate as described under the estimation of sugars taking into consideration the dilution of the urine and express the result in gms. % of glucose.

N.B.—The precautions to be observed are the same as those described under the estimation of sugars.

6. *Albumin.*

I. Esbach's method.

Principle.—A definite quantity of urine is mixed with a definite quantity of Esbach's reagent. The albumin is precipitated. From the amount of the ppt. the quantity of albumin is estimated.

Reagent.—Dissolve 1 gm. of picric acid and 2 gms. of citric acid in 100 c.c. of water.

Method.—Fill the albuminometer to mark U with urine. Add Esbach's reagent to mark R. Stopper tube and invert it slowly several times to mix the fluids. Allow the tube to stand upright for 24 hrs.

Calculation.—The graduations on the albuminometer indicate the gms. of albumin per litre. Express the result in gm. %.

II Aufrecht's method.—This is a modification of the above method and the principle is the same.

Aufrecht's reagent.—1·5 gms. of picric acid, and 3 gms. of citric acid dissolved in water to make up to 100 c.c. of soln.

Method.—Fill the albuminometer with urine to the letter U and add the reagent to the letter R. Close the end of the tube with a rubber cork and mix the fluids by gentle repeated inversions. Place the tube in a centrifuge for 3 minutes at 2000 to 2500 RPM.

Calculation.—The ppt. of albumin on the graduated scale indicates exactly the % of albumin.

7. *Creatinine.*

Folin's method.

Principle.—A measured amount of urine is treated with picric acid and NaOH. The creatinine combines with the picric acid in the cold to form an orange colored compound. A known amount of creatinine is similarly treated and the solutions compared in colorimeter.

Reagents.

(1) *Standard creatinine solution.* Dissolve 1 gm. of pure dry creatinine in sufficient O·1 N. HCl to make 1000 c.c. Mix well. The soln. contains 1 mg. of creatinine per c.c.

(2) *Picric acid.* 1%.

Method.—Measure 1 c.c. of urine into a 100 c.c. vol. flask and in a second similar flask place 1 c.c. of standard creatinine soln. To each flask add 20 c.c. of 1% picric acid soln. from a burette followed by 1·5 c.c. of 10% NaOH. Mix gently and allow to stand for 15 minutes. Dilute to mark with water and mix by inversion. Compare the unknown against the standard in a colorimeter, setting the standard at 20 mm.

Calculation.—With the standard at 20 mm. the quantity of creatinine in the sample of urine is $\frac{2000}{U}$ mgs per 100 c.c.

N.B.—For the result to be accurate, it is necessary that the creatinine in the quantity of urine added should be approximately 1 mgm. per c.c. A range of 75 to 125 mgs. per 100 c.c. may be accurately estimated. If the creatinine content of the urine is found to be beyond this range, as found by one estimation, the volume of urine taken for analysis should be altered so as to contain about 1 mgm. and this fact allowed for in calculation.

8. *Acidity and Ammonia.*—*A. Acidity* (*Folin's method.*—Titratable acidity.)

Principle.—The urine is titrated with standard sod. hydroxide soln., using phenolphthalein as the indicator. Pot. oxalate is added to precipitate the Calcium which would otherwise interfere with the end point due to the precipitation of calcium phosphate on neutralisation of the urine.

Method.—Place 25 c.c. of the urine in a 250 c.c. conical flask and add about 5 gms. of powdered pot. oxalate and 1 to 2 drops of phenolphthalein soln. to the fluid. Shake the mixture vigourously for one to two minutes and titrate it immediately with 0.1 N. NaOH until a faint but unmistakable pink color remains permanent, on further shaking. From the burette reading calculate the titratable acidity of the urine under examination. The result is expressed as follows:

The titrable acidity of the urine is equivalent to X c.c. of 0·1 N NaOH per 100 c.c.

B. Ammonia.

Formal titration method.

Principal.—Neutral ammonium salts and amino acids react with an excess of neutral formaldehyde to give hexamethylenetetramine, the acid being liberated.

$$4NH_4Cl+6HCHO=N_4(CH_2)_6+6H_2O+4HCl.$$

From the amount of liberated acid, which can be estimated by titration, the amount of ammonia in the form of neutral salts can be calculated.

Method.—To the mixture just after neutralization in the urinary titratable acidity determination, add 10 c.c. of neutral formalin soln. Mix well and titrate with 0.1 N NaOH to a permanent pink color.

Calculation.—One c.c. of 0.1 N NaOH is equivalent to 1·7 mgms. of ammonia. Multiply the number of c.c. of 0·1 N NaOH required to neutralise after the addtion of formalin by 1·7 and by 4 to get the no. of mgs. of NH_3 (plus amino acid ammonia expressed as NH_3) in 100 c.c. of urine examined.

9. *Uric Acid.*

Colorimetric estimation (Benedict)

Principal.—The urine is diluted and treated with Bendict's arseno-phospho-tungstate reagent and with sod. cyanide. A deep blue color is obtained which is compared with that obtained with a standard soln. of uric acid similarly treated.

Reagent.

(1) *Benedict's uric acid reagent.*—**(poison)**—In about 600 c.c. of water, dissolve 100 gms. of sod. tungstate. Add 50 gms. of pure arsenic acid, 25 c.c. of 85% H_3PO_4 and 20 c.c. of Con. HCl. Boil for 20 minutes Cool and dilute to 1000 c.c.

(2) *Sodium cyanide* 5% **(deadly poison)**

(3) *Standard uric acid soln*—0·2 mg. in 10 c.c. prepared by diluting a stock standard soln. of uric acid in lithium carbonate (1 mg. per c.c.)

Method.—Label two 50 c.c. vol. flasks, S and U. Dilute the urine 20 times.

Into S measure 10 c.c. of standard uric acid soln. (0·2 mg. of uric acid) Into U measure 10 c.c of diluted urine. To each add 5 c.c. of the cyanide soln. from the burette—**never from a pipette**—as it is a deadly poison. To each add 1 c.c. of Benedict's uric acid reagent, also from a burette—as this is also a poison.

Mix the contents, allow to stand for 5 minutes. Fill up to the mark with distilled water, mix well and compare in a colorimeter with the standard at 20 mm.

The reading of U should be between 14 and 26 mm. If not the estimation should be repeated with a 1 in 40 or 1 in 10 dilution of the urine as the case may be.

If U is the reading, mgs. of uric acid per 100 c.c. of urine under examination is.

$\frac{40}{U}$X number of times urine is diluted.

LESSON 15.

Blood Quantitative analysis

Blood analysis is an indispensable adjunct to the study of the body, both in health and disease. A number of different methods are available for the determination of the various constituents of blood and some satisfactory methods are given below. If serious errors are to be avoided, exact manipulation of the apparatus is necessary and the student is advised to cultivate the correct method of using volumetric glass ware etc. References on the above subject are given under quantitative analysis for urine.

1. Preparation of the protein free blood filtrate.

Method of Folin and Wu.

Principal.—The proteins of whole blood or plasma or serum are removed by the precipitation with tungstic acid and filtration. The

filtrate is suitable for the determination of chlorides, urea, sugar, nonprotein nitrogen, creatinine and uric acid.

Procedure.—Transfer 5 c.c. of blood to a 100 c.c. conical flask. Add from a burette exactly 35 c.c. of water. Mix. Add 5 c.c. of 10% Sod. tungstate soln. Mix. Finally add, slowly with shaking, 5 c.c. of 2/3N sulphuric acid. Mix the contents well by shaking. Let stand for 10 minutes. The color should change from red to dark brown, otherwise it denotes that coagulation is not complete. In such cases, add 10% sulphuric acid drop by drop, shaking well after each addition until the dark brown color sets in.

Pour the mixture on a dry folded filter paper in a funnel large enough to hold it all. Cover the funnel to reduce evaporation. Collect the filtrate in a clean dry container. If the first few drops are not absolutely clear return this portion to the funnel.

10 c.c. of the above filtrate represents 1 c.c. of blood.

2. *Estimation of Chlorides.*

Principle.—See estimation of total chlorides in gastric digest.

Reagents.—1. *Silver nitrate in Nitric acid.* 1 c.c. ≡ 1mg. NaCl.

2. *Standard thiocyanate* 1 c.c. ≡ 1 c.c. silver nitrate.

Method.—Measure 10 c.c. of the protein free blood filtrate into a china dish. To this add 10 c.c. of acid silver nitrate soln. and stir thoroughly. Allow the mixture to stand for 5 minutes for the silver chloride to coagulate. Add about 3 drops of freshly prepared iron alum solution. Titrate with standard thiocyanate solution from a 5 c.c. burette, until a salmon red color is obtained, which persists for 15 seconds. During the titration gently rotate the dish to mix the thiocyanate.

Calculation.—If T is the volume of the thiocyanate added.
(10–T) 100 = mg. of NaCl in 100 c.c. of blood.

3. *Urea.*

Van Slykes Urease-aeration method.

Principle.—3 c.c. of blood are aerated with urease soln. and a phosphate buffer soln. The mixture is allowed to stand for 5 minutes. The urea is converted into ammonia. The mixture is made alkaline with Pot. Carbonate and the ammonia blown by a current of airin to a measured amount of standard acid. This is titrated with a standard alkali and from the result the amount of urea present is calculated.

Reagents.—(1) Phosphate buffer soln.
(2) Urease Soln.
(3) Standard HCl 0·01 N.

(4) Standard NaOH 0·01 N.
(5) Saturated K_2CO_3 Soln.
(6) Caprylic alcohol.
(7) Methyl red.

Method.—Before commencing the estimation, study the apparatus fitted up and its working (for the description, the tubes are described as, A, B and C in the direction of the air current).

A, is a wash bottle containing 10% sulphuric acid to remove the ammonia from air.

Measure 3 c.c. of phosphate buffer and 1 c.c. of urease soln. into B. Add. 3 c.c. of oxalated blood and 5 drops of Caprylic alcohol. Mix carefully.

Into C measure 20 c.c. of 0·01 N acid. Add 1 drop of Carprylic alcohol. Connect up A and B only and send a slow current of air through the apparatus for 8 minutes and a rather more rapid current for 2 minutes. This keeps the contents of B well mixed up for the time. Stop air current and disconnect B from A. Remove the stopper and introduce into B about 4 c.c. of Sat. Pot carbonate soln. and about 3 gms. of solid Sod. carbonate. Stopper immediately. Connect up the complete apparatus and restart a rather slow air current. (**N.B.**—The apparatus should be rapidly assembled as soon as K_2CO_3 Soln. is added). After two minutes gradually quicken the air current as far as possible but taking special care not to risk any spurting of alkali from B into C. Maintain this strong current for 15 minutes. Disconnect the apparatus. Wash down the inside and outside of the bubbling tube, after removing the stopper, with distilled water into the acid. Add 4 drops of methyl red to the acid and titrate with 0·01 N NaOH. From result calculate mgms. of urea in 100 c.c. of blood.

Calculation.—If 20 c.c. of 0·01 N acid requires x c.c. of 0·01 N NaOH for back titration the amount of urea in 100 c.c. of blood = (20-x)10.

4. *Sugar.*

Folin and Wu's method.

Principle—. The protein free blood filtrate is heated with alkaline Copper sulphate soln. using a special tube to prevent the reoxidation of the cuprous oxide by the atmospheric oxygen. The cuprous oxide formed is treated with a phosphomolypdic acid soln. a blue color being obtained which is compared with that of a standard.

Reagents.

(1) *Standard sugar solns.*

(*a*) Stock soln. 1% glucose in saturated Benzoic acid. From this the following sugar solns. are freshly prepared before use by appropriate dilutions with water.

(b) A working standard containing 0.1 mgm. of glucose per c.c.

(c) „ „ „ 0.2 mgms.

(d) „ „ „ 0.3 mgms.

(2) *Alkaline copper sulphate soln.*—Dissolve 40 gms. sodium carbonate (anhydrous) in about 400 c.c. of water. Add and dissolve 7.5 gms. of Tartaric acid, Transfer the soln. to a litre flask. Add 4.5 gms. of crystalline $CuSO_4$. dissolve and make up to the mark.

(3) *Phosphomolybdic acid soln.*—To 35 gms. of Molybdic acid and 5 gms. of Sod. tungstate add 200 c.c. of 10% sod. hydroxide and 200 c.c. of water. Boil vigorously for about half an hour, cool and dilute to about 350 c.c. in a 500 c.c. vol. flask, add 125 c.c. conc. Phosphoric acid and dilute to the mark.

Method.—Transfer 2 c.c. of protein free filtrate to a Folin-Wu sugar tube, graduated at 25 c.c. and to other similar tubes add 2 c.c. of sugar standard b.c. and d. To each tube and 2 c.c. of alkaline copper sulphate soln. The mixture now reaches the constricted part of the tube. Transfer the tubes to a water bath which has already been kept boiling. Keep in the water bath exactly for 8 minutes. Remove from this after this period and immediately cool under running water without shaking. To each tube add 2 c.c. of Phosphomolybdic acid reagent. After about one minute dilute to the mark with water and mix well, taking care to see that the fluid in the bulb at the end of the tube mixes well with the rest of the fluid. Use the standard which most nearly matches the unknown. Compare in a colorimeter.

Calculation.

$$\frac{\text{Reading of S}}{\text{Reading of U}} \times \frac{\text{mgms. of glucose in standard (i.e. 0.2, O.4, or O.6)}}{\text{O.2}} \times 100.$$

= mgms. glucose per 100 c.c. of blood.

The readings of the standard and the unknown should be within a few mms. of each other for the result to be reliable. If it is not so, another suitable standard should be selected and the estimation repeated.

5. Creatinine.

Folin's method.

Principle.—Same as given under the estimation of creatinine in urine.

Reagents.

(2) *Alkaline picrate.*—To 25 c.c. of sat. aqueous solution of picric acid and 5 c.c. of 10% NaOH and mix. This reagent must be freshly prepared before use.

(2) *Creatinine solutions*

(*a*) *Stock soln. of standard creatinine.*—Prepared by diluting 6 c.c. of standard creatinine soln. used in the estimation of creatinine in urine (i.e. 1 mg. of creatinine per c.c.) to 1000 c.c. with O.1 HCl and preserved with a few drops of toluene....5 c.c. of this contains 0·03 mg of creatinine.

(*b*) **Working standards.**—Prepared by diluting the stock standard as follows:—

(i) 5 c.c. of stock standard + 15 c.c. of water.
(ii) 10 c.c. ,, ,, + 10 c.c. ,,
(iii) 15 c.c. ,, ,, + 5 c.c. ,,
(iv) 20 c.c. ,, ,, used undiluted.

The normal range (1 to 4 mgms. per 100 c.c. of blood) is covered by the working standard (i) and (ii) The other standards need be used only when a high creatinine content (4 to 8 mgms. per 100 c.c. of blood) is anticipated in diseased conditions.

Method.—Label 2 small flasks U and S. Into U measure 10 c.c. of blood filtrate. Into S measure 10 c.c. of working standard (i).

To both add 5 c.c. of alkaline picrate soln.

Mix carefully and allow to stand for 10 minutes. Then compare in a colorimeter setting the standard at 20 mm. Comparison should be finished within 15 minutes of adding the alkaline picrate.

Calculation.—With the standard (i) at 20 mm.

$\frac{20 \times 1.5}{U}$ = mg. of creatinine in 100 c.c. of blood.

The readings of the standard and unknown should be near each other. This may be obtained by choosing a suitable standard. If standard (ii) is used, multiply $\frac{20 \times 1.5}{}$ by 3 to obtain mg. creatinine per 100 c.c. of blood. If standard (iii) by 4.5 and standard (iv) by 6.

6. Nonprotein Nitrogen.

Kjeldahl's method.

Principle.—The nitrogenous compounds in a given volume of protein free blood filtrate are converted into ammonium sulphate by boiling with H_2SO_4. Copper sulphate being added to aid the oxidation and Pot. persulphate to raise the boiling point. The mixture is diluted with water and made alkaline by the addition of sodium hydroxide and the ammonia lead into a measured amount of standard acid. The amount

of this acid that is neutralised by the ammonia is found by subsequent titration with standard alkali. Knowing the amount of ammonia formed from the volume of blood filtrate taken the percentage of nonprotein nitrogen in blood can be readily calculated.

Method.—Measure 20 c.c. of protein free blood filtrate into a 500 c.c. flask with a large neck (kjeldahl flask) Add 2 c.c. of pure Sulphuric acid, 1 gm. of pot persulphate, and 2 drops of saturated copper sulphate. Heat over a micro burner under a hood using a fume absorber. Use fairly large flame at first until the greater part of the water has boiled off. When the volume of the fluid has been considerably reduced, the flame should be adjusted so that the mixture boils gently. It is better to use a small flame close to the flask than a large one away. After a time the fluid goes nearly black and subsequently slightens in color until it is faint blue or green in color. Heating must be continued for atleast 5 minutes after this stage has been reached.

Remove the flame and allow to cool until the flask can be held in the hand. Then add 20 c.c. of distilled water and shake, gently heating if necessary, to obtain a solution. Dilute to about 200 c.c. with water and fit up the distillation apparatus.

Distillation.—Into a 250 c.c. conical flask take 20 c.c. of 0·1 N HCl. Place it below the nozzle of the condenser. Adjust the height of the flask so that the nozzle just dips beneath the surface of the standard acid. Add 2 pieces of granulated zinc to the Kjeldahl flask and c.c. of 10% NaOH. Fit up the apparatus and turn on the water circulation through the jacket of the condenser.

Gently bring to boil the fluid in the Kjeldahl flask. Ammonia distills over and is neutralised by the acid. Maintain boiling till at least 50 c.c. of water distills over. Always take care to see that the nozzle is only just below the surface of the acid as otherwise the acid may be sucked back.

Now lower the conical flask so that the nozzle is at least 1½" above the surface of the acid. Wash the outside of the nozzle by a jet of distilled water. Continue boiling for about 2 minutes. Stop boiling and remove the conical flask.

Titrate with standard sodium hydroxide 0·1N, using Tashiri's indicator (contains Methyl red and Methylene blue.

Calculation.—1 c.c. of 0·1 N acid ≡ 1·7 mg. NH_3 ≡ 1·4 mg N_2
If X c.c. of 0·1 N NaOH is required for back titration the amount of acid, neutralised by the NH_3 = (20-X) c.c.

∴ Nitrogen liberated as ammonia from 20 c.c. of filtrate = 1.4 (20-X) mg.

20 c.c. of filtrate corresponds to 2 c.c. of blood.

∴ The nonprotein nitrogen content of blood = 70 (20-X) mg per 100 c.c.

7. *Uric Acid.*

Method of Brown.

Principle.—Protein free filtrate is treated with a special uric acid reagent in the presence of optimal amounts of cyanide-urea solution. The color developed is compared with that of a uric acid standard treated similarly.

Reagents.

(1) *Sodium cyanide Soln.* 12%—Prepared fresh and only as much as is required. Handle this very carefully. It is a very deadly poison. Measure it only from a burette. **Never use a pipette.**

(2) *Urea soln.*—50%.

(3) *Uric acid reagent.*—Dissolve 100 gms. of sod. tungstate and 20 gms. of anhydrous disodium hydrogen phosphate in about 150 c.c. of water with the aid of heat. Mix 25 c.c. of Con. H_2SO_4 with about 75 c.c. of water and pour the warm solution slowly and with shaking into the flask. Boil gently for one hour under a reflex condenser. Cool and dilute to 1000 c.c.

Uric acid standard.—Dilute 1 c.c. of stock soln. standard containing 1 mg. uric acid to 400 c.c. with water. This diluted standard contains 0·005 mg uric acid in 2 c.c.

Method.—Transfer 2 c.c. of protein free blood filtrate to a cylinder graduated at 10 c.c. In a similar cylinder place 2 c.c. of the standard uric acid soln. containing 0·005 mg. uric acid. To each tube add 2 c.c. of cyanide soln. from a burette. Mix by rotation and add 2 c.c. of urea solution to each tube. Again mix. Finally add 1 c.c. of uric acid reagent, mix and allow to stand at room temp. for 50 minutes. Dilute to the 10 c.c. mark with water, stopper and mix by inversion. Read in the colorimeter in the usual way.

Calculation.

$$\frac{\text{Reading of Standard}}{\text{Reading of Unknown}} \times 0{\cdot}005 \times \frac{10}{2} \times 100$$

= Mg. of uric acid per 100 c.c. of blood.

With the standard at 20, mg. of uric acid in 100 c.c. of blood is $\therefore \frac{50}{\text{Reading of Unknown}}$

Jaya & Co., (Paramount Press), Madras-5.